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Genome-wide analysis reveals differences in brain gene expression patterns associated with caste and reproductive status in honey bees (*Apis mellifera*)

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Abstract

A key characteristic of eusocial species is reproductive division of labour. Honey bee colonies typically have a single reproductive queen and thousands of sterile workers. Adult queens differ dramatically from workers in anatomy, physiology, behaviour and lifespan. Young female workers can activate their ovaries and initiate egg laying; these 'reproductive' workers differ from sterile workers in anatomy, physiology, and behaviour. These differences, however, are on a much smaller scale than those observed between the queen and worker castes. Here, we use microarrays to monitor expression patterns of several thousand genes in the brains of same-aged virgin queens, sterile workers, and reproductive workers. We found large differences in expression between queens and both worker groups (~2000 genes), and much smaller differences between sterile and reproductive workers (221 genes). The expression patterns of these 221 genes in reproductive workers are more queen-like, and may represent a core group of genes associated with reproductive physiology. Furthermore, queens and reproductive workers preferentially up-regulate genes associated with the nurse bee behavioural state, which supports the hypothesis of an evolutionary link between worker division of labour and molecular pathways related to reproduction. Finally, several functional groups of genes associated with longevity in other species are significantly up-regulated in queens. Identifying the genes that underlie the differences between queens, sterile workers, and reproductive workers will allow us to begin to characterize the molecular mechanisms underlying the evolution of social behaviour and large-scale remodelling of gene networks associated with polyphenisms.

Keywords: caste, eusociality, genomics, longevity, microarrays, reproduction

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Introduction

One of the key transitions in the evolution of social behaviour in insects is the shift to reproductive division of labour (Smith & Szathmary 1995; Keller 1999). In solitary species, a single female performs all required tasks (egg laying, brood care, foraging), but in eusocial species, females differentiate into either a queen or a worker. Queens are reproductively active but do not perform other colony tasks, while workers specialize on brood care or foraging, and usually do not perform both tasks simultaneously. The differentiation between queens and workers is a dramatic example of polyphenism, in which the same genome can give rise to vastly different phenotypic outcomes (Wheeler 1986).

The ovarian groundplan hypothesis proposes that ovarian development, brood care, and foraging are uncoupled in eusocial insects, such that ovary development is maintained in queens, brood care is performed in young workers

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(which can also be competent to activate their ovaries) and foraging is performed by older workers, which have the lowest capacity to become reproductively active (West-Eberhard 1996). Thus, queens and workers involved in brood care may be physiologically similar, while foragers may be physiologically distinct. Indeed, in honey bees, young bees involved in brood care (nurse bees) and queens have low levels of juvenile hormone, while foragers have high levels (Robinson *et al*. 1991). Recently, it has been proposed that this re-organization of basic groundplans may be apparent at the molecular level as well (i.e. Linksvayer & Wade 2005; Toth & Robinson 2007). Thus, nurse bees may share certain physiological traits with queens, and have similar gene expression patterns. Furthermore, it would be predicted that if workers become reproductively active, gene expression patterns would shift to become more queen-like. Clearly, these models are relatively simplistic, since genes and gene networks are generally highly pleiotropic, which makes it challenging to find simple associations. These fundamental questions in the evolution of social behaviour can best be addressed by monitoring genome-wide differences in gene expression, to capture the global expression patterns in the midst of complex gene interactions and networks.

In honey bees (*Apis mellifera*), each colony normally has only one reproductively active queen, and tens of thousands of nonreproductive workers (Winston 1987). The developmental trajectories that determine if female larvae develop into queens or workers are established early during larval development. Young larvae (less than 3 days old) destined to become queens are provided with nutrient-rich food, which results in changes in hormone titres, altered physiology and anatomy (Page & Peng 2001). For example, queens have 150–180 egg-producing ovarioles per ovary, while workers have 3–26 (Snodgrass 1956; Sakagami & Akahira 1958). As adults, this differential development results in large differences in behaviour. Workers undergo behavioural maturation in which they perform different tasks as they age, performing brood care when they are young (nurse bees) and eventually transitioning to foraging behaviour (forager bees) (Fahrbach & Robinson 1995; Robinson *et al*. 1997). In contrast, queens do not perform in-hive tasks or forage, and instead specialize on reproduction, laying up to 2000 eggs/day. These caste differences are accompanied by large differences in lifespan: queens live approximately 2 years, while workers live ~6 weeks during the summer, and 3–4 months over the winter (reviewed in Page & Peng 2001).

Workers are typically sterile, but they can become reproductively active under certain circumstances, depending on social, nutritional, and genotypic factors (Hoover *et al*. 2003; Amdam *et al*. 2004; Lattorff *et al*. 2005; Amdam *et al*. 2006; Hoover *et al*. 2006). Pheromones produced by queens and developing larvae inhibit ovary development in workers (Mohammedi *et al*. 1998; Hoover *et al*. 2003). Workers also need adequate nutrition, during larval and early adult development in order to activate ovaries (Hoover *et al*. 2006; Schafer *et al*. 2006). Specific strains of bees (anarchistic bees and *Apis mellifera capensis*) can activate their ovaries fairly readily, even in the presence of a queen (Barron *et al*. 2001; Lattorff *et al*. 2005), as can bees selected for a high pollen hoarding phenotype (Amdam *et al*. 2006). Reproductively active workers have developed ovaries with mature eggs and may initiate egg-laying behaviour; it is not clear if laying workers participate in general hive tasks, although anarchistic bees are less active (Dampney *et al*. 2004). Laying workers also have lower levels of juvenile hormone (JH); whereas in sterile workers, JH levels increase precipitously as bees mature into foragers (Robinson *et al*. 1992).

