

Genome-wide analysis of alternative reproductive phenotypes in honeybee workers

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Abstract

A defining feature of social insects is the reproductive division of labour, in which workers usually forego all reproduction to help their mother queen to reproduce. However, little is known about the molecular basis of this spectacular form of altruism. Here, we compared gene expression patterns between nonreproductive, altruistic workers and reproductive, non-altruistic workers in queenless honeybee colonies using a whole-genome microarray analysis. Our results demonstrate massive differences in gene expression patterns between these two sets of workers, with a total of 1292 genes being differentially expressed. In nonreproductive workers, genes associated with energy metabolism and respiration, flight and foraging behaviour, detection of visible light, flight and heart muscle contraction and synaptic transmission were overexpressed relative to non-reproductive workers. This implies they probably had a higher whole-body energy metabolism and activity rate and were most likely actively foraging, whereas same-aged reproductive workers were not. This pattern is predicted from evolutionary theory, given that reproductive workers should be less willing to compromise their reproductive futures by carrying out high-risk tasks such as foraging or other energetically expensive tasks. By contrast, reproductive workers mainly overexpressed oogenesis-related genes compared to nonreproductive ones. With respect to key switches for ovary activation, several genes involved in steroid biosynthesis were upregulated in reproductive workers, as well as genes known to respond to queen and brood pheromones, genes involved in TOR and insulin signalling pathways and genes located within quantitative trait loci associated with reproductive capacity in honeybees. Overall, our results provide unique insight into the molecular mechanisms underlying alternative reproductive phenotypes in honeybee workers.

Keywords: Altruism, *Apis mellifera*, microarray, reproductive division of labour

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Introduction

The reproductive division of labour in eusocial insects, in which the workers usually forego all reproduction in order to help rear siblings, represents one of the most spectacular examples of altruism in nature, as well as a classic Darwinian paradox (Darwin 1859; Ratnieks & Wenseleers 2008; Ratnieks *et al.* 2011). Indeed, it is not trivial to explain how a genetic trait that confers steril-

ity on its bearer would ever be able to spread in a population (Ratnieks *et al.* 2011). Over the last decades, however, great progress has been made in understanding what evolutionary factors drive worker sterility in insect societies. For example, based on inclusive fitness theory (Hamilton 1964), it is now understood that the incentive for workers to try to reproduce is set by a cost/benefit ratio in which factors such as relatedness, colony-level costs, and the presence of 'policing' mechanisms, which result in the selective removal of worker-laid eggs, all play a role (Wenseleers & Ratnieks 2006; Ratnieks & Wenseleers 2008). Policing is best known

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from the honeybee, where *c.* 98% of all worker-laid eggs are policed by other workers (Ratnieks & Visscher 1989; Wenseleers & Ratnieks 2006) and only *c.* 0.01–0.1% of the workers try to reproduce by activating their ovaries in presence of the queen (Ratnieks 1993). The presence of worker policing, in turn, has been explained based on the fact that honeybee queens mate with multiple drones (Palmer & Oldroyd 2000), and that this causes the workers to be collectively more related to the queen's offspring than to the offspring of other workers (Ratnieks 1988; Wenseleers & Ratnieks 2006).

Despite the fact that the evolution of altruism and worker sterility is now understood fairly well at an ultimate level, at a more proximate, mechanistic level many important questions remain. In fact, despite the postulation of 'genes for altruism' by hundreds of studies in evolutionary biology, very little is known about the actual underlying molecular mechanisms and gene regulatory networks that control worker reproduction and worker sterility, even in the honeybee – the best studied species in this respect (Thompson *et al.* 2006, 2008; Grozinger *et al.* 2007).

In honeybees, each colony usually has one reproductively active queen, and tens of thousands of mostly sterile workers (Ratnieks 1993). The development of young larvae into either queens or workers depends on the amount and type of food received during early larval development, which activates, among others, various epigenetic developmental switches (Kucharski *et al.* 2008). This leads to a high juvenile hormone (JH) titre in queen larvae (Rembold 1987), and via the insulin/insulin-like (IIS) and TOR (target of rapamycin) signalling pathways (Wheeler *et al.* 2006; Corona *et al.* 2007; Patel *et al.* 2007; de Azevedo & Hartfelder 2008), resulting in the complete development of the queen's ovaries, consisting of *c.* 150–180 ovarioles per ovary (Sakagami & Akahira 1958; Snodgrass 1984). Future worker bees, on the other hand, are fed with less royal jelly and more sugars, which leads to lower JH titres (Rachinsky & Hartfelder 1990; Rachinsky *et al.* 1990) and to the destruction of nearly all ovarioles by apoptosis (Capella & Hartfelder 1998), resulting in the formation of only 3–26 ovarioles per ovary (Snodgrass 1984). In presence of the queen or young female larvae, the workers generally do not activate their ovaries (Winston 1987; Ratnieks 1993). In these situations, pheromones released by the queen and brood result in an inhibition of worker ovary activation (Mohammedi *et al.* 1998; Hoover *et al.* 2003; Slessor *et al.* 2005; Maisonnasse *et al.* 2010). Nevertheless, upon loss of the mother queen, and upon subsequent failure to rear a replacement queen, as many as 30% of the workers develop their ovaries (Miller & Ratnieks 2001), with the exact proportion depending on various social, nutritional and

genetic factors (Hoover *et al.* 2003, 2006; Amdam *et al.* 2004, 2006; Schafer *et al.* 2006).

Interestingly, in adult honeybees, and in contrast to bumblebees and many other insects (Bloch *et al.* 2000), oogenesis and ovarian activation in adult workers is not influenced by JH (Robinson *et al.* 1991, 1992; Robinson & Vargo 1997). Instead, JH regulates, together with vitellogenin, the age-related division of labour, including the transition from nurse to forager (Robinson & Vargo 1997; Robinson & Huang 1998; Guidugli *et al.* 2005). On the other hand, ecdysteroids are known to regulate vitellogenin synthesis in honeybee larvae (Barchuk *et al.* 2002) and the titres of the ecdysteroid 20-hydroxyecdysone (20E) appear to be slightly elevated in egg laying workers in queenless honeybee colonies (Robinson *et al.* 1991), but see Hartfelder *et al.* (2002) for a corollary.

Our hypothesis is that environmental information is perceived in the workers' antennae and that this input signal is further processed in the central nervous system. This, on its turn, generates signals towards the rest of the body, which control the activation of the ovaries. However, during these processing and signalling events other physiological systems, such as the metabolism might be targeted as well. The aim of this study was to obtain a more detailed picture of gene regulatory networks involved in worker reproduction and sterility. Therefore, we used a whole-genome microarray to identify the genes which are differentially expressed between nonreproductive 'altruistic' honeybee workers and reproductive 'selfish' ones in a queenless honeybee colony. In contrast to earlier studies, the main novelties are, first, our study used a whole-genome microarray vs. earlier EST-based microarrays (e.g. Whitfield *et al.* 2003; Thompson *et al.* 2006; Grozinger *et al.* 2007). Second, we analysed normal, wild-type European honeybees, *Apis mellifera carnica*, as opposed to special strains of 'anarchistic' bees or bees selected for a high 'pollen hoarding' phenotype, which have unusually high proportions of egg-laying workers, or bees with higher numbers of ovarioles respectively (Barron *et al.* 2001; Amdam *et al.* 2006; Thompson *et al.* 2006, 2008). Third, we looked at gene expression throughout the whole body, as opposed to brain gene expression (Grozinger *et al.* 2007). Fourth, we analysed bees collected from natural, though hopelessly queenless colonies with a normal age structure and kept under entirely natural conditions, unlike caged bees (Grozinger *et al.* 2007). Fifth, we focused on relatively older bees (18 days) than used in earlier studies (e.g. 4 days in Thompson *et al.* 2006, 2008 and 10-day old in Grozinger *et al.* 2007) allowing us to compare nonreproductive workers with reproductive ones that had fully mature ovaries.

