PROCEEDINGS OF THE ROYAL SOCIETY B

Proc. R. Soc. B (2005) **272**, 1145–1152
doi:10.1098/rspb.2005.3060
Published online 1 June 2005

Differential gene expression in queen–worker caste determination in bumble-bees

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Investigating how differential gene expression underlies caste determination in the social Hymenoptera is central to understanding how variation in gene expression underlies adaptive phenotypic diversity. We investigated for the first time the association between differential gene expression and queen–worker caste determination in the bumble-bee *Bombus terrestris*. Using suppression subtractive hybridization we isolated 12 genes that were differentially expressed in queen- and worker-destined larvae. We found that the sets of genes underlying caste differences in larvae and adults failed to overlap greatly. We also found that *B. terrestris* shares some of the genes whose differential expression is associated with caste determination in the honeybee, *Apis mellifera*, but their expression patterns were not identical. Instead, we found *B. terrestris* to exhibit a novel pattern, whereby most genes upregulated (i.e. showing relatively higher levels of expression) in queen-destined larvae early in development were upregulated in worker-destined larvae late in development. Overall, our results suggest that caste determination in *B. terrestris* involves a difference not so much in the identity of genes expressed by queen- and worker-destined larvae, but primarily in the relative timing of their expression. This conclusion is of potential importance in the further study of phenotypic diversification via differential gene expression.

Keywords: *Bombus terrestris*; caste determination; caste evolution; gene expression; social insects; suppression subtractive hybridization

1. INTRODUCTION

The evolution of sterile castes in social insects represents one of the major transitions in evolution (Maynard Smith & Szathmáry 1995). In common with other major transitions, such as the divergence of the germ line and somatic cells in multicellular organisms, it has involved units of biological organization at one level (individuals) cooperating to form a new unit of biological organization at a higher level (the colony), accompanied by the emergence of a reproductive division of labour among the lower-level units. In the advanced social Hymenoptera (ants, bees, wasps), this reproductive division of labour involves a polyphenism whereby adult females occur in two, adaptively interdependent castes. Females are morphologically, physiologically and behaviourally specialized as either reproductive queens or non-reproductive workers. With few exceptions (Julian *et al.* 2002; Volny & Gordon 2002; Helms Cahan & Keller 2003), whether female larvae develop into queens or workers depends not on genotypic differences, but on their environment at critical periods of caste determination (Wilson 1971; Wheeler 1986). Therefore, caste determination and differentiation must involve the differential expression of genes shared by queens and workers. Hence, the social Hymenoptera present a unique opportunity to investigate, in the fundamental context of one of evolution's major transitions, how variation in gene expression underlies adaptive morphological and behavioural diversity.

Comparative studies of caste-related gene expression provide essential insights into the nature, origin and evolution of caste polyphenism in social insects (Evans & Wheeler 1999, 2001; Robinson 2002*a*). One key question to be answered is whether those genes whose differential expression originally underlay reproductive differentiation in female adults of 'primitively' eusocial insects (those having queens and workers that do not differ in morphology) are also involved in larval caste determination in advanced social taxa (Evans & Wheeler 1999). Answering this question could reveal whether, at the proximate level, the evolution of a distinct morphological worker caste involves genes originally regulating reproductive differences among adult females. To date, however, the identity of differentially expressed genes associated with queen–worker caste determination in larvae has only been investigated in the advanced social honeybee *Apis mellifera* (Corona *et al.* 1999; Evans & Wheeler 1999, 2000; Hepperle & Hartfelder 2001). Related investigations have examined caste-associated gene expression in honeybee pupae and adults (Piulachs *et al.* 2003; Guidugli *et al.* 2004) and how variation in gene expression underlies behavioural differences among adult honeybee workers (Ben-Shahar *et al.* 2002; Kucharski & Maleszka 2002; Robinson 2002*b*; Whitfield *et al.* 2003), soldier caste differentiation (Miura *et al.* 1999; Miura 2001) and differences between worker and soldier castes in termites (Scharf *et al.* 2003), wing polymorphism in

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Received 25 October 2004
Accepted 4 February 2005

reproductive and sterile ant castes (Abouheif & Wray 2002), and physiological differences between alate and dealate queens in red fire ants (Tian *et al.* 2004).