Identifying the genes underlying caste differences has been of great interest, but previous studies focused on relatively small numbers of genes. Gene expression differences in worker and queen honey bee larvae were assessed using macroarrays (Evans & Wheeler 2001b), subtractive hybridization (Evans & Wheeler 1999), differential display (Hepperle & Hartfelder 2001), semiquantitative real-time polymerase chain reaction (PCR) with selected candidate mitochondrial genes (Corona *et al*. 1999) or transferrin (do Nascimento *et al*. 2004), or quantitative real-time PCR (qRT–PCR) with genes involved in the insulin-signalling pathway (Wheeler *et al*. 2006). Adult differences in gene expression have been explored recently, using candidate genes involved in oxidative processes (Corona *et al*. 2005), or insulin signalling pathways (Corona *et al*. 2007). Studies comparing queens and workers in other social Hymenoptera have included bumble bees (*Bombus terrestris,* Pereboom *et al*. 2005), a eusocial stingless bee (*Melipona quadrifasciata,* Judice *et al*. 2004; Judice *et al*. 2006) and ants (*Lasius niger,* Graff *et al*. 2007). All of these analyses involve relatively small (< 700) numbers of unique genes, and most involved whole-body RNA extracts.

Recently, genes associated with worker reproduction have been considered in more detail. Anarchistic bees are a strain of honey bees that have high rates of ovary development among workers, even in the presence of a queen (Barron *et al*. 2001). Microarray studies on young anarchistic and wild-type adult bees found that two genes (major royal jelly proteins 2 and 7) were expressed at significantly higher levels in the heads of wild-type bees (Thompson *et al*. 2006). These genes are expressed primarily in the hypopharyngeal glands, and are associated with the production of brood food (Drapeau *et al*. 2006). In *A. m. capensis*, workers can activate their ovaries and lay parthenogenic eggs. Associative mapping of this trait has found that a single locus is involved in this thelytokous parthenogenesis (Lattorff *et al*. 2005). Finally, comparisons of strains of bees selected for high and low pollen hoarding demonstrated that workers of the high pollen hoarding strain have more

ovarioles, higher vitellogenin levels, and increased likelihood to activate their ovaries (Amdam *et al*. 2004; Amdam *et al*. 2006). Thus, genes associated with foraging or pollen foraging may also be associated with worker reproduction.

Several types of genes are predicted to be differentially regulated between queen and worker castes. First, given the differences in larval metabolism and adult longevity between queens and workers, genes associated with oxidative–reductive processes are predicted to be differentially regulated. Indeed, this has been determined for a subset of oxidoreductase genes in worker honey bee larva (Corona *et al*. 1999; Evans & Wheeler 1999, 2001b), bumble bee larvae (Pereboom *et al*. 2005) and adult stingless bees (Judice *et al*. 2004) although the correlation is not as clear in adult honey bees (Corona *et al*. 2005). Second, insulin signalling regulates growth, longevity and reproduction in multiple species (reviewed in Wu & Brown 2006), and thus is likely to be involved in regulating similar pathways in honey bees. Interestingly, reproduction and longevity are typically considered to be negatively correlated (reviewed in Carey 2001), but in honey bees these processes are apparently uncoupled, since queens are extremely reproductively active and long-lived. Differences in expression of insulin-pathway genes have been identified in honey bee worker and queen larvae (Wheeler *et al*. 2006) and adults (Corona *et al*. 2007), and differences in pollen hoarding appear to genetically map to loci containing insulin signalling genes (Hunt *et al*. 2007).

Previous studies of caste differences or worker reproduction have involved relative small numbers of genes and multiple tissue sources, which limit the interpretability of the results. Here, we compared brain gene expression patterns of same-aged virgin queens, sterile workers, and reproductive workers using microarrays. These microarrays represent approximately 7000 unique genes (Whitfield *et al*. 2002) and have been annotated to reflect the recent sequencing of the honey bee genome (The Honey Bee Genome Sequencing Consortium 2006). Performing large-scale pattern analysis on multiple groups of genes is likely to yield a more robust result than characterizing small groups of selected genes, and could also lead to the identification of novel genes or pathways associated with caste differences and reproduction.

In this study, we address the following main questions. First, are gene expression patterns in reproductive workers altered to become more 'queen-like'? Second, is there evidence for the ovarian groundplan hypothesis, that is, are genes associated with the nursing behavioural state also associated with reproduction or caste differences? Third, what functional categories of genes are associated with caste differences? Are these related to categories of genes associated with longevity, such as metabolism or insulin signalling? Finally, we use qRT–PCR to assess the validity of the array results using an independent set of queens and workers.