The specific aims of our study were, first, the identification of the major gene classes that are differentially expressed between reproductive and nonreproductive honeybee workers. This was done using detailed gene ontology (GO) – enrichment analyses. Second, by comparing our results with those of earlier gene expression studies (Grozinger *et al.* 2003, 2007; Thompson *et al.* 2006, 2008; Kocher *et al.* 2008; Alaux *et al.* 2009a) as well as with QTL-studies on the reproductive capacity of worker bees (Oxley *et al.* 2008; Linksvayer *et al.* 2009), we show how the altered physiology of reproductive and non-reproductive worker bees is reflected in expression differences in a wide range of pathways, including ovary maturation, metabolic rate and activity levels, neural processes and many others. Third, since evolutionary theory predicts that reproductive workers should tend to avoid carrying out risky tasks (Franks & Scovell 1983; Bourke 1988), and behavioural studies showed that non-reproductive honeybee workers display higher work rates than reproductive ones, and an earlier onset of foraging (Hillesheim *et al.* 1989; Martin *et al.* 2002; Dampney *et al.* 2004; Oldroyd & Beekman 2008), we tested whether such a pattern can also be distilled from our data. Finally, our study tried to generate a list of potential key regulatory switches involved in activating or suppressing ovary development in honeybee workers, based on previous analyses and gene classes generally known for their key-regulating functions, e.g. ecdysteroid induced genes (Beckstead *et al.* 2005), neuropeptides (Hummon *et al.* 2006; Boerjan *et al.* 2010), odorant binding proteins and receptors (Foret & Maleszka 2006) and others.

Materials and methods

Sample collection

Two bee colonies (*A. mellifera carnica*), headed by an instrumentally inseminated single-mated queen (obtained from the DLR Fachzentrum für Bienen und Imkerei, Mayen, Germany) were kept at the experimental beekeeping facility of the Laboratory of Zoophysiology of Ghent University, Belgium. To induce worker reproduction, both colonies were dequeened in the beginning of August 2008, and any newly built queen-rearing cells were removed in order to maintain the queenless condition. Broodframes with emerging brood were placed in an incubator at 34 °C and high relative humidity. For 5 days, newly emerged bees were marked with a paintdot on their thorax using a day-specific colour code (Posca paint-markers), and then introduced into their queenless colony of origin. Eighteen-day-old workers were recaptured in the early

morning and immediately anesthetized on ice and dissected to score ovary-development by making two lateral incisions in the abdomen. Note that the dissection pad was cooled with ice. If the length of the largest oocyte was greater than 1.1 mm (c. 50% the size of a freshly laid egg), the ovary was scored as developed; if the length of the largest oocyte was smaller than 0.3 mm, the ovary was scored as undeveloped. Dissections always took less than 35 s per sample. All samples were immediately frozen in liquid nitrogen and stored at –80 °C until RNA-extraction which was performed on individual whole bees. Intermediate stages of oocyte development were not studied. From each colony, eight bees with fully developed ovaries and eight bees with undeveloped ovaries were analysed. Subsequently, we will refer to these bees as reproductive and nonreproductive ones (note we did not monitor egg-laying behaviour). RNA-extraction was carried out on individual bees with the RNeasy – lipid tissue kit (Qiagen) using manufacturers' guidelines on individual bees. RNA was eluted in two batches of 50 and 30 µL RNase free water, both of which were pooled for further analysis.

Microarray analysis

Microarray hybridizations were performed by the VIB MicroArray Facility (Flemish Institute for Biotechnology), using a newly developed Agilent whole-genome honeybee microarray, which contained 15 187 probes targeting all but 177 of the 11 062 coding sequences represented in the Prerelease 2 version of the honeybee Official Gene Set (OGSPrls2) available on Beebase (genomes.arc.georgetown.edu/downloadFASTA.html) (details in Data S1). RNA concentration and purity were determined using the Nanodrop ND-1000 (Nanodrop Technologies) and RNA integrity was assessed using a Bioanalyser 2100 (Agilent). Per sample, an amount of 1 µg of total RNA spiked with 10 viral polyA transcript controls (Agilent RNA Spike-In Kit) was converted to double stranded cDNA in a reverse transcription reaction. Subsequently, the sample was converted to antisense cRNA, amplified and labelled with Cyanine 3-CTP (Cy3) or Cyanine 5-CTP (Cy5) in an *in vitro* transcription reaction according to the manufacturer's protocol (Agilent). A mixture of purified, labelled cRNA (Cy3 label: 14 pmol; Cy5 label: 10 pmol) was hybridized on the Agilent 8 × 15 K honeybee array, followed by (manual) washing according to the manufacturer's procedures. To assess the raw probe signal intensities, arrays were scanned using the Agilent DNA Micro-Array Scanner with SureScan High-Resolution Technology and probe signals were quantified using Agilent's Feature Extraction software (version 10.1.1.1).

All samples were hybridized using a direct comparison balanced block dye swap design, and each two-colour array was performed with samples dissected on the same day, originating from bees collected from the same colony. In total, we compared RNA extracts of eight reproductive and eight nonreproductive individual workers collected each from two experimental colonies, resulting in the analysis of a total of 16 two-colour arrays.

Differential expression analysis

To test for differential expression we first prefiltered the Agilent Feature Extraction unprocessed signals to retain only those probes which had at least one significant hybridisation in one of the channels of the arrays. This resulted in a dataset containing 14 248 probes representing 10 334 genes. Next, the red and green channels were normalised within and between arrays using loess and quantile normalisation in the Bioconductor package *limma* (Smyth 2005). For the genes with two available probes, we only retained the probe with the highest average expression, since that could be measured with the greatest accuracy and the least amount of noise. Finally, differential expression was assessed using empirical Bayes moderated *t*-tests carried out in *limma* (Smyth 2005) on the combined dataset of both colonies. In this analysis, colony was included as a random blocking variable and *p*-values were false discovery rate (FDR) corrected using the Benjamini–Hochberg method (Benjamini & Hochberg 1995). All genes with an adjusted *p*-value smaller than 0.05 were considered as differentially expressed and were used for further functional analysis. The gene expression data from this study have been deposited at ArrayExpress (<http://www.ebi.ac.uk/arrayexpress>, access nr. E-TABM-1003). We performed additional quantitative real time PCR (qRT PCR) analyses in order to validate the microarray. Sample collection, methods and results are available in the Supporting information.

Functional analysis

First, genes of the OGSPrls2 were annotated with GO information collected from Uniprot and DAVID, from *Drosophila* orthologs included in Flybase and Uniprot, from a Blast2Go search (Gotz *et al.* 2008), as well as based on the presence of conserved PFAM protein domains with known function (see Data S1). Subsequently, we tested which biological process GO terms were significantly enriched among the sets of genes that were differentially expressed in reproductive and non-reproductive workers using the Bioconductor *TopGO* package, using the modified hypergeometric test-based

method 'elim' (Alexa *et al.* 2006). When a given gene was annotated with several different GO-terms, the term that was maximally enriched was given priority in the annotation of its putative function.

Overlap with other studies and candidate gene sets

Exact hypergeometric one-tailed Fisher-exact tests were used to determine the significance of overlap between the sets of genes that were differentially expressed in reproductive and nonreproductive workers in our dataset and various other candidate gene sets (Table S2). In addition, to test whether the overlap in the expression pattern of the different studies (upregulated, downregulated or not differentially expressed) was in the predicted direction, we used one-tailed Spearman Rank Correlation tests. Candidate gene sets included (1) differentially expressed genes identified in reproductive vs. nonreproductive and anarchistic vs. wild-type bees (Thompson *et al.* 2006, 2008; Grozinger *et al.* 2007), (2) genes which were inferred to be involved in determining ovary activation in honeybee queens (Kocher *et al.* 2008), (3) genes that are known to respond to exposure to QMP (Grozinger *et al.* 2003), or BP (Alaux *et al.* 2009a), (4) genes contained within various quantitative trait loci (QTL) linked to worker reproductive potential (OvA1–OvA4 linked to the anarchistic worker phenotype, Oxley *et al.* 2008 and L1–L2 linked to variation in worker ovariole numbers, Linksvayer *et al.* 2009). We also looked at genes known to be regulated by 20 hydroxyecdysone (20E) (Beckstead *et al.* 2005) and genes coding for neuropeptide hormones (Hummon *et al.* 2006) and their receptors, because they regulate a wide range of physiological processes, including reproduction (Hartfelder *et al.* 1995, 2002; Lindemans *et al.* 2009). For the association with the candidate gene sets, we only included genes which were above background in both studies as the relevant gene universe. To calculate the overlap with QTL studies, we only included the genes which had been successfully mapped to a particular chromosome in the Amel 4.0 assembly.