Social insects exhibiting a less advanced level of sociality provide an excellent set of model systems to complement these studies. An appropriate species for investigation is the bumble-bee *Bombus terrestris*, which represents an intermediate stage between primitive and advanced sociality. Adult queens in *B. terrestris* are always larger than workers, but do not differ morphologically from them (e.g. both queens and workers have a sperm receptacle; M. J. Duchateau, personal communication). However, queens do differ physiologically and behaviourally from workers in that they accumulate glycogen and fat in their fat body, leave the nest to mate and are capable of overwintering (Michener 1974; Röseler 1991). Previous research has shown that caste membership in *B. terrestris* is determined at two stages in female larval development: if a colony queen is present and is producing a putative, caste-signalling pheromone, initially bipotential first- and second-instar larvae (i.e. larvae capable of developing into adults of either caste) are irreversibly determined towards development as workers; if such a queen is absent, larvae develop as normal queens provided that, in their fourth (last) instar, they also receive sufficient food (Röseler 1991; Cnaani & Hefetz 2001; Cnaani *et al.* 1997, 2000; Hartfelder *et al.* 2000; Bortolotti *et al.* 2001; Bourke & Ratnieks 2001; Pereboom *et al.* 2003). In addition, *B. terrestris* workers can be reproductive, since the end of the annual colony cycle (the so-called competition phase) is characterized by the emergence of dominant, reproductive (egg-laying) workers and subordinate, non-reproductive workers (Duchateau & Velthuis 1988; Bloch 1999; Bloch & Hefetz 1999). Therefore, by comparing the gene expression profiles underlying differences between adult queens and workers with those underlying differences between reproductive and non-reproductive adult workers, it is, in principle, possible to distinguish between differences owing to the presence or absence of reproduction and those owing to caste membership *per se*.

In this study, we isolated and sequenced genes whose differential expression is associated with caste determination in female *B. terrestris* larvae. Capitalizing on the specific features of *B. terrestris*, we sought to test two previously untested hypotheses. First, by investigating whether these genes are also differentially expressed between adult queens and workers and between reproductive and non-reproductive adult workers in *B. terrestris*, we tested whether the same genes underlie caste differences in larvae and adults. Second, by comparing the genes isolated in *B. terrestris* with those known to be associated with caste determination in *A. mellifera* larvae (Corona *et al.* 1999; Evans & Wheeler 1999, 2000; Hepperle & Hartfelder 2001), we tested whether the identity of relevant genes is conserved across these related taxa and whether any conserved genes share patterns of differential expression. Overall, we therefore sought to investigate whether, in the evolution of advanced sociality, genes that originally underlay reproductive differences between adults have been co-opted for use in caste determination during larval development.

2. MATERIAL AND METHODS

(a) *Sample collection*

All colonies used in the study were maintained in nest-boxes according to standard protocols (Duchateau & Velthuis 1988) in controlled-temperature rooms at the Institute of Zoology, London. We monitored colonies and the production of brood daily to evaluate colony development. Using suppression subtractive hybridization (SSH; Diatchenko *et al.* 1996) to enrich for differentially expressed genes, we created two paired subtracted cDNA libraries as follows: (i) from worker- and queen-destined larvae in the first and second instars (early-instar larvae); (ii) from worker- and queen-destined larvae in the fourth instar (last-instar larvae). Additionally, to investigate gene expression in adult phenotypes, we collected adult colony queens, and dominant, reproductive and subordinate, non-reproductive adult workers.

(i) *Larval phenotypes*

In June 2001, we raised colonies from *Bombus terrestris dalmatinus* queens obtained from stocks maintained at the Department of Behavioural Biology, University of Utrecht, and derived from a commercial supplier (Koppert Biological Systems, Berkel and Rodenrijs, the Netherlands; for stock maintenance methods see Duchateau 2000). We obtained early-instar ($n=427$) and last-instar ($n=123$) worker-destined larvae in July 2001 from 10 queenright colonies (i.e. colonies with a queen) producing worker brood only. Last-instar queen-destined larvae ($n=161$) were collected from the same 10 colonies in July and August 2001 after they had started producing queen brood. Larval instar, sex and caste were confirmed by determining body mass and by examining adults eclosing from a non-sampled brood from the same larval batches as the sampled larvae (Cnaani *et al.* 1997; Pereboom 1997). The number of non-sampled larvae (controls) remaining in the colony was always equal to or greater than the number of sampled larvae.

We removed the queen from five additional colonies on the eighth day after the start of the third brood (when a queen is still laying diploid, female eggs) in order to stimulate the development of new queens (Pereboom *et al.* 2003). We sampled early-instar queen-destined larvae ($n=90$) originating from the last batches of diploid eggs laid in the 7 days preceding the queens' removal. For the construction of library one (early instar), we only used larvae from batches that produced 100% queens in the control batch.