Materials and methods

General bee rearing

Bees for microarray study. For the microarray studies, the honey bees used were of mixed European descent, closest to the European subspecies *Apis mellifera ligustica*, and were reared at the Simon Fraser University Bee Facility (Burnaby, Canada). Bees were derived from two source colonies (colonies A and B). To obtain sterile and reproductive workers, we removed frames containing late-stage pupae from each of the two source colonies and placed them in an incubator 1 day before adult emergence (33 °C). We collected bees ~20 h after emergence and placed them in small (10 × 10 × 7 cm) Plexiglas cages. Cages were kept in a humidity-controlled (30% RH), dark incubator at 33 °C; manipulations were performed under red light. Bees were fed a honey/pollen paste *ad libitum* (45% honey, 45% pollen, 10% water) and provided with water, both of which were changed daily. Cages of bees were collected onto dry ice when they were 10 days old. Worker heads and abdomens were separated and labelled. Ovaries of bees were dissected and scored as undeveloped, intermediate, or fully developed, according to a modified scale from Velthuis (1970). Only those bees with undeveloped and fully developed ovaries were used in subsequent analyses.

Queens were grafted from eggs and young larvae derived from the two source colonies according to standard beekeeping procedures (Graham 1992). Queen cells were placed in small cages in a queen-bank colony, and the queens emerged from pupation in the cages. Queens were maintained as virgins, to avoid the confounding effects mating might have on the comparison with workers. Queens were collected 10 days after emergence and placed on dry ice and stored at –80 °C. Note that queens and workers were collected at the same time of day (afternoon), to avoid circadian rhythm effects.

Bees for qRT-PCR study. In order to verify differences in gene expression from the array study for a set of interesting candidate genes, we tested expression using a different method (qRT–PCR) and an independent set of bees from a different genetic background (*Apis mellifera carnica*) reared at the North Carolina State University (NCSU) Bee Research Facility. The source colony used for this experiment was headed by a queen (Glenn Apiaries) instrumentally inseminated with semen from a single drone (Laidlaw 1987). Because male bees are haploid, the average coefficient of relatedness among offspring of such an instrumentally inseminated queen is 0.75. Queens were produced by grafting larvae as before, but before emergence as adults, queen cells were placed with 35 one-day-old workers in a small (10 × 10 × 7 cm) Plexiglas cages. In total, 10 queens were used, each placed in a separate cage. Additionally,

two cages of 35 one-day-old workers from the source colony were created. Cages were kept in a humidity-controlled (30% RH), dark incubator at 33 °C; manipulations were performed under red light. Bees were fed a honey/pollen paste and provided with water as above. The two cages of workers and the 10 cages with queens were collected by submergence in liquid nitrogen when the bees were 10 days old. Note that all of the caged workers in these experiments had undeveloped ovaries.

Brain dissection. Whole bee heads were partially lyophilized to facilitate brain dissection (Schulz & Robinson 1999). Dissections were performed over dry ice so the tissue never thawed. Since ocelli and the subesophageal ganglion frequently fractured during dissection, these were removed during all dissections, while the remainder of the brain was included.

Microarrays

For the arrays, RNA from individual worker and queen brains were compared. Brains were dissected from freeze-dried heads, and RNA was extracted using a PicoPure kit (Arcturus). The RNA was amplified (using the Amino Allyl MessageAmp kit, Ambion Inc), resulting in final yields of approximately 100 μg. For each sample, 5 μg of RNA was labelled with Cy3, while another 5 μg was labelled with Cy5 (Amersham Biosciences). The samples were then hybridized against each using a loop design (Fig. 1a) as described by Grozinger *et al*. (2003). Note that the directionality of the dye labelling was switched between replicates to avoid the effects of dye biasing. The arrays were scanned using an Axon 4000B scanner (Axon Instruments), and Cy3 and Cy5 intensities for the expressed sequence Tags (EST) were normalized using a Lowess curve fit and a regionally normalization according to block using R/MAANOVA software (http://www.jax.org/staff/churchill/labsite/software/Rmaanova/index.html). ESTs with low intensities (< 300 intensity units on the scanner) were discarded. Additional array spots were removed, including spots corresponding to negative and positive controls. EST clones that had produced multiple bands or no bands during the PCR amplification used for synthesizing the arrays, wingless virus genes, and those ESTs found to be expressed at 1.5× higher levels in hypopharyngeal gland than in the worker brain were removed (Whitfield *et al*. 2003). Seven replicates were completed in total; two replicates were derived from worker bees in cage 1 of colony A, two replicates from cage 2 of colony A, two replicates from cage 1 of colony B, and one replicate from cage 2 of colony B. A total of 22 arrays were used; one array was duplicated. The gene expression data from these studies meet Minimum Information About a Microarray Experiment (MIAME) standards and have been deposited at ArrayExpress (www.ebi.ac.uk/arrayexpress).