Results

Differentially expressed genes in reproductive vs. nonreproductive workers

In total, 1292 genes were differentially expressed (adjusted *p*-value <0.05), 740 of which were upregulated in reproductive workers and 552 were upregulated in nonreproductive workers (Fig. 1). Out of these, 318 genes showed a more than 1.5-fold upregulation, 293 in reproductive workers and 25 were upregulated in non-

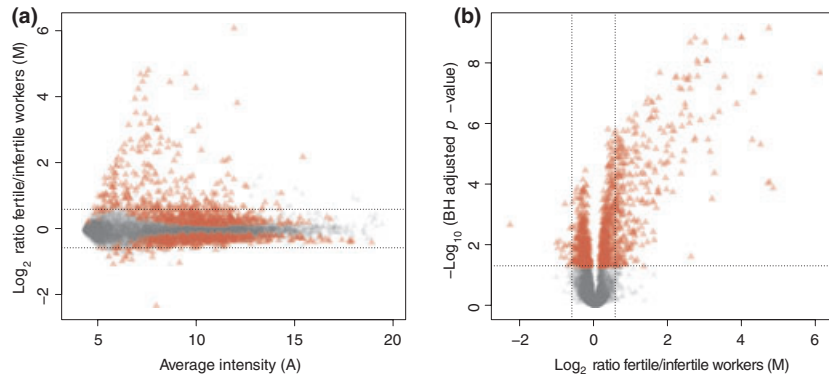


Fig. 1 Sets of genes which were differentially expressed between reproductive and nonreproductive workers, displayed as an MA plot (a) and a Volcano plot (b). Significantly differentially expressed genes (Benjamini–Hochberg adjusted p -values < 0.05) are highlighted in red; the Log-ratio cutoff lines show genes that were more than 1.5-fold differentially expressed.

reproductive workers (Fig. 1). Overall, a total of 1144 out of the 1292 (89%) differentially expressed genes could be annotated with GO information and were subjected to further functional analysis, 669 of which upregulated in reproductive workers and 475 in nonreproductive ones. Results of technical and biological validation experiments by means of qRT PCR are presented in the Supporting information.

Overlap with other microarray studies

There was no significant overlap between the genes that were differentially expressed in our study and those found to be differentially expressed in the brains of reproductive vs. nonreproductive workers Grozinger *et al.* 2007 (tests 1–3, Table S2), or in the brains and abdomens of anarchistic vs. wild-type honeybees (Thompson *et al.* 2006, 2008) (tests 4–6, Table S2). Nevertheless, a complete list of genes which were found to be differentially expressed in the same way in Grozinger *et al.* (2007) and Thompson *et al.* (2006, 2008) are provided in Table S1 and a selection of genes is displayed in Figs 2 and 3. The weak association with the study of Grozinger *et al.* (2007) may be due to the fact that it looked at differences in brain gene expression in 10-day-old, caged bees, whereas we used RNA extracts of whole bodies of 18-day-old bees, which were not caged but obtained from natural, queenless colonies. The nonsignificant association with the studies of Thompson *et al.* (2006, 2008) was expected given the small number of differentially expressed genes found in these studies, 4 and 7, respectively, and the fact that these studies used much younger bees, of only 4 days old and of a specific anarchistic strain. In addition, there is no correlation with the study of Brito *et al.* comparing normal and CO₂ treated worker bees (test 76, Table S2, Brito *et al.* 2010).

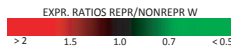
In contrast to the above studies, we did find a significant overlap with the microarray study of Kocher *et al.* (2008) on differential gene expression in honeybee queens with variable ovary activation. In particular, there was a significant association between genes being upregulated in nonreproductive workers in our study and those being upregulated in virgin relative to laying queen ovaries (test 11, Fisher exact test, $p = 0.02$, Table S2). In addition, there was a nearly significant trend for genes which were upregulated in reproductive workers compared to genes which are upregulated in the ovaries of laying relative to mated queen (test 7, Fisher exact test, $p = 0.06$, Table S2) and in the brains of mated relative to virgin queen (test 22, Fisher exact test, $p = 0.07$, Table S2). Furthermore, there was a significant association between the expression status of genes in our study and that in laying relative to mated, non-laying queen ovaries in Kocher *et al.* 2008 (test 9, Spearman rank correlation test, $p = 0.009$, Table S2). For examples see Figs 2 and 3 and Table S1.

Interestingly, there was also a significant negative association between genes being up- or downregulated in reproductive workers and the tendency to be up- or downregulated in response to exposure to either QMP (test 30, Spearman rank correlation test, $p = 0.02$, Table S2) or BP (test 33, Spearman rank correlation test, $p = 0.04$, Table S2). This supports the role of QMP and BP in determining ovary activation in honeybee workers (Mohammedi *et al.* 1998; Hoover *et al.* 2003; Slessor *et al.* 2005; Maisonnasse *et al.* 2010). For examples see Figs 2 and 3 and Table S1.

Functional enrichment analyses

GO-term enrichment analysis demonstrated that 171 and 135 biological process GO-terms were significantly enriched among the genes which were upregulated in