(ii) *Adult phenotypes*

Newly emerged queens produced by the first series of colonies in August 2001 were mated with *B. t. dalmatinus* males obtained from Koppert Biological Systems. After a period of seven months of artificial hibernation in boxes with peat dust at 0–4 °C and 80–100% relative humidity, 40 of these queens were allowed to found new colonies (see Duchateau 2000). From 15 of those colonies, we sampled the queen and the first 10 (non-reproductive) workers to eclose ($n=150$) in May 2002. We obtained dominant, laying workers ($n=91$) and subordinate, non-laying workers ($n=92$) in May and June 2002 from an additional 10 queenright colonies (9–10 workers per colony) that had entered their competition phase. Dominant workers were identified by being observed to behave aggressively (Duchateau & Velthuis 1988) within a period of 10 min, but were not removed until they were actually observed to lay eggs. In dominant workers, the rate of aggression is

sufficiently high that a worker showing no aggressive behaviour in 10 min can be confidently classified as subordinate (J. J. M. Pereboom, personal observations; Lopez-Vaamonde *et al.* 2003). All removed individuals were immediately frozen at −80 °C.

(b) *Selection of differentially expressed genes*

(i) *RNA extraction and mRNA purification*

We extracted total RNA from homogenized pooled individuals of each of the phenotypes using TRIZOL reagent (Invitrogen) followed by treatment with DNase I to remove any residual genomic DNA. For library one, we pooled approximately 30 individuals and, for library two, we pooled four to six individuals to maximize our chances of detecting differential expression of genes that may show variation between individuals in the exact timing of expression. Sampled larvae originated from two to five different colonies, and individuals were selected to represent the full range of body sizes for early and last larval instars, respectively. Messenger RNA (mRNA) was purified from total RNA using a Nucleotrap kit (BD Clontech).

(ii) *SSH and cDNA library construction*

We synthesized complementary DNA (cDNA) from 2 μg mRNA and implemented SSH performing forward and reverse subtractions following the PCR-Select cDNA subtraction protocol (BD Clontech). We then ligated the PCR-amplified products into the pCR2.1 vector (T/A cloning kit, Invitrogen) according to the manufacturer's instructions. Single recombinant colonies of *Escherichia coli* were transferred to individual wells of standard 96-well microtitre plates each holding 150 μl LB medium, containing 20% glycerol and 50 μg ml^{-1} ampicillin. Colonies were allowed to grow in the medium at 37 °C overnight, and the libraries were then stored in this form at −80 °C.

(iii) *Differential screening*

We used differential screening (Jin *et al.* 1997) to eliminate false positives (sequences falsely appearing to be differentially expressed by chance). Arrays of *E. coli* colonies derived from libraries were spotted onto duplicate nylon membranes (Hybond NX, Amersham) and cultured on LB agar medium (containing 50 μg ml^{-1} ampicillin) at 37 °C overnight. Colonies were denatured (0.5 M NaOH, 1.5 M NaCl) and neutralized (0.5 M Tris–HCl (pH 7.4), 1.5 M NaCl), and DNA was fixed to the membrane by baking at 80 °C.

Hybridization probes for differential screening of libraries were made from subtracted cDNA populations derived from SSH. Each part of a paired subtraction was radio-labelled separately by incorporation of (α-32P)-dATP using random priming (Random Primed DNA Labeling Kit, Roche). All prehybridizations, hybridizations and washes were carried out at 65 °C in glass bottles in a rotating incubator. Membranes were pre-hybridized for 30 min in Church buffer and, after addition of the denatured probe, hybridized overnight (Sambrook & Russell 2001). After hybridization, membranes were washed four times for 3 min in low-stringency wash buffer (2× SSC, 0.1% SDS), four times for 5 min in 1× SSC, 0.1% SDS wash buffer, and four times for 5 min in high-stringency wash buffer (0.1× SSC, 0.1% SDS). Membranes were wrapped in plastic and exposed to X-ray film (Kodak BioMax MS) with intensifying screens for 3–72 h, depending on the strength of the signal. Membranes were stripped of probe by washing in 0.1% SDS at 95 °C. Each duplicate membrane was hybridized in turn with each probe to control for possible differences in *E. coli* colony growth on the duplicates.

(iv) *Sequencing and identification of selected sequences*

We PCR-amplified candidate clones (selected by differential screening) using M13-forward and -reverse primers and sequenced both strands on an ABI-Prism 377 semi-automated sequencer. DNA sequence identification was achieved by comparing nucleotide and deduced amino acid sequences with published sequences of other organisms by means of homology searches of major databases (GenBank, EMBL, Swiss-Prot) using the Blast-X and Blast-N algorithms (Altschul *et al.* 1997). We set a cut-off for significant sequence homology at a probability score (expectation value or *E*-value, describing the probability of finding homologues by chance) of less than 10^{-3}.