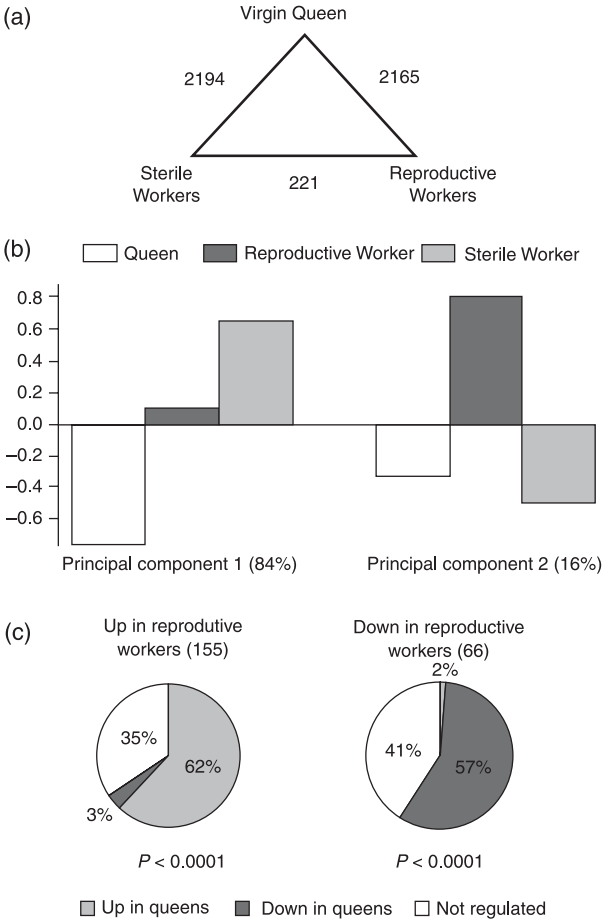

Fig. 1 General gene expression patterns associated with caste and worker reproduction. (a) Individual bee brains from the three different groups were compared on microarrays using a loop design, as shown. A Bayesian statistical approach (Townsend & Hartl 2002) was used to identify genes that differed significantly at the 97.5% confidence level. The numbers of genes expressed at significantly different levels between the three groups are shown. (b) The expression patterns of the 221 genes found to be significantly different between the sterile and reproductive workers were subjected to a principal component analysis (using GENESPRING software). (c) The sets of genes found to be significant up- or down-regulated in reproductive workers compared to sterile workers were compared to the sets of genes found to be significantly up- or down-regulated between queens and sterile workers. Chi-squared analyses were used to determine if there were significant biases in expression patterns. Analyses were based on the null hypothesis that genes up- and down-regulated in queens vs. sterile workers would be equally represented in the 'up-regulated in reproductive workers' list, or in the 'down-regulated in reproductive workers' list.

With the completion of the genome (The Honey Bee Genome Sequencing Consortium 2006), it was possible to match many of the ESTs to known or predicted genes (e.g. Whitfield *et al*. 2006). The data were analysed using the new genomic annotation. In total, 5547 genes/ESTs were

used in the analysis, 2673 of which remained as unannotated ESTs. Significant differences in gene expression between the three groups (sterile workers, reproductive workers, and virgin queens) was determined at the 97.5% confidence level using BAGEL (Bayesian analysis of gene expression levels) software (for review of methodology, see Townsend & Hartl 2002; Meiklejohn & Townsend 2005). This statistical analysis method provides similar results as ANOVAS for analysis of honey bee brain microarray data (Grozinger *et al*. 2003; Whitfield *et al*. 2003). The lists of significantly different genes can be found in Table S1, Supplementary material. Principal components analysis on the set of genes that were expressed at significantly different levels between sterile and reproductive workers was performed using GENESPRING software (Silicon Genetics). Genes were annotated into functional groups using *Drosophila* Gene Ontology (e.g. Whitfield *et al*. 2002; Grozinger *et al*. 2003; Whitfield *et al*. 2003; Whitfield *et al*. 2006). Significant biases to up- or down-regulation of expression of specific functional categories or groups of genes were determined using a chi-squared analysis.

Quantification of queen and worker brain gene expression by qRT-PCR

RNA was isolated from dissected heads using an RNeasy RNA extraction kit (QIAGEN), yielding 0.6–2 μg/brain. Complementary DNA was synthesized from 100 ng RNA. Gene expression levels were measured using qRT-PCR with an ABI PRISM 7900 sequence detector and the SYBR green detection method (Applied Biosystems). A housekeeping gene, *eIF3-S8*, was used as a loading control. Expression of *eIF3-S8* did not vary in previous bee brain microarray studies (Grozinger *et al*. 2003; Whitfield *et al*. 2003), or in this array study (queen = 1.02, sterile worker = 0.98, reproductive worker = 0.99; expression level is relative to average expression across all three groups). For each sample, duplicate qRT-PCRs were performed and averaged. A standard curve was generated for each primer using dilutions of genomic DNA, to calculate the relative quantities of RNA levels for each sample. A dissociation curve and negative control [cDNA reaction without reverse transcriptase (RT) enzyme] were used to ensure primer specificity and lack of genomic DNA contamination.

Candidate genes were chosen based on significant differences on arrays and interest in the functional category (i.e. representative genes associated with immune signalling were selected). Primers were developed using PRIMEREXPRESS software (Applied Biosystems). The sequences for the primers are in Table S2, Supplementary material.

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). For each individual brain sample, the ratio of the expression level of the gene of interest to that of the control gene (*eIF3-S8*) was calculated. In total, 10 queen brains and 10 worker brains were analysed. Significant differences in expression levels between workers and queens were determined using a one-tailed *t*-test. For the figures, the mean expression ratios were normalized to the worker brains.

Vitellogenin RNA quantification by qRT–PCR

Bees from one untreated cage for colony B were analysed. Worker bee abdomens were dissected under ice-cold RNAlater (QIAGEN). Ovaries were scored for developmental stage. Eight bees with high ovary development (stage 3 or 4, Pernal & Currie 2000) and eight bees with low ovary development (stage 0 or 1, Pernal & Currie 2000) were used. For the selected bees, the ovaries and intestines were removed, and RNA was extracted from the remaining carcass (with associated fat bodies) using an RNeasy RNA extraction kit (QIAGEN), yielding ~20 μg/abdomen. Complementary DNA was synthesized from 100 ng RNA. Expression of *vitellogenin* was quantified relative to the control genes *eIF3-S8* and *actin*. The *vitellogenin* primer sequences are in Table S2, *actin* primers were as in Amdam *et al*. (2006).