Name of gene or fly ortholog	BEEBASE	FC	P _{adj}	M	QTL	QMP	BP	ECD	AS	colony 1	colony 2	Putative function	
triglyceride lipase	GB11256-RA	0.2	2.1E-03									glycerolipid metabolism	
diacylglycerol kinase family member 1	GB16799-RA	0.8	3.3E-03			✓						glycerolipid metabolism	
glycerol-3-phosphate dehydrogenase	GB11613-RA	0.8	1.5E-02									glycerolipid metabolism, upreg. in foragers	
lipophorin receptor	GB16983-RA	0.8	2.0E-02									lipid transport	
Z band alt. spliced PDZ-motif protein 66	GB10901-RA	0.5	2.4E-02									energy metabolism	
xylulokinase	GB18997-RA	0.8	1.4E-02									glycolysis	
ADP-dependent glucokinase	GB13862-RA	0.8	1.1E-02									glycolysis, upreg. in foragers	
glucose-6-phosphate isomerase	GB16429-RA	0.9	3.0E-02										
enolase	GB15039-RA	0.9	4.7E-02			✓							
citrate synthase	GB12573-RA	0.8	8.0E-03										
aconitate hydratase	GB12488-RA	0.8	1.5E-03										
isocitrate dehydrogenase γ	GB15716-RA	0.8	4.6E-03										
dihydroipoamide succinyltransferase	GB13073-RA	0.8	8.6E-03										
succinate dehydrogenase A	GB17439-RA	0.8	1.0E-04										
isocitrate dehydrogenase β (NAD+)	GB18960-RA	0.9	2.9E-02										
cytochrome c oxidase subunit 6A1	GB10253-RA	0.9	1.5E-02										
V-ATPase subunit I	GB11840-RA	0.9	1.1E-02			O2							
NADH dehydrogenase 1β subcomplex 7	GB15252-RA	0.8	4.9E-02										
Rieske iron-sulfur protein	GB13508-RA	0.9	3.8E-02										
ATPase IR	GB17812-RA	0.8	1.1E-02										
plasma membrane calcium ATPase	GB13865-RA	0.9	3.5E-02										
Na/K-transporting ATPase subunit α	GB20055-RA	0.7	5.1E-03										
arginine kinase	GB10973-RA	0.8	5.8E-03			✓							
coracle	GB13343-RA	0.8	1.6E-04										
krotzkopf verkehrt	GB12511-RA	0.8	6.5E-03										
organic cation/carnitine transporter	GB17795-RA	0.7	5.7E-03			P3							
α-glucosidase	GB30223-RA	0.7	1.9E-02										
nose resistant to fluoxetine protein 6	GB16461-RA	0.8	1.2E-02			P3							
insulinase domain containing gene	GB11385-RA	0.8	1.3E-02			P2							
MAK10 homolog	GB11992-RA	0.9	2.9E-02			P2							
protein kinase C δ	GB13673-RA	0.8	8.4E-03			✓							
shaker	GB18814-RA	0.8	4.3E-04										
hyperkinetic	GB10006-RA	0.8	1.5E-04										
wings up A	GB17075-RA	0.8	2.2E-03										
myosin regulatory light chain 2	GB13399-RA	0.8	9.7E-03			T							
myosin heavy chain 1	GB11965-RA	0.8	3.9E-04										
α-actinin	GB11028-RA	0.9	3.8E-02										
ryanodine receptor	GB15169-RA	0.8	4.4E-02										
tropomyosin 1	GB30512-RC	0.8	3.2E-03			P2							
excitatory amino acid transporter 3	GB16911-RA	0.8	3.2E-02										
G protein-coupled receptor kinase 1	GB15959-RA	0.9	4.6E-02			✓							
shaker cognate b	GB19350-RA	0.8	1.7E-02			✓							
netrin receptor unc5	GB14364-RA	0.6	4.6E-02										
cycle	GB11309-RA	0.8	2.6E-02										
slowpoke	GB17365-RA	0.9	1.3E-02										
quiver	GB15765-RA	0.8	3.2E-02			P2							
clock	GB17107-RA	0.9	4.0E-02										
beadec	GB14109-RA	0.8	1.7E-02										
venom dipeptidylpeptidase IV	GB14496-RA	0.7	1.2E-02			L1							
obstructor d	GB16370-RA	0.6	2.5E-02										
klarsicht	GB30140-RA	0.7	9.2E-03										
no receptor potential A2	GB14619-RA	0.7	1.0E-02										
arrestin 1	GB16006-RA	0.8	8.1E-03			O1							
G protein β subunit 1	GB12662-RA	0.8	1.3E-02										
photoreceptor dehydrogenase	GB11685-RA	0.8	4.6E-02			✓							
discs large 1	GB30138-RE	0.8	4.3E-02										
cytochrome P450 305D1	GB11943-RA	0.5	1.2E-02										
neurotransmitter transporter 5	GB18205-RA	0.8	4.0E-02										
GABA neurotransmitter transporter-1B	GB16752-RA	0.8	4.9E-02			✓							
ligand-gated chloride channel homolog 3	GB12078-RA	0.6	1.1E-02										
CG9297-PA	GB12536-RA	0.6	9.6E-03										
nicotinic acetylcholine receptor α2	GB18518-RA	0.8	2.0E-02			✓							
stoned B	GB17165-RA	0.7	2.8E-03										
synaptotagmin 13	GB15417-RA	0.7	5.2E-03										
syntaxin 5	GB18673-RA	0.8	3.7E-02										
synapsin	GB12564-RA	0.8	3.0E-02										
synaptotagmin 7	GB19709-RA	0.8	3.7E-02										
synaptotagmin 4	GB11060-RA	0.8	2.7E-02										
capon	GB10113-RA	0.8	2.6E-02			O3	✓						
bitesize	GB13617-RA	0.9	2.6E-02			✓							
Down syndrome cell adhesion molecule	GB30209-RA	0.9	3.4E-02			✓							
cadherin-N	GB12853-RB	0.8	6.4E-03										
unzipped	GB14145-RA	0.8	1.8E-02										
chaoptic	GB11660-RA	0.8	1.2E-02										
dynamitin	GB15728-RA	0.9	6.0E-03										
unnamed	GB15906-RA	0.7	9.8E-03			✓							
RAS	GB15876-RA	0.8	3.4E-02										
calcium and integrin-binding protein 1	GB16769-RA	0.7	2.7E-02			G							
DNA-dep. protein kinase catalytic subunit	GB11176-RA	0.8	3.4E-02			G							
E3 ubiquitin-protein ligase MARCH3	GB11494-RA	0.6	2.7E-03			T							
unnamed	GB19925-RA	0.7	1.4E-02			✓							
allatostatin	GB30252-RA	0.7	3.0E-03										
neuropeptide F	GB16364-RA	0.8	4.5E-02										
MVP neuropeptide	GB19071-RB	0.8	2.6E-02										
prohormone 4	GB18206-RA	0.8	2.2E-02										
allatostatin A receptor	GB19021-RA	0.8	4.5E-02										
orexin receptor type 2	GB19597-RA	0.7	3.2E-02			✓							
diuretic hormone 44 receptor 1	GB10976-RA	0.8	1.9E-02										
odorant receptor 156	GB30392-RA	0.5	8.1E-03										
odorant rece. tor 2	GB19990-RA	0.7	3.8E-02			✓							
odorant receptor 10	GB16894-RA	0.9	4.8E-02			✓							
odorant binding protein 10	GB15549-RA	0.7	3.9E-02			✓							
sensory neuron membrane protein	GB15549-RA	0.7	3.9E-02										
cabut	GB10114-RA	0.7	3.7E-02			L1							
nuclear receptor / transcr. factor ftz-f1	GB16873-RA	0.9	4.6E-03										
kliongin	GB10469-RA	0.8	7.5E-03										
glycoprotein 150	GB10945-RA	0.8	8.9E-04										
ecdysone-inducible gene L2	GB17369-RA	0.7	2.6E-02			G							
inwardly rectifying K-channel	GB17183-RA	0.8	2.1E-03										
transcription factor bric-a-brac	GB13762-RA	0.8	8.4E-03			O3							



Energy metabolism & respiration
Digestion & feeding behaviour
Flight behaviour
Muscle contraction
Heart muscle contraction
Regulation of flight muscle contraction
Regulation of heart contraction
Motor axon guidance
Rhythmic behaviour, upreg. in foragers
Rhythmic behaviour
Aggression related, upreg. in foragers & soldiers
Venom protein
Chitin biosynthesis
Detection of visible light
Phototransduction
Phototransduction
Positive phototaxis
Brain-expressed xenobiotic detoxifying enzyme
Neurotransmitter transport
Neurotransmitter transport
Neurotransmission
Neurotransmission
Synaptic transmission
Synaptic transmission, upreg. in soldiers
Synaptic transmission
Synaptic vesicle transport, upreg. in soldiers
Axon extension involved in axon guidance
Axon extension involved in axon guidance
Axonogenesis, upreg. in foragers
Memory
Transcription factor
Transcription factor, signal transduction
Integrin-mediated signaling
Serine/threonine protein kinase
Ubiquitin-mediated proteolysis, endocytosis
Unknown
Neuropeptides, upreg. in foragers
Neuropeptides, upreg. in nectar foragers
Neuropeptide receptor, reg. JH synthesis
Neuropeptide receptor, reg. JH synthesis & feeding
Neuropeptide receptor, reg. osmotic balance
Odorant receptor
Queen mandibular pheromone co-receptor
Brain-specific odorant binding protein
Response to pheromone
Germ band shortening
Ecdysone-regulated ovary regression
Growth regulation, caste determination
Ovarian follicle cell migration
Neg. reg. of insulin sign. pathway
Germ band shortening
Female gonad development

reproductive and nonreproductive workers, respectively (Tables 1 and S3, representative genes annotated with some of these GO-terms are shown in Figs 2 and 3).