(c) *Confirmation of differential expression*

We isolated total RNA from pooled individuals of queen or worker phenotypes at different life-history stages (10 queen- and 10 worker-destined early-instar larvae; 3 queen- and 6 worker-destined last-instar larvae; 2 adult colony queens and 10 adult non-reproductive workers; 10 adult reproductive workers and 10 adult non-reproductive workers), with the larval phenotypes from the same set of colonies used for the construction of the corresponding subtracted libraries. For each phenotype we denatured and loaded 10 μg of total RNA on a 1.5% denaturing agarose gel (Sambrook & Russell 2001). After electrophoresis, samples were visualized by ethidium bromide staining, and gel images recorded using a CCD camera (Syngene GeneGenius system). RNA was transferred to nylon membrane by Northern blotting (Sambrook & Russell 2001) and fixed by baking at 80 °C.

We prepared Northern blots bearing RNA from each of the eight phenotypes. These were probed in turn with 17 unique sequences selected from the candidates isolated by differential screening that were successfully sequenced as described in the results. Hybridization probes were PCR-amplified from selected clones using M13-forward and -reverse primers. After agarose gel electrophoresis, probes were extracted from the gel using 'QIAquick' gel extraction (Qiagen) and radio-labelled with (α-32P)-dATP by random priming. Hybridization and washing conditions and exposure to X-ray film were as described above. Scanned images of the RNA gels and the autoradiographs were analysed on a Macintosh computer using the public domain software Nih Image (http://rsb.info.nih.gov/nih-image/). We calculated the relative optical density (OD) of the autoradiograph signals from pairs of phenotypes using gel plotting macros. These values were then normalized in proportion to the OD of the mRNA fluorescence on the RNA gel image to control for differences in RNA loading. We considered a minimum twofold difference (arbitrarily chosen) in OD between pairs of phenotypes as a criterion for differential expression.

(d) *Data deposition*

Differentially expressed sequences have been deposited in GenBank (dbEST) under accession numbers DN048368 (dbEST Id. 27769598) to DN048383 (dbEST Id. 27769613).

3. RESULTS

(a) *Confirmation of caste of sampled larvae*

For the construction of the two cDNA libraries, the sex and caste of adults developing from non-sampled larvae confirmed the presumed sex and caste of the sampled larvae. The 10 queenright colonies providing early- ($n=427$) and last-instar ($n=123$) worker-destined larvae for RNA-extraction subsequently produced only workers ($n\geq427$ and $n\geq123$, respectively) from the control batches as expected. In the queen production stage, the same 10 colonies produced only queens ($n\geq161$) from the control batches taken from the same groups of larvae from which last-instar queen-destined larvae ($n=161$) had been sampled. The body mass of a representative sample (including smallest and largest individuals) of the sampled last-instar worker- and queen-destined larvae was 273.3 ± 61.4 mg ($n=30$) and 867.9 ± 183.3 mg ($n=30$), respectively, which confirms their caste membership (in the last instar, worker-destined larvae weigh 70–380 mg and queen-destined larvae weigh 250–1300 mg; Cnaani *et al.* 1997; Pereboom 1997). In the five queenless colonies, all unsampled early-instar larvae ($n=202$) from the same batches as the sampled queen-destined larvae ($n=90$) developed as queens.

(b) *Differential screening and sequence identification*

We successfully constructed both parts of library one, but differential screening and sequencing suggested SSH was unsuccessful for the worker part of library two, despite our repeating the SSH procedure twice. Library one thus consisted of 960 clones and library two (queen part) of 480 clones. We screened all clones from both libraries using differential screening (figure 1) and selected 100 candidate clones (at random from those clones showing the strongest differential hybridization) from library one and 50 candidate clones (on the same basis) from library two (queen part) to be sequenced. Of 129 successfully sequenced clones, 37 (28.7%) showed no significant homology to any known DNA or deduced amino acid sequences. Some of the remaining 92 sequences were abundant, with multiple clones present in the subtracted libraries (e.g. hexamerin, 10/92 clones; CG-5520 protein, 9/92 clones; chymotrypsin, 11/92 clones).