Results

Caste strongly influences brain gene expression

Brain gene expression differences between queens, sterile workers and reproductive workers were analysed using a Bayesian statistical approach (Townsend & Hartl 2002) to identify genes that differed significantly at the 97.5% confidence level. There were large differences in gene expression between the queens and sterile workers (2194 genes) and the queens and reproductive workers (2165, Fig. 1a, Fig. S1, Supplementary material). There was a substantial amount of overlap between the genes that differed between queens and both worker groups: 669 genes were significantly up-regulated in queens vs. both worker groups, and 1005 genes were significantly down-regulated (1674 genes total). Expression of only 221 genes was significantly different between sterile and reproductive workers.

Brain expression patterns in reproductive workers shift to become 'queen-like'

The general pattern of expression of the 221 genes that differed between sterile and reproductive workers was analysed using a principal components analysis (Fig. 1b). The majority of the variation (85%) was explained by the first principal component, in which the sterile workers and queens were on two extremes and the reproductive workers were intermediate. This result suggests that reproductive workers were up-regulating genes that were similarly

up-regulated in queens (compared to sterile workers), and were down-regulating genes that were down-regulated in queens (compared to sterile workers). To test this, the sets of genes that were significantly different between reproductive workers and sterile workers were compared to the sets of genes that were significantly different between queens and sterile workers (Fig. 1c). Of the 155 genes up-regulated in reproductive workers, 62% were also up-regulated in queens, while only 3% were down-regulated in queens; this was a significant bias ($P < 0.0001$, chi-squared test). Of the 66 genes down-regulated in reproductive workers, 57% were also down-regulated in queens, while only 2% were up-regulated; again, this was a significant bias ($P < 0.0001$, chi-squared test).

Queen-worker differences correlate with effect of queen pheromone

Previous studies identified sets of genes in the worker brain that were significantly regulated by exposure to queen mandibular pheromone (QMP; Grozinger *et al*. 2003). QMP is released by the queen, and is a primary regulator of worker behaviour (Slessor *et al*. 2005). Since QMP exposure inhibits ovary development in worker bees (Hoover *et al*. 2003), it is possible that QMP-down-regulated genes are associated with ovary activation. Thus, we hypothesized that in reproductive workers, genes that are down-regulated by QMP would preferentially be up-regulated. We compared the genes that were significantly different between the queens vs. both worker groups (caste genes), and those that were significantly different between sterile and reproductive workers (Table 1). Contrary to our prediction, we found that there was no bias in terms of the reproductive worker-regulated genes. However, we found that there was a significant bias for genes up-regulated by QMP to be also up-regulated in queens vs. workers, and genes down-regulated QMP to be down-regulated in queens vs. workers ($P < 0.01$, chi-squared test).

Nursing-related genes associated with caste and reproduction

Previous studies identified sets of genes in the worker brain that were up-regulated in the brains of nurses vs. forager (nurse-related genes) or up-regulated in forager vs. nurse brains (forager-related genes, Whitfield *et al*. 2003). We compared these two sets of genes to those genes that were significantly different between the queens vs. both worker groups (caste genes), and those that were significantly different between sterile and reproductive workers. We found that there was a significant bias for the nurse-related genes to be up-regulated in queens vs. workers than to be down-regulated (Tables 1, $P < 0.01$, chi-squared test). We also found that there was a significant bias for nurse-related genes to be up-regulated in reproductive workers vs. down-regulated, compared to sterile workers ($P = 0.03$).

Table 1 Regulation of genes associated with honey bee behaviour. Previous microarray studies (Whitfield *et al.* 2003) identified genes whose expression was significantly up-regulated in the brains of nurses vs. foragers (nurse related, 1165 genes) or foragers vs. nurses (forager related, 1109 genes). Previous array studies (Grozinger *et al.* 2003) also identified genes whose expression levels in worker brains were significantly up- or down-regulated by exposure to QMP (1054 up-regulated, 1233 down-regulated). Note that for comparison with the array results described here, these gene/EST lists were collapsed into the corresponding predicted genes from the genome. These nurse-related, forager-related and QMP-regulated sets of genes were compared to the sets of genes found to be significantly up- or down-regulated in queens vs. both sterile and reproductive workers, and the set of genes found to be up- or down-regulated in reproductive workers ('rep worker') vs. sterile workers. Chi-squared analysis was performed to determine if there were significant biases in expression patterns. For example, to determine if nurse-related genes were preferentially up- or down-regulated in queens vs. workers, the null hypothesis was that the sets of up- or down-regulated genes would be equally represented in the nurse-related list

	Queen > Worker (669 genes)	Queen < Worker (1005 genes)	Chi-squared test, *P* value	Rep < Sterile Worker (155 genes)	Rep < Sterile Worker (66 genes)	Chi-squared test, *P* value
Nurse-related (1165 genes)	179	193	< 0.01	29	7	0.03
Forager-related (1109 genes)	137	214	0.39	35	17	0.61
Up-regulated by QMP (1054 genes)	166	79	< 0.01	19	5	0.31
Down-regulated by QMP (1233 genes)	94	210	< 0.01	26	14	0.43