Genes upregulated in reproductive workers were mostly enriched for GO-terms relating to oogenesis, mitosis, meiosis and the cell cycle (*c.* one-third of all enriched GO-terms). For examples see Fig. 3 and Table S1. Genes annotated with GO-terms concerning mRNA production, processing and turn-over which is necessary for the storage of maternal mRNA in mature oocytes, as well as genes annotated with GO-terms related to epigenetic control of gene expression (e.g. histone demethylation and acetylation), including, most notably, DNA methyltransferase 3 (Fig. 3 and Table S3) were also overrepresented. The latter might hint at the presence of germline DNA methylation and genomic imprinting (Queller 2003; Kucharski *et al.* 2008; Elango *et al.* 2009). For examples see Figs 2 and 3 and Table S1.

Among the genes upregulated in nonreproductive workers, genes annotated with GO-terms related to increased energy metabolism and respiration were greatly overrepresented, including for example six genes involved in the citric acid cycle, 10 genes involved in oxidative phosphorylation, several genes involved in glycolysis and ATP metabolic processes. Additional enriched terms are lipid transport and metabolism with differentially expressed genes such as triglyceride lipase, which was fivefold upregulated and is important for burning triglycerides (Canavoso *et al.* 2001) and the lipophorin receptor, which is involved in shuttling lipids out of the fat body, and which has previously been shown to be upregulated in the fat body and other tissues of nonreproductive honeybee workers (Guidugli-Lazzarini *et al.* 2008) (Tables 1 and S3, Fig. 2). In addition, genes involved in flight, foraging and locomotory behaviour, phototransduction, the detection of visible light, positive phototaxis and flight and heart muscle contraction were overexpressed in sterile workers, as well as genes involved in the regulation of the tracheal system (Tables 1 and S3, examples

shown in Fig. 2). With respect to brain and neuron functioning, nonreproductive workers overexpressed genes involved in synaptic transmission, neurotransmitter transport, axon extension, memory and response to external stimuli and pheromones (Tables 1 and S3, Fig. 2). Lastly, the genes upregulated genes in sterile workers were also greatly, *c.* eightfold, enriched for neuropeptide precursors known to be associated with foraging behaviour or the modulation thereof (test 36, Fisher exact test, $p = 0.004$, Table S2, Fig. 2).

Overlap with QTL studies

Genes that were upregulated in reproductive workers were 1.6-fold enriched within QTL OvA3, a region known to be associated with differences in worker reproductive behaviour (Oxley *et al.* 2008; Linksvayer *et al.* 2009) (test 43, Fisher exact test, $p = 0.02$, Table S2), which was previously shown to be linked with variation in worker egg-laying in strains of anarchistic bees (Oxley *et al.* 2008). This association came about despite the fact that OvA3 was not significantly enriched for genes with oogenesis-related GO-terms (test 73, Fisher exact test, Table S2). Genes upregulated in nonreproductive workers, in turn, were significantly enriched for genes lying within QTL regions Pln1 to Pln4 (test 71, Fisher exact test, $p = 0.03$, Table S2), which is known to be associated with differences in foraging predisposition and sucrose responsiveness (Hunt *et al.* 2007). Particularly the *c.* twofold, enrichment for genes lying within QTL Pln2 on chromosome 1 (test 59, Fisher exact test, $p = 0.03$, Table S2) is noteworthy. There were no significant associations between our complete set of differentially expressed genes and any of the analysed QTLs (Table S2).

Discussion

Overall, our study revealed that there were significant differences in gene expression between reproductive and nonreproductive workers in a vast number (1292)

Fig. 2 Selection of genes which were upregulated in sterile workers, illustrating genes corresponding to enriched classes of GO BP terms (Tables 1 and S2) and genes of special interest. Beebase identifiers, putative function, fold change (FC, reproductive/nonreproductive) and *FDR*-adjusted p values (p_{adj}) are also mentioned, as well as whether the genes were confirmed to be upregulated in sterile workers in other microarray studies (M: G = upregulated in the brains of sterile workers in Grozinger *et al.* 2007), T = upregulated in wild-type vs. anarchistic workers in the studies of (Thompson *et al.* 2006, 2008)), mapped to QTL loci linked to worker reproductive potential and foraging predisposition (O1–O4: QTLs linked to the anarchy phenotype, Oxley *et al.* 2008; L1–L2: QTLs linked to variation in worker ovariole numbers, Linksvayer *et al.* 2009; P1–P4: QTLs linked to pollen foraging and sucrose responsiveness, Hunt *et al.* 2007), were known to be upregulated by exposure to queen mandibular pheromone (QMP, Grozinger *et al.* 2003) or brood pheromone (BP, Alaux *et al.* 2009a) and whether the gene's fly ortholog was known to be 20-hydroxyecdysone (20E)-responsive (ECD, Beckstead *et al.* 2005). We also mention genes which are differentially expressed between virgin queens, mated queens and laying queens (K, Kocher *et al.* 2008). Top-20 differentially expressed genes are highlighted in red. The 8 arrays performed per colony are arranged on the X-axis based on a hierarchical clustering (tree not shown).

Table 1 Selected biological process GO-terms which were enriched among the genes that were differentially expressed in reproductive and nonreproductive workers

Class: differentially expressed in		Reproductive workers								
Nonreproductive workers		GO nr.	NS	FE	P-level	GO biological process term	GO nr.	NS	FE	P-level
Foraging/flight related						Oogenesis, mitosis and meiosis				
Regulation of striated muscle contraction	0006942	2	7.7	0.02	0007307	Eggshell chorion gene amplification	5	5.6	0.001	
Muscle contraction	0006936	15	6.2	0.0003	0003006	Reproductive developmental process	43	1.5	0.04	
Heart function	0007507	15	2.0	0.01	0048477	Oogenesis	37	1.5	0.04	
Flight behaviour	0007629	4	3.3	0.03	0008069	Ovarian follicular epithelium	3	3.6	0.04	
Adult locomotory behaviour	0008344	10	2.9	0.002	0030727	Germarium-derived germ-line cyst formation	2	5.6	0.04	
Detection of visible light	0009584	7	4.4	0.03	0007067	Mitosis	34	3.2	0.00001	
Chitin-based cuticle biosynthetic process	0008362	4	3.2	0.03	0007143	Female meiosis	9	3.0	0.002	
Energy metabolism and respiration					0051321	Meiotic cell cycle	19	2.8	0.03	
Citric acid cycle	0006099	6	3.8	0.004						
Oxidative phosphorylation	0006119	10	1.9	0.04						
ATP metabolic process	0046034	8	2.1	0.03						
Gener. of precursor metabolites and energy	0006091	21	2.0	0.03						
Reg. of tube diameter, open tracheal system	0035158	6	7.6	0.00006						
Lipid transport	0006869	7	3.2	0.05						
Aging	0007568	11	1.9	0.03						
Brain and behaviour						Brain and behaviour				
Synaptic transmission	0007268	24	1.9	0.002	0045475	Locomotor rhythm	8	3.5	0.002	
Axon extension involved in axon guidance	0048846	3	8.1	0.004	0009629	Response to gravity	3	8.3	0.004	
Memory	0007613	6	2.2	0.04	0007423	Sensory organ development	31	1.4	0.04	
Neurotransmitter transport	0006836	15	2.0	0.01	0008355	Olfactory learning	7	2.9	0.01	
Proboscis extension reflex	0007637	2	9.5	0.02						
Response to external stimulus	0009605	28	2.1	0.0008						
Response to nutrient levels	0031667	7	2.7	0.01						
Response to pheromone	0019236	2	7.7	0.02						
Response to temperature stimulus	0009266	8	2.9	0.006						
Response to organic substance	0010033	15	1.7	0.03						
Chemosensory behaviour	0007635	8	2.0	0.04						

These data demonstrate that nonreproductive workers had much higher work rates than fertile ones, and that they were most likely actively foraging, whereas reproductive bees of the same age were not (NS = number of genes associated with GO-term which were differentially expressed; FE = fold enrichment, *p*-level based on TopGO method 'elim'). For a complete list, see Table S2.

of genes, and together with the various enrichment analyses that we presented, this provides detailed insight into the physiology of both sets of workers. Below we discuss the implications of some of the associations that we found, and point out particular candidate genes which would deserve further study.