(c) *Northern blots: patterns of expression*

Sixteen sequences of the 129 successfully sequenced clones were selected for probing the Northern blots (table 1). The selected sequences were chosen to include: (i) all sequences ($n=4$) found to show significant homology to genes known to be differentially expressed across castes in *A. mellifera*, (ii) a set ($n=9$) of sequences with significant homology to non-*Apis* genes, and (iii) a set ($n=3$) of sequences with no known homologues. From the Northern blots, we found that four sequences showed no detectable expression and 12 sequences exhibited differences in expression between queen- and worker-destined larvae, including five sequences exhibiting differences in expression of molecular size variants (table 1; figure 2). Such variants may represent multiple loci encoding similar protein products, or they may be the result of alternative splicing of the mRNA from a single locus (Boue *et al.* 2003). Results of the Northern blots were not always consistent with results from differential screening.

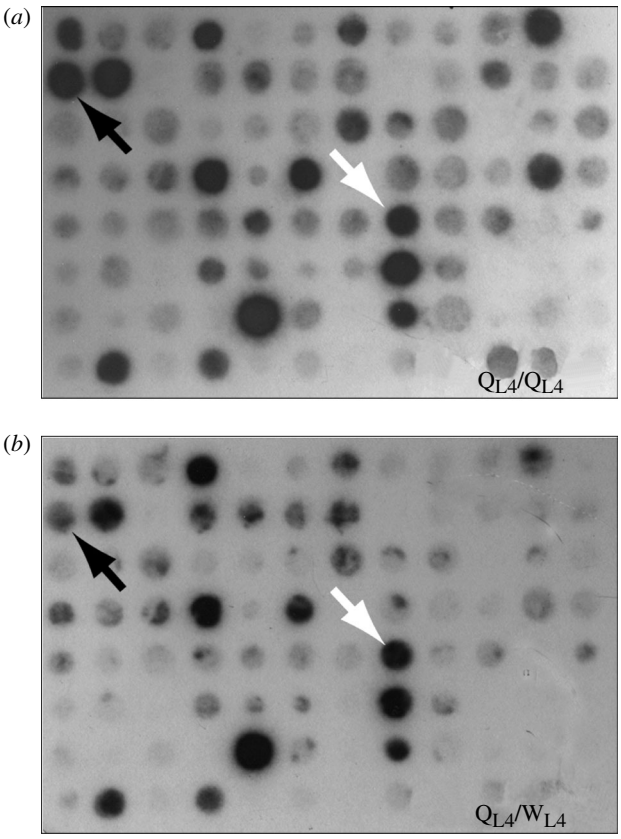

Figure 1. Array of 96 potentially differentially expressed clones from the last-instar queen library (library two, Q_{L4}), first probed with cDNA from last-instar queen-destined larvae (*a*; Q_{L4}), and stripped and re-probed with cDNA from last-instar worker-destined larvae (*b*; W_{L4}). The black arrow indicates a clone expressed at a higher level in last-instar queen-destined larvae compared with worker-destined larvae (i.e. stronger signal after hybridization to queen cDNA). A white arrow indicate clones expressed equally in worker- and queen-destined larvae (i.e. false positives).

The 16 candidate sequences had been selected based on displaying relatively higher levels of expression in early-instar queen-destined larvae ($n=6$), early-instar worker-destined larvae ($n=6$) or last-instar queen-destined larvae ($n=4$). However, in the Northern blots, 9 sequences showed a higher level of expression in early-instar queen-destined larvae, and 12 showed a higher level of expression in last-instar worker-destined larvae. The reasons for these discrepancies are unknown, but similar discrepancies have been recorded previously (Cao *et al.* 2004). They do not affect our conclusions because results from Northern blots provided clear evidence of differential expression.

In the early instars, most sequences (9/12) displayed relatively higher levels ($\geq$twofold difference) of expression in queen-destined larvae than in worker-destined larvae, but, in the last instar, all 12 sequences were expressed at higher levels in worker-destined larvae than in queen-destined larvae (with the exception of the second band in clone no. Q_{L1} 39; table 1). In those cases where sequences were expressed in both larvae and adults (8/12), four displayed higher levels of expression in adult workers compared with queens (Cytochrome Oxidase I, Hypothetical Protein, ATP-synthase beta chain, and the unknown sequence from clone no. Q_{L4} 14; table 1) and one sequence