Table 2 Caste differences in functional categories of genes. Sets of genes in different functional categories were identified using the *Drosophila* Gene Ontology nomenclature. Expression patterns within these sets of genes were characterized to determine how many genes were significantly up- or down-regulated in queens compared to both worker groups. Significant biases ($P < 0.01$) in expression patterns were determined using a chi-squared test and denoted in the table as '**'. The null hypothesis was that the sets of up- or down-regulated genes would be equally represented gene in family lists

Gene family	Total	Queen up-regulated (699)	Queen down-regulated (1005)	*P* value (< 0.01)
Transcription factor	95	8	19	NS
Oxidoreductase	88	25	4	**
Peptidases	81	25	8	**
Mitochondria	76	20	4	**
Transporter	62	8	11	NS
Stress response	12	2	1	NS
Immune response (Defensins)	11	5	0	**
Heat shock	7	0	0	NS
Antioxidant	5	2	0	NS

NS, non significant.

Vitellogenin levels in sterile and reproductive workers

Another measure of reproductive potential in worker bees is the level of *vitellogenin* RNA expression (Amdam *et al*. 2004). Vitellogenin is a yolk protein that is synthesized in the fat bodies and required for egg maturation (Brandt *et al*. 2005). When *eIF-S8* was used as a control gene, *vitellogenin* levels were not significantly different between sterile workers and reproductive workers (sterile workers: 1.0 ± 0.2, reproductive workers 1.0 ± 0.2; mean ± standard error, $N = 8$ bees; $P = 0.3$, one-tailed *t*-test). When actin was used as a control gene, however, reproductive workers had significantly lower *vitellogenin* levels (sterile workers: 1.0 ± 0.1, reproductive workers 0.6 ± 0.1; mean ± standard error, $N = 8$ bees; $P = 0.02$, one-tailed *t*-test). If actin is used as a control gene, *eIF-S8* levels are significantly lower in the fat bodies of reproductive workers (data not shown).

General caste differences

Based on scans of the *Drosophila* Gene Ontology Biological Processes gene categories, we identified sets of genes with directional biases to be either up- or down-regulated in queens vs. workers, which then was confirmed by a chi-squared analysis (Table 2). Certain groups of genes appeared to be preferentially up-regulated in queens compared to workers, including groups associated with metabolism (oxidoreductases and mitochondrial genes) and immune function (peptidases and defensins). There is a tendency to up-regulate antioxidants, but this is not significant, likely because of the low number of genes in this category. Other large groups of genes (transcription factors and transporters) or genes with potentially similar functions (stress response and heat shock) are not significantly regulated.

Since insulin signalling is involved in longevity (Wu & Brown 2006) and expression differences in insulin signalling genes have been associated with queen-worker differences (Corona *et al*. 2007), we looked at expression of these genes in more detail. *Tor* encodes a protein kinase associated with nutrient/insulin signalling and longevity (Kapahi & Zid 2004), while *chico* is an insulin receptor substrate whose expression is also associated with longevity (Clancy *et al*. 2001). The *Tor-like* gene (GB11176, fly homologue CG5092) and the *insulin receptor* (GB18331) were significantly up-regulated in queens, while expression of *Tor* (GB11213) and *chico* (GB11037) were not significantly different.

Candidate caste genes

We tested several candidate genes derived from our microarray study using qRT–PCR (Table 3). Since the original microarray study reared workers in cages in an incubator while the queens were reared in colonies, we sought to further control the environment by rearing both workers and queens in cages in the laboratory, and also ensure that the results were reproducible using a different set of bees derived from a different genetic background. We tested genes that were associated with immune response (*Gnb3*, transferrin), oxidative processes (*sodh,* Parker *et al*. 2004), neural plasticity (*NCC69-like*), and sex-determination (*Dmrt93*); all of these were significantly regulated in both the arrays and qRT–PCR. We also tested genes associated with circadian rhythm (*per*) and vision (*black*) which were found to be significantly regulated in the arrays. Expression of *per* did not differ between workers and queens in the qRT-PCR studies, while *black* was up-regulated in workers rather than down-regulated. Perhaps exposure to light or synchronized worker behaviour in the field colonies altered expression of these genes in the queens in the array studies.

We also tested expression of *telomerase*, which was not on the arrays but was recently identified from the sequenced honey bee genome (Robertson & Gordon 2006). Decreased

Table 3 Candidate genes involved in caste differences. Candidate genes identified from the microarray studies were tested using qRT–PCR. Mean expression levels in queen (Q) vs. worker (W) or queen vs. sterile worker (SW) brains were converted to $\log_2$ scale and are shown from qRT–PCR and array analysis, respectively. Genes expressed at significantly different levels (using a one-tailed *t*-test) are indicated (**$P < 0.01$. *$P < 0.05$). Putative function is based on *Drosophila* Gene Ontology classification