Differential activity patterns of reproductive and nonreproductive workers

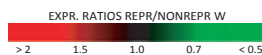
Overall, the outcome of the GO enrichment analysis suggests that reproductive and nonreproductive workers greatly differed not only in their reproductive potential, but also in their generic activity patterns. In particular, in nonreproductive workers, genes associated with increased energy metabolism and respiration, flight and foraging behaviour, detection of visible light, flight and heart muscle contraction and synaptic transmission were all overexpressed, implying they probably had a higher whole-body energy metabolism and activity rate and were most likely already actively foraging, whereas same-aged reproductive workers were not. In addition, some of the differences we found are characteristic for the neural remodelling that occurs upon the transition from in-hive to outside tasks (Whitfield *et al.* 2003, 2006). In fact, several of the genes involved in these functions, including *synaptotagmin 4* (regulating neurotransmitter release), *bitesize* (involved in eye and muscle development) and *unzipped* (involved in axon guidance), have all been shown to be overexpressed in brains of honeybee worker engaging in defensive behaviour (Alaux *et al.* 2009b), and, in the case of *unzipped*, also in brains of paper wasps (Toth *et al.* 2010). As additional evidence, however indirect, a venom gland protein (dipeptidylpeptidase IV) and *beadex*, an aggression-related gene previously shown to be upregulated in foragers and soldiers (Alaux *et al.* 2009b) are also upregulated in nonreproductive workers and hint to forage or defence tasks. Also a gene involved in motor axon guidance (*netrin receptor unc5*) for muscle coordination, 4 genes involved in the regulation of circadian rhythmic behaviour (including *cycle* and *slowpoke*, which both have been shown to be upregulated in foragers, Alaux *et al.* 2009a; Toth *et al.* 2010) were all upregulated in nonreproductive workers and are typical for foragers (Fig. 2). Additional experiments on dissected neural tissue will provide additional insights. Finally, genes related to digestion and feeding behaviour were also upregulated in nonreproductive workers, for example α -glucosidase, which is one of only five proteins with a function in carbohydrate metabolism known to be expressed in the hypopharyngeal glands of worker bees (Santos *et al.* 2005; Alaux *et al.* 2009b), and thought to be involved in the process-

ing of nectar by forager bees (Kubo *et al.* 1996; Wolschin & Amdam 2007; Alaux *et al.* 2009b). Taken together, it therefore seems likely that nonreproductive workers had higher metabolic and implied activity rates, and were most likely actively foraging, whereas same-aged reproductive workers were not. This clearly supports our hypothesis that reproductive workers should tend to avoid carrying out risky tasks, so as to not compromise their reproductive futures (Franks & Scovell 1983; Bourke 1988), and is in line with earlier behavioural studies that have documented reduced work rates and an earlier onset of foraging in honeybee strains with a high reproductive potential (Hillesheim *et al.* 1989; Martin *et al.* 2002; Dampney *et al.* 2004; Oldroyd & Beekman 2008).

Overexpression of foraging-related neuropeptides in nonreproductive workers

In further support of nonreproductive workers having a greater foraging predisposition, we also found that genes upregulated in nonreproductive workers were greatly, *c.* eightfold, enriched for neuropeptide precursors known to be associated with foraging behaviour or the modulation thereof (test 36, $p = 0.004$, Table S2). In particular, sterile workers overexpressed the 4 neuropeptide encoding transcripts: allatostatin, neuropeptide F, MVPV neuropeptide and prohormone 4 (Fig. 2). Allatostatin, which in several insects is known to inhibit JH production in the corpora allata, is upregulated in foragers in both the honeybee (Alaux *et al.* 2009a) and in paper wasps (Toth *et al.* 2010). Neuropeptide F has been identified as one of the 100 genes most predictive for honeybee foraging (Alaux *et al.* 2009a). Finally, MVPV neuropeptide and prohormone 4 have both been shown to be associated with nectar rather than pollen foraging (Brockmann *et al.* 2009) and the latter has also been shown to be upregulated in foragers relative to nurses (Whitfield *et al.* 2003). In addition to the aforementioned neuropeptide precursors, nonreproductive workers also overexpressed 3 neuropeptide receptors: diuretic hormone 44 receptor 1, the allatostatin A receptor and the allatotropin receptor (Weaver & Audsley 2009), as well as the nuclear receptor and transcription factor *ftz-fl*. The allatostatin and allatotropin receptors are known to regulate JH synthesis and may be implicated in the marked change in the JH hemolymph titres seen upon the transition from in-hive to outside tasks (Robinson & Vargo 1997; Robinson & Huang 1998). *Ftz-fl* may be implicated in ecdysone-regulated ovary regression in nonreproductive workers, given that it has previously been shown to be important in honeybee caste determination and that its expression has been shown to be repressed by exposure to ecdysteroids (Hepperle & Hartfelder 2001).