Table 1. Relative expression levels (A versus B, i.e. phenotype A versus phenotype B) measured as the optical density of autoradiograph signals of one phenotype (X_A), divided by the sum of the optical densities of signals of both phenotypes (X_A+X_B). (1.00, Expressed in phenotype A only; 0.67 or >0.67, expressed two or more times more highly in phenotype A than in phenotype B; 0.5, no differential expression; 0.33 or <0.33, expressed two or more times more highly in phenotype B than in phenotype A; 0.00, expressed in phenotype B only (twofold or greater differences are printed in bold type). *, no signal detected in either phenotype. ‡, Northern analysis was performed in duplicate, using separate blots. Expression was assayed in early-instar queen- and worker-destined larvae (Q_{L1} and W_{L1}), in last-instar queen- and worker-destined larvae (Q_{L4} and W_{L4}), in adult colony queens and non-reproductive adult workers (Q_{adult} and W_{adult}) and in reproductive and non-reproductive adult workers (W_{rep} and $W_{non\text{-}rep}$. *First column*: clone numbers refer to the subtracted library from which the clone was retrieved. Italics indicate clones for which Northern results were inconsistent with the subtracted library from which they were captured. *Second column*: GenBank accession number and dbEST Id (between brackets) of the submitted sequences. *Third column*: sequences are named according to results of BLAST homology searches; the GenBank accession number and *E*-values of the closest hit are provided between brackets (*E*-values express the probability of finding homologues by chance; significance at $E<10^{-3}$). Sequences with no significant homologues are referred to as 'unknown sequence'. Gene functional group classification is based on the gene ontology classification used in Adams *et al.* (2000). A number of phenotypes produced multi-banded patterns as indicated.)

clone number	GenBank acc. no. (dbEST Id.)	sequence similarity (GenBank acc. no.; *E*-value)	functional group		larval phenotypes		adult phenotypes	
					Q_{L1} versus W_{L1}	Q_{L4} versus W_{L4}	Q_{adult} versus W_{adult}	W_{rep} versus $W_{non\text{-}rep}$
W_{L1} 79	DN048368 (27769598)	larval cuticle protein‡ (O02387; $E=3\times10^{-11}$)	structural protein		**0.68**	**0.09**	*	*
Q_{L4} 20	DN048376 (27769606)	hexamerin‡ (AY601637; $E=5\times10^{-53}$)	storage protein	band 1	*	**0.02**	*	*
				band 2	*	**0.24**	*	*
Q_{L1} 39	DN048371 (27769601)	unknown sequence		band 1	*	**0.21**	*	*
				band 2	*	**0.86**	*	*
Q_{L1} 93	DN048374 (27769604)	unknown sequence		band 1	**0.92**	**0.02**	*	*
				band 2	*	**0.00**	*	*
W_{L1} 21	DN048379 (27769609)	60-S ribosomal protein (AY072286; $E=5\times10^{-14}$)	protein synthesis	band 1	**0.97**	**0.00**	*	*
				band 2	**0.80**	**0.11**	0.35	0.66
W_{L1} 28	DN048380 (27769610)	chymotrypsin‡ (P00768 $E=4\times10^{-31}$)	enzyme		**0.97**	**0.01**	0.46	**0.27**
W_{L1} 67	DN048381 (27769611)	cytochrome oxidase I‡ (AY181169; $E=2\times10^{-32}$)	electron transport		**0.94**	**0.11**	**0.33**	**0.14**
W_{L1} 23	DN048378 (27769608)	hypothetical protein (AABL0106743; $E=7\times10^{-11}$)	unknown		**0.89**	**0.00**	**0.00**	0.38
W_{L1} 68	DN048382 (27769612)	peroxiredoxin (AY438331; $E=3\times10^{-34}$)	enzyme		**0.92**	**0.25**	**0.69**	**0.11**
Q_{L4} 34	DN048377 (27769607)	fatty acyl CoA-desaturase (AF417841; $E=5\times10^{-44}$)	enzyme		**0.87**	**0.00**	0.45	0.55
Q_{L4} 18	DN048375 (27769605)	ATP synthase beta subunit (AY580209; $E=2\times10^{-93}$)	electron transport		**0.92**	**0.00**	**0.29**	0.53
Q_{L4} 14	DN048383 (27769613)	unknown sequence		band 1	0.65	**0.20**	**0.14**	0.46
				band 2	*	*	**0.00**	*
				band 3	*	*	**0.00**	*
Q_{L1} 42	DN048370 (27769600)	imaginal disk growth factor (Q9W303; $E=3\times10^{-30}$)	enzyme		*	*	*	*
Q_{L1} 32	DN048369 (27769599)	super oxide dismutase 1 (AY4624198; $E=4\times10^{-20}$)	enzyme		*	*	*	*
Q_{L1} 40	DN048372 (27769602)	CG 5520 protein (AE003763; $E=3\times10^{-4}$)	signal transduction		*	*	*	*
Q_{L1} 43	DN048373 (27769603)	CG31605-PA (AE003619; $E=1\times10^{-13}$)	immunity		*	*	*	*