Fly homologue	qRT-PCR $\log_2$ (Q/W)	Arrays $\log_2$ (Q/SW)	Significant in qRT-PCR?	Honey bee annotation (GB)	FlyBase ID	Function
CG7509	4.1	0.9	**	GB13055-PA	FBgn0035575	Cell adhesion, transmission of nerve impulse
Ncc69-like	2.7	1.5	**	GB15653-PA	FBgn0051547	Cation/amino acid transporter
Transferrin	1.6	0.7	**	GB19745-PA	FBgn0022355	Iron homeostasis, immune response
Gnbp3	0.9	0.9	**	GB19961-PA	FBgn0040321	Immune function
Sodh-1	0.4	1.3	**	GB14284-PA	FBgn0024289	Sorbitol dehydrogenase 1, immune function?
EGF-R	0.4	0.4	*	GB12207-PA	FBgn0003731	Epidermal growth factor, developmental processes
Period	0.2	–0.8	NS	GB19264-PA	FBgn0003068	Circadian rhythm
Black	–0.8	0.7	**	GB16827-PA	FBgn0000153	Glutamate decarboxylase activity, visual cues
Dnrt93	–1.2	–1.0	**	GB18260-PA	FBgn0038851	Transcription factor, sex determination

telomerase activity and telomere length are associated with cell senescence and ageing in mammals (Stewart & Weinberg 2006). While bees, flour beetles *Tribolium castaneum*, and silk moths *Bombyx mori* have clear *telomerase* homologues, *Drosophila* does not have a *telomerase* homologue and uses different mechanisms for maintaining telomere length (Melnikova & Georgiev 2005). Telomere length in *Drosophila* also does not appear to be strongly associated with longevity (Walter *et al*. 2007). As predicted by their longer lifespan, *telomerase* gene expression in queens is higher than in workers (workers = 1.00 ± 0.05, queens = 1.24 ± 0.07, $N = 8$, one-tailed *t*-test, $P = 0.01$).

Discussion

Here we present the first genome-wide comparison of gene expression patterns associated with caste differences and worker reproduction in a social insect. According to the ovarian groundplan hypothesis, workers engaged in brood care may be more similar to queens in terms of physiology than foragers, and recently it has been predicted that these similarities may be apparent at the level of gene expression (West-Eberhard 1996; Linksvayer & Wade 2005; Toth & Robinson 2007). The results of our microarrays support these models, since we found that both queens and reproductive workers preferentially up-regulate expression of genes associated with the nursing behavioural/physiological state. Furthermore, while sterile and reproductive workers did not differ greatly in terms of brain gene expression, expression of these genes clearly shifted to become more 'queen-like', suggesting that there may be a core group of genes associated with reproductive physiology that is also differentially expressed between castes. Finally, the physiological differences between queens and workers (such as longevity) are predicted to be due to differential expression of certain classes of genes (such as those associated with oxidative processes or insulin signalling) and indeed, we have observed the expected differences in our microarray studies, and we have also found strong caste differences in expression of genes associated with immune function. Thus, these studies not only provide new candidate genes and gene pathways associated with caste differences and reproduction, but also provide insight into the models of evolution of eusociality.

As predicted by the ovarian groundplan hypothesis (West-Eberhard 1996), we did find a bias to up-regulate nursing-related genes in queens relative to workers, and in reproductive workers relative to sterile workers. Since none of the bees in this study were engaged in reproductive behaviour (there was no opportunity for egg laying), these associations are likely due to similarities in physiology, rather than behaviour. A related model is the 'reproductive ground plan', which hypothesizes that pollen-hoarding behaviour in foragers is derived from maternal provisioning

behaviour associated with reproduction (Amdam *et al*. 2004; Amdam *et al*. 2006). In support of this model, levels of juvenile hormone, vitellogenin levels, ovariole number, ovary activation and age-at-onset of foraging are all correlated, but these correlations are not consistent across all strains of bees (Amdam *et al*. 2007). In our studies, we found that nurse-related genes were preferentially up-regulated in reproductive workers vs. sterile workers, which is not consistent with the link between reproduction and foraging. Perhaps genes specifically associated with pollen foraging and/or hoarding are linked to reproduction; this cannot be tested with our current data set. Overall, however, the nursing and foraging genes represented a fairly small sample of the 'caste' genes (~20%) so it seems likely that additional networks of genes are involved.

The number of genes differing between reproductive and sterile worker brains was relatively small. In previous microarray studies comparing nurses and foragers or workers exposed to queen mandibular pheromone (Grozinger *et al*. 2003; Whitfield *et al*. 2003), more than 2000 genes were found to be significantly different. It is possible that ovary activation simply does not involve large restructuring of neurones in the worker brain, and thus gene expression differences are minimal. Greater differences may have been observed if we considered other tissues, such as fat bodies or ovaries. Perhaps only a few major regulatory genes need to alter expression for adult worker bees to switch between sterility and reproduction. Indeed, a comparison of expression differences between wild-type and anarchistic workers (which readily activate their ovaries) found only two genes whose expression in the head was significantly different (Thompson *et al*. 2006), and these genes are expressed primarily in the hypopharyngeal glands used to make brood food (Drapeau *et al*. 2006). Comparisons of other behaviourally distinct groups of bees (guards and undertakers, for example) also found relatively small differences in brain gene expression (Cash *et al*. 2005). It is possible that the sterile workers used in our study were trending towards the nurse or forager behavioural state, and thus the individual variation could have obscured the differences. It is also possible that the sterile workers were in the initial stages of ovary activation which could have reduced the observed differences in gene expression. Morphologically, the ovaries were clearly undeveloped, but expression of *vitellogenin* RNA, which encodes a protein required for egg maturation, was expressed at higher levels in sterile bees (when actin was used as a control gene). When different strains of bees are considered, those with higher *vitellogenin* levels are more likely to activate their ovaries (Amdam *et al*. 2006), but our studies suggest that within a strain of bees, *vitellogenin* RNA levels are not tightly correlated with activated ovaries. *Vitellogenin* is also expressed at high levels in nurse bees for brood food production and juvenile hormone regulation (Amdam *et al*. 2003; Guidugli *et al*. 2005), and thus may be involved in other processes.