Name of gene or fly ortholog	BEEBASE	FC	<i>p</i> _{adj}	M	QTL	QMP	BP	ECD	K	colony 1	colony 2	Putative function
transcription factor E2F	GB18882-RA	6.8	7.9E-09									
minichromosome maintenance 6	GB19871-RA	2.2	3.1E-03									
myb protein	GB12498-RA	1.8	2.0E-05	G	O3				✓			eggshell chorion gene amplification
transcription factor DF	GB13495-RA	1.5	3.0E-05									
myb-interacting protein 120	GB10684-RA	1.8	1.8E-03			✓						eggshell chorion gene amplification
double parked	GB12839-RA	1.6	5.3E-05		O3							chorion gene amplification, regulation of oviposition
piefke	GB15091-RA	1.2	3.5E-02		O3		✓					female gonad development
single-minded	GB11417-RA	2.2	1.8E-03			✓						genital disc development
huckebein	GB30303-RA	19.2	3.3E-06									germ cell migration
stathmin	GB18507-RA	3.0	3.8E-04		L1							germ cell migration, steroid biosynthesis
farnesyl pyrophosphate synthase	GB15337-RA	26.1	6.6E-10									steroid biosynthesis
mevalonate decarboxylase	GB16296-RA	1.2	2.8E-02		O3							steroid biosynthesis
cholesterol O-acetyltransferase	GB16016-RA	1.5	3.0E-03			✓		✓				steroid biosynthesis
without children	GB19476-RA	1.2	9.2E-03									ecdysteroid biosynthesis
cytochrome P450 314A1	GB13998-RA	3.8	7.8E-06									egg chamber growth, 20E biosynthesis
fringe glycosyltransferase	GB17604-RA	1.7	3.7E-02									egg chamber formation
cbl	GB16551-RA	1.4	2.5E-05									germ line cyst formation, reg. insulin sign. pathway
slimfast	GB15921-RA	1.6	7.3E-03		O3	✓						lipid metabolism, growth regulation
tuberous sclerosis complex 1	GB12676-RA	1.3	5.9E-05									reg. TOR & insulin sign. pathways
tyrosine phosphatase 61F	GB14529-RA	1.2	3.1E-02						✓			reg. of insulin sign. pathway
shaggy	GB30241-RB	1.2	2.5E-02									follicle cell development, reg. insulin sign. pathway
FAM47C	GB19763-RA	28.4	1.3E-04									germ line protein
DNA methyltransferase 3	GB14232-RA	2.1	2.1E-06									de novo DNA methylation, germ line imprinting
homeobox protein six4	GB10752-RA	3.8	1.4E-04									gonad development
immunoglobulin CG42343	GB12833-RA	2.7	3.9E-04	G								mitotic cell cycle
G2/mitotic-specific cyclin-A	GB14847-RA	9.1	5.9E-07									mitotic spindle organisation
kinesin 8	GB10037-RA	15.6	1.4E-09		O3							mitotic spindle organisation
chromator	GB10259-RA	1.2	9.5E-04		O3	✓						mitotic spindle organisation
t-complex chaperonin 5	GB10587-RA	1.2	3.2E-03				✓					nurse cell to oocyte transport
decapping protein 1	GB14691-RA	2.3	4.3E-05									
maelstrom	GB17844-RA	14.3	2.1E-07									
vasa	GB14804-RA	6.1	2.3E-02									oocyte axis specification
oo18 RNA-binding protein	GB12560-RB	5.1	7.0E-08									
exuperantia	GB19360-RA	2.1	3.3E-02									
kinesin A	GB18655-RA	7.9	2.0E-09		L2							
serine/threonine protein kinase 6 (aurora)	GB14418-RA	5.4	2.1E-07									
kinesin 14	GB13977-RA	3.8	1.9E-05									
Rac GTPase-activating protein 1	GB18839-RA	2.1	3.6E-03	G	O4							oocyte meiosis
bifocal	GB16223-RA	1.5	1.7E-03									
mini spindles	GB10660-RA	1.5	2.4E-02									
targeting protein for Xkfp2	GB14990-RA	15.6	1.4E-09									oocyte meiosis
serine/threonine protein kinase JIL-1	GB13245-RA	1.4	9.3E-03					✓				oocyte meiosis
germinal histone H4	GB14107-RA	10.3	3.8E-07									oocyte nucleosome assembly
chromatin assembly factor 1 subunit A	GB15706-RA	1.9	2.8E-04	G								nucleosome assembly, DNA replication
yellow-g	GB10842-RA	25.7	8.8E-05									
hoepel1	GB14978-RA	22.1	2.6E-08									
drop dead	GB15247-RA	4.3	1.7E-04									
egg-derived tyrosine phosphatase	GB11379-RA	2.7	7.3E-07									
star	GB13389-RA	2.7	5.7E-06									oogenesis
eggless	GB17661-RA	2.0	8.1E-07									
heat shock protein 1α (Hsp83/Hsp90)	GB14494-RA	1.8	1.4E-02					✓				
ovo protein	GB11352-RA	1.6	6.7E-06									
DNA topoisomerase 1	GB15849-RA	1.5	1.8E-04		L1							
domino	GB10524-RB	1.4	1.4E-02									
brain tumor	GB12558-RA	2.1	1.5E-03					✓				
ecdysteroid-regulated gene E74	GB10759-RA	1.2	4.0E-02					✓				oogenesis
broad-complex	GB14070-RA	1.2	4.7E-02					✓				
dacapo	GB12878-RA	2.6	1.2E-04				✓					
Src oncogene at 64B ortholog	GB14772-RA	1.8	8.8E-06				✓					oogenesis
poly A binding protein cytoplasmic 1	GB11055-RA	1.2	3.8E-02				✓					
protein on ecdysone puffs	GB13256-RA	1.5	1.8E-04				✓		✓			oogenesis
megator	GB11086-RA	1.6	6.0E-06				✓					oogenesis, caste determination
odorant binding protein 9	GB13938-RA	5.0	4.3E-05		O1							ovarian odorant binding proteins
odorant binding protein 7	GB30243-RA	2.4	1.0E-02									
squid	GB12840-RA	1.3	1.6E-03									ovarian follicle cell migration
ecdysone 20-monooxygenase	GB30218-RA	4.2	3.2E-06									ovarian follicle cell development, 20E biosynthesis
supernumerary limbs	GB10096-RA	1.5	1.1E-04					✓				ovarian follicle cell development
buffy	GB15274-RA	5.6	3.3E-08									ovarian follicle development
heat shock protein DnaJ (Hsp40)	GB19172-RA	1.3	1.2E-03	G								protein folding
lipid storage droplet-1	GB15498-RA	1.7	6.4E-04	G				✓				lipid transport & storage
wech	GB19249-RA	68.5	2.0E-08									ovary muscle functioning
vitellogenin	GB13999-RA	1.9	2.6E-01	G,T								yolk protein precursor
yolkless	GB16571-RA	6.5	2.8E-06									vitellogenin receptor, vitellogenin transport
α-methylidopa hypersensitive protein	GB30066-RA	1.8	9.8E-03									dopamine sensitive protein
ecdysteroid-regulated gene E93/mbilk-1	GB16065-RA	26.5	8.0E-05					✓				mushroom body-specific transcription factor
gustatory receptor 3	GB16407-RA	2.1	1.5E-03									gustatory receptor
defective proboscis extension response	GB16778-RA	1.3	2.7E-02									proboscis extension reflex
pteropsin	GB12200-RA	1.7	4.0E-02			✓		✓				nonvisual opsin
PDF receptor	GB14562-RA	1.6	2.4E-03									circadian rhythm
estrogen-related receptor	GB11125-RA	1.3	1.1E-03					✓				hormone receptor, upregulated in nurses
ETHR ecdysis-triggering hormone receptor	GB13260-RA	1.5	4.7E-02		O3							neuropeptide receptor
prohormone 2	GB10356-RA	1.2	1.1E-02		L1	✓						neuropeptide
metabotropic GABA-B receptor subtype 2	GB13604-RA	1.4	5.4E-04				✓					neurotransmission
unnamed	GB10975-RA	2.4	4.0E-04	G								transcription factor
upstream of N-ras	GB11497-RA	1.5	6.2E-06				✓					transcription factor
TATA-box-binding protein	GB17946-RA	1.4	8.2E-06			✓						transcription factor
unnamed	GB16814-RA	8.3	3.2E-06				✓					cysteine peptidase
FAM60A	GB18347-RA	1.3	1.6E-02	G								unknown



Oogenesis & oviposition related

Brain related

By contrast, only one neuropeptide precursor, the *Apis*-specific prohormone 2 (Fig. 3), was upregulated in reproductive workers. It is not known to modulate foraging behaviour (Brockmann *et al.* 2009) but has been shown to be downregulated by exposure to QMP (Grozinger *et al.* 2003), which may suggest a role in the regulation of reproduction. Finally, in reproductive workers not only the orphan nuclear oestrogen-related receptor α , which is an upstream factor of vitellogenin synthesis, was upregulated, but also the gene for ecdysis-triggering hormone (ETH) receptor, suggesting an additional function for the latter. The ETH receptor was recently found to be expressed in the *corpora allata* in *Bombyx mori*, pointing to a role in the regulation of JH synthesis (Yamanaka *et al.* 2008).

Genes lying within QTLs linked to worker reproductive potential and foraging predisposition

The overlap with QTLs associated with increased reproductive potential offers a useful approach to identify candidate genes linked to worker reproduction. It enables a targeted approach to reconstruct the genetic architecture of candidate genes, which are potentially *cis*-regulated. Candidate genes contained within the QTLs include *myb*, a transcription factor that regulates eggshell chorion formation, *double parked*, involved in the regulation of oviposition, *piefke*, involved in female gonad development and mevalonate decarboxylase, which is involved in steroid biosynthesis (Fig. 3). Additional genes of interest are *slimfast*, involved in the regulation of growth and the TOR signalling pathway, which is involved in queen-worker caste determination (Wheeler *et al.* 2006; Corona *et al.* 2007; Patel *et al.* 2007; de Azevedo & Hartfelder 2008), and the gene coding for ecdysis-triggering hormone receptor (Fig. 3).

Similarly, we also found genes upregulated in nonreproductive workers to be enriched for genes lying within the foraging-related QTLs Pln 1–4 (Table S2; Hunt *et al.* 2007). Candidate genes that might explain

this association include *tropomyosin 1*, involved in heart muscle contraction, *quiver*, involved in rhythmic behaviour, an organic cation/carnitine transporter, involved in the regulation of appetite, nose resistant to fluoxetine protein 6, involved in digestion and defecation, an insulinase domain containing gene (insulin breakdown) and MAK10 homolog (regulation of the TOR signalling pathway and nutrient sensing) (Fig. 2).