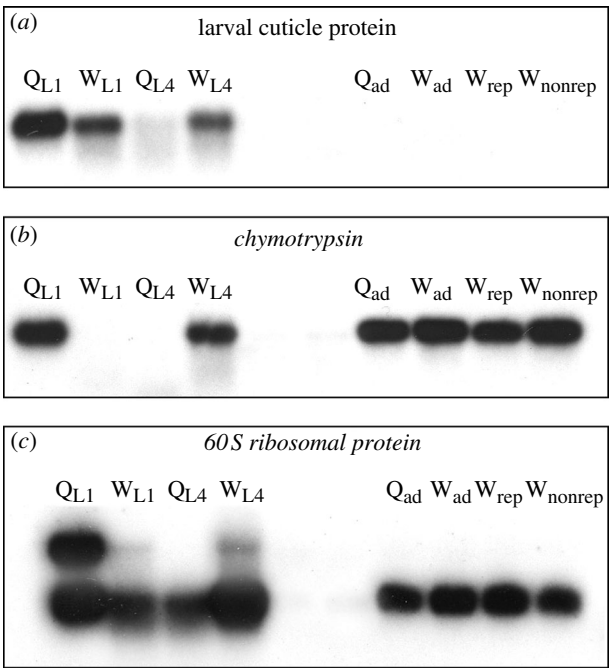

Figure 2. Autoradiographs of three representative Northern blot results. (*a*) Larval cuticle protein was upregulated in early-instar queen-destined larvae (Q_{L1}) and in last-instar worker-destined larvae (W_{L4}), but showed no expression in adult phenotypes (Q_{ad} (Q_{adult} in table 1) versus $W_{non\text{-}rep}$). (*b*) Chymotrypsin was exclusively expressed in early-instar queen-destined larvae and last-instar worker-destined larva, and showed expression at approximately similar levels in all adult phenotypes. (*c*) 60S Ribosomal protein showed multiple bands in early-instar queen-destined larvae (Q_{L1}), early-instar worker-destined larvae (W_{L1}) and last-instar worker-destined larvae (W_{L4}). Relative levels of expression are given in table 1, where all additional abbreviations are defined.

(Peroxiredoxin) displayed higher levels of expression in adult queens compared with workers. Three sequences (Chymotrypsin, Cytochrome Oxidase I and Peroxiredoxin) were expressed at higher levels in non-reproductive workers compared with reproductive workers.

The genes associated with caste membership and/or being reproductive (adult queens versus non-reproductive workers) and being reproductive alone (reproductive versus non-reproductive adult workers) tended not to share patterns of differential expression (table 1). Those genes showing higher levels of expression in adult workers versus queens, but no differences in expression between reproductive and non-reproductive workers (Hypothetical Protein, ATP-synthase beta subunit, and unknown sequence Q_{L4} 14) are presumably associated with membership of the worker caste only. The only gene suggesting an association with the absence of reproduction is Cytochrome Oxidase I, being upregulated in adult workers (versus queens) and in non-reproductive workers (versus reproductive workers). No genes appeared to be associated with the presence of reproduction alone.

All four genes (Hexamerin, Larval Cuticle protein, ATP-synthase beta subunit and Cytochrome Oxidase I) previously known to be differentially expressed between queen- and worker-destined larvae of the honeybee, *A. mellifera* (Corona *et al.* 1999; Evans & Wheeler 1999, 2000), and identified as candidates for differential expression in *B. terrestris* by differential screening, were confirmed to be differentially expressed in *B. terrestris* larvae using Northern blots. Hexamerin shared the same expression pattern in *A. mellifera* and *B. terrestris*, being upregulated in last-instar worker-destined larvae in both species. However, the remaining three sequences were expressed at higher levels in last-instar queen-destined *A. mellifera* larvae, but in *B. terrestris* they were expressed at higher levels in last-instar worker-destined larvae. In *B. terrestris*, these genes were also expressed at higher levels in early-instar queen-destined larvae.