Interestingly, we found a significant bias for QMP-regulated genes to be associated with caste differences. These genes were previously identified in a microarray study of the brains of young workers exposed to QMP (Grozinger *et al*. 2003). Queens preferentially up-regulated genes that were up-regulated by QMP, and down-regulated genes that were down-regulated by QMP. Since the queen is producing QMP, it is possible that she is responding to her own pheromone. It seems unlikely, however, that queens and workers would have a similar brain gene expression response to the pheromone. More likely, there is some common physiology between queens (relative to workers) and workers exposed to QMP. QMP keeps young workers more nurse-like, both in terms of behaviour, physiology, and brain gene expression (Robinson *et al*. 1998; Grozinger *et al*. 2003). Both nurse bees and queens have low levels of juvenile hormone (Robinson *et al*. 1991), and nurses have high levels of *vitellogenin* (Amdam *et al*. 2003); queens also had higher expression of *vitellogenin* than workers on our arrays (data not shown). Thus, the biased expression of QMP-regulated genes may again suggest some correlation between the nurse physiological state and the queen-worker caste difference, as predicted by the ovarian groundplan hypothesis.

One of the most striking differences between honey bee queens and workers is in longevity; queens can live for 1–2 years, while workers live for only 6 weeks in the summer (Carey 2001; Page & Peng 2001). Several types of genes have been linked to longevity, including those involved in oxidative processes, insulin signalling, and immune function (DeVeale *et al*. 2004; Hughes & Reynolds 2005), while *telomerase* activity has been shown to be involved in ageing in mammals (Geserick & Blasco 2006). Its function in insects is not clear (Robertson & Gordon 2006), although males have shorter telomeres than longer-lived females in the ant species *Lasius niger* (Jemielity *et al*. 2007). We found a general bias in queens to up-regulate genes associated with oxidative processes (oxidoreductases and mitochondrial genes) and immune function (defensins and peptidases). We also found that *telomerase* expression was significantly up-regulated, as were genes involved in insulin signalling. These results are consistent with previous work demonstrating up-regulation of oxidative pathways, mitochondrial genes, and insulin signalling in larval and adult honey bee queens (Corona *et al*. 1999; Evans & Wheeler 1999, 2001a, b; Parker *et al*. 2004; Corona *et al*. 2005; Corona & Robinson 2006; Wheeler *et al*. 2006; Corona *et al*. 2007). The general up-regulation of immune genes in queens compared to workers has not been previously characterized, although in the microarray study comparing *Lasius niger* queens and workers, three of the genes up-regulated in queens may play a role in innate immune responses (Graff *et al*. 2007). Immune function has not been directly assessed in workers

and queens, but our results suggest that queens are more resistant to pathogens and diseases than workers.

Honey bees are a fascinating system in which to study the genetic foundation of polyphenism, reproduction, behaviour and longevity. Clearly, these are complex processes involving networks of several hundred or thousands of genes, and dissecting the specific pathways involved will be challenging. However, with the sequencing of the honey bee genome (Honey Bee Genome Sequencing Consortium 2006), the availability of functional genomics resources (Whitfield *et al*. 2002), a growing number of functional genomics studies (Grozinger *et al*. 2003; Whitfield *et al*. 2003; Cash *et al*. 2005; Thompson *et al*. 2006; Whitfield *et al*. 2006), and the ability to manipulate and uncouple aspects of honey bee physiology and behaviour (Robinson *et al*. 2005), it is now possible to use honey bees as a tractable system to test mechanistic and evolutionary theories of behaviour.

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This study was a collaborative effort between the research groups of Christina Grozinger (North Carolina State University) and Mark Winston (Simon Fraser University). The Grozinger lab uses functional genomics, chemical ecology and behavioural studies to dissect the molecular mechanisms underlying chemical communication and reproduction in honey bees. Dr. Yongliang Fan is a research associate in the Grozinger lab, focusing on functional genomics in honey bees, comparative genomic studies with other hymenopteran species, and chemical ecology. The primary research interest of the Winston lab was pheromonal regulation of honey bee behavior and physiology, though recently Mark Winston has retired from research to become the Academic Director of the Morris J. Wosk Center for Dialogue at Simon Fraser University. Dr. Shelley Hoover was Mark's last graduate student who studied honey bees; her research interests include elucidating the social, physiological, and genetic factors that regulate worker reproduction.

Supplementary material

The following supplementary material is available for this article:

Fig. S1 Clustering analysis of microarray data.

Table S1 Significantly regulated gene lists. Significant differences in gene expression between the three groups (sterile workers, reproductive workers, and virgin queens) was determined at the 97.5% confidence level using a Bayesian statistical analysis approach (Townsend & Hartl 2002; Grozinger *et al*. 2003). This table catalogues the lists of genes found to be significantly up- or down-regulated in the pairwise comparisons of the three sample groups.

Table S2 Sequences of primers used in qRT–PCR. Primers were developed using PRIMEREXPRESS software (Applied Biosystems). Primer sequences are depicted in the 5′ to 3′ orientation

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