Additional candidate genes linked to worker ovary activation

With respect to key switches involved in the activation of ovary development in reproductive workers, several genes involved in steroid biosynthesis were upregulated in reproductive workers. This includes one of the seven known copies of farnesyl pyrophosphate synthase (The Honeybee Genome Sequencing Consortium 2006), which was 26-fold upregulated, as well as cholesterol O-acyltransferase, *without children*, cytochrome P450 314A1 and ecdysone 20-monooxygenase (Fig. 3, Table S1). Many genes that were ecdysteroid-induced were also upregulated, including the brain expressed ecdysteroid-regulated gene *E93/mbk-1* (Park *et al.* 2003), which was 27-fold upregulated and has previously been inferred to be important in the detection of QMP and shown to correlate with worker ovary size (Kocher *et al.* 2010), ecdysteroid-regulated gene *E74*, broad-complex, lipid storage droplet-1, and several others (Fig. 3). A dopamine-responsive gene, α -methyl-dopa hypersensitive protein is noteworthy as well. As expected, given the involvement of these pathways in, among others, honeybee caste determination, components of the TOR and insulin signalling pathways were upregulated, including *cbl*, the QMP downregulated gene *slimfast*, tuberous sclerosis complex 1, tyrosine phosphatase 61F and *shaggy* (Fig. 3) (Wheeler *et al.* 2006; Corona *et al.* 2007; Patel *et al.* 2007; de Azevedo & Hartfelder 2008). Furthermore, *megator* was upregulated in reproductive workers, which is also known to be involved in honeybee caste determination (Barchuk

Fig. 3 Selection of genes which were upregulated in reproductive workers, illustrating genes corresponding to enriched classes of GO BP terms (Tables 1 and S2) and genes of special interest. Beebase identifiers, putative function, fold change upregulation in reproductive workers (FC) and *FDR*-adjusted *p* values (p_{adj}) are also mentioned, as well as whether the genes were confirmed to be upregulated in reproductive workers in other microarray studies (M: G = upregulated in the brains of reproductive workers in Grozinger *et al.* 2007), T = upregulated in wild-type vs. anarchistic workers in the studies of Thompson *et al.* (2006, 2008)), mapped to QTL loci known to be linked to worker reproductive potential and foraging predisposition (O1–O4: QTLs linked to the anarchy phenotype, Oxley *et al.* 2008; L1–L2: QTLs linked to variation in worker ovariole numbers, Linksvayer *et al.* 2009; P1–P4: QTLs linked to pollen foraging and sucrose responsiveness, Hunt *et al.* 2007), were known to be downregulated by exposure to queen mandibular pheromone (QMP, Grozinger *et al.* 2003) or brood pheromone (BP, Alaux *et al.* 2009a) and whether the gene's fly ortholog was known to be 20-hydroxyecdysone (20E)-responsive (ECD, Beckstead *et al.* 2005). We also mention genes which are differentially expressed between virgin queens, mated queens and laying queens (K, Kocher *et al.* 2008). Top-20 differentially expressed genes are highlighted in red. The 8 arrays performed per colony are arranged on the X-axis based on a hierarchical clustering (tree not shown).

et al. 2007) and downregulated by exposure to QMP (Grozinger *et al.* 2007) needs mentioning. Interestingly, *vitellogenin* was differentially expressed in only one of the two colonies (in colony 1: $p_{\text{adj}} = 0.035$, but not in colony 2: $p_{\text{adj}} = 0.83$). Nevertheless, the vitellogenin receptor *yolkless*, which is involved in the transport of vitellogenin into the developing ovary (Guidugli-Lazzarini *et al.* 2008), was sixfold upregulated in reproductive workers of both colonies (Fig. 3).

Additional candidate genes linked to worker sterility

Although genes related to differences in metabolism and in worker ovary activation represent the vast majority of differentially expressed genes in our study, we also found several genes which are potential key determinants in controlling worker reproduction at an early stage in the gene regulatory pathway (Figs 2 and 3). For example, *A. mellifera* odorant receptor 2 (Fig. 3) was significantly upregulated in nonreproductive workers and has previously been shown to be a queen pheromone co-receptor (together with *AmOr11*, Wanner *et al.* 2007), and known to be upregulated by exposure to QMP (Grozinger *et al.* 2007). This suggests that workers that are more sensitive to QMP may also be less likely to activate their ovaries when the colony becomes queenless. Similarly, odorant receptor 156, which was 2.1-fold upregulated in nonreproductive workers, and odorant binding protein 10 (*obp10*), which is known to be upregulated by exposure to QMP (Grozinger *et al.* 2007), might be implicated in the response to queen or brood pheromone and the regulation of worker reproduction.

Ecdysteroid-regulated genes upregulated in nonreproductive workers represent another interesting class of genes which might represent key switches in inducing worker sterility, for example *cabut*, involved in germ band shortening and associated with a QTL locus that has been shown to be linked with variation in worker ovariole numbers (Linksvayer *et al.* 2009) and the aforementioned orphan nuclear receptor *ftz-f1* as well as *Klingon*, involved in honeybee caste determination (Hepperle & Hartfelder 2001; Guidugli *et al.* 2004). Furthermore, *ecdysone-inducible gene L2*, which negatively regulates the insulin signalling pathway and the inwardly rectifying K^+ -channel, involved in germ band shortening is upregulated in nonreproductive workers (Fig. 2, Table S1), which suggests functions of insulin-like peptides in repressing ovary activation. The transcription factor *bric-a-brac*, involved in female gonad development, may also represent a possible switch in maintaining sterility in nonreproductive workers (Fig. 2, Table S1).

Conclusion

Overall, our study revealed that there were massive differences between reproductive and nonreproductive workers in whole-body gene expression for 1292 out of 11 062 (12%) of the genes represented in the honeybee genome. This dataset provides unprecedented insight into the molecular players underlying alternative reproductive insect phenotypes. One surprising conclusion stemming from these data was that reproductive and nonreproductive workers not only differed in their reproductive potential, as shown by the differential expression of various oogenesis-related genes, but also in the expression of various genes that are related to differences in their likely activity patterns. This was shown by the fact that nonreproductive workers overexpressed genes involved in flight behaviour, muscle contraction, respiration, heartbeat, vision and phototaxis (Tables 1 and S3), as well as foraging-related neuropeptides and genes linked to foraging-related QTLs (Table S2). This finding supports the theoretical prediction stating that laying workers should be selected to exploit the colony by carrying out low-risk tasks, so as to not compromise their reproductive futures (Franks & Scovell 1983; Bourke 1988), and is consistent with the somewhat reduced work rate (i.e. engagement in in-hive tasks as well as in foraging and defence) and foraging predisposition of reproductive workers documented by direct behavioural observations in honeybee workers (Hillesheim *et al.* 1989; Dampney *et al.* 2004; Oldroyd & Beekman 2008).

Even though our study was mainly aimed at documenting downstream gene expression changes that occur in reproductive vs. nonreproductive bees, we also discovered many candidate genes linked to the early steps of worker ovary activation. Future experiments on separate tissues can provide insights into the complete physiological pathways underlying worker sterility. Overall, this set of genes should provide a rich resource for future functional genomic studies, and should help towards the discovery of some of the key genetic determinants of the suppressed fertility of honeybee and other social insect workers.

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Data accessibility

All microarray data are available at <http://www.ebi.ac.uk/arrayexpress>, access nr. E-TABM-1003.

Supporting information

Additional supporting information may be found in the online version of this article.

Tables S1 List of genes which were differentially expressed between reproductive and nonreproductive workers, together with functional annotation information

Tables S2 Association tests between our differentially expressed gene sets and various other candidate gene sets, calculated using exact hypergeometric one-way Fisher exact tests or one-way Spearman rank correlation tests

Tables S3 Biological processes gene ontology (GO) terms over-represented among the sets of genes that were upregulated in reproductive and nonreproductive workers, calculated using *TopGO*

Tables S4 Results of technical (same samples of the microarray analysis) and biological (different samples) validation experiment by means of quantitative Real time Polymerase Chain Reaction (qRT-PCR), using protocols described in Data S1

Tables S5 Primers for the validation experiments using qRT-PCR, specific for the candidate reference genes and the target genes

Data S1 Methods.

Data S2 Results.

Data S3 References.

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