4. DISCUSSION

Using SSH, we isolated 12 genes whose differential expression is associated with caste determination and differentiation of queen- and worker-destined bumble-bee (*B. terrestris*) larvae. Differential expression was confirmed in both the early instars and the last instar, consistent with previous research that demonstrates these instars to be the sensitive periods for queen–worker caste determination in *B. terrestris* (see §1). The sets of genes associated with caste-related expression in larvae tended not to be differentially expressed between adult queens and workers (7/12), or between reproductive and non-reproductive workers (9/12). By comparing both adult queens with workers, and reproductive with non-reproductive adult workers, we were able to discriminate one gene associated with the absence of reproduction, and three genes associated with membership of the worker caste. Sequence analysis showed that four sequences differentially expressed in queen- and worker-destined *Bombus* larvae are differentially expressed in queen- and worker-destined honeybee (*A. mellifera*) larvae, although the expression pattern of these genes was not always the same. An unexpected finding in *B. terrestris* was that, in queen-destined larvae, genes expressed in early instars were downregulated in the last instar, whereas, in worker-destined larvae, the same genes were not expressed in early instars and were upregulated in the last instar (e.g. Chymotrypsin; figure 2*b*). Overall, the differentially expressed genes isolated in *B. terrestris* did not appear to fall preferentially into any one functional group (table 1). In addition, the underlying reasons for the observed expression differences in specific genes were not obvious, and therefore they remain to be determined. For example, although early-instar queen-destined larvae exhibited higher expression of the Larval Cuticle protein gene than early-instar worker-destined larvae (table 1), this could not be attributed to differences in the growth rates of these instars, which are absent (Cnaani *et al.* 1997).

In this study, the first hypothesis we sought to test was whether genes differentially expressed between queen- and worker-destined larvae are also differentially expressed between adult queens and workers, and between reproductive and non-reproductive adult workers. Half (6/12) of the genes we identified as associated with caste-determining processes in larvae were not expressed differentially or not expressed at detectable levels in adult queen and worker phenotypes. This suggests that, in *B. terrestris*, different genes often underlie caste differences in larvae and adults. Although larval and adult phenotypes would be expected not to share the differential expression of some genes

(e.g. genes associated with ovary activation in adults), it is conceivable that some genes (e.g. regulatory genes associated with the switch to development as one phenotype or another) do share patterns of differential expression across larvae and adults. Our results suggest that the latter class of genes do not represent the majority.

The second hypothesis we sought to test was whether genes associated with caste determination in *B. terrestris* larvae are identical with those known to be associated with caste determination in *A. mellifera* larvae (Corona *et al.* 1999; Evans & Wheeler 1999, 2000; Hepperle & Hartfelder 2001) and whether such genes share patterns of differential expression. We found four genes to be differentially expressed in queen- and worker-destined larvae of both *B. terrestris* and *A. mellifera*. This occurred even though recent molecular evidence suggests independent origins of eusociality in *Apis* and *Bombus* (Cameron & Mardulyn 2001). However, the precise pattern of differential gene expression was not identical. First, three of the four genes upregulated in last-instar worker-destined *B. terrestris* larvae were upregulated in last-instar queen-destined *A. mellifera* larvae. Second, in *A. mellifera*, last-instar queen-destined larvae downregulate many of the genes expressed by young bipotential larvae, but last-instar worker-destined larvae show similar expression profiles to those of young bipotential larvae (Evans & Wheeler 2000). In *B. terrestris*, however, genes expressed in early-instar queen-destined larvae are downregulated in early-instar worker-destined larvae. Third, in contrast to *B. terrestris* larvae, *A. mellifera* larvae express a distinct set of worker- or queen-related genes late in development (Evans & Wheeler 2000).

An unanticipated result of this study was our finding that genes differentially expressed in larvae were upregulated in queen-destined larvae early in development and upregulated in worker-destined larvae late in development. Explaining this novel pattern of gene expression remains a challenge for future studies. Together with the contrasting pattern of differential gene expression in *B. terrestris* and *A. mellifera*, it suggests that caste determination in *B. terrestris* involves a difference not so much in the type of genes expressed by queen- and worker-destined larvae, but primarily in the relative timing of their expression during development. This conclusion is of potential importance in the further study of caste evolution and, hence, of the evolution of other forms of phenotypic diversification via differential gene expression. In *Bombus*, comparative studies encompassing both species that lack a sensitive period of caste determination in the early larval instars (e.g. *Bombus hypnorum*; Röseler 1991), and the obligate social parasites (formerly *Psithyrus* species) that lack a worker caste altogether (Alford 1975), as well as experimental manipulations of gene expression using RNAi techniques (e.g. Beye *et al.* 2002), should prove fruitful for advancing still further our understanding of how differential gene expression underpins caste determination.

We thank Marie José Duchateau for providing bumble-bee stocks, Jay Evans for helpful advice and encouragement at the start of this project, Sam Martin for advice on SSH, and two anonymous referees for commenting on an earlier version of this paper. The Leverhulme Trust provided financial support for this work through a research grant to A.F.G.B., W.C.J. and R.L.H.

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