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 2002a). 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 & Malezska 2002; Robinson 2002b; 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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reproductive and sterile ant castes (Abouheif & Wray 2002), and physiological differences between alate and deplete 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; Rösele 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 (Rösele 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; Dianenko 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 (Sambrook & Russell 2001). After hybridization, membranes were pre-hybridized for 30 min in Church buffer and, after addition of the denatured probe, hybridized overnight (Sambrook & Russell 2001). Hybridization probes for differential screening of libraries were 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 = 427 and n = 123, respectively) from the control batches as expected. In the queen production stage, the same 10 colonies produced only queens (n = 161) 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 (Pereboom et al. 1997; Cnaani et al. 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, QL4), first probed with cDNA from last-instar queen-destined larvae (QL4), and stripped and re-probed with cDNA from last-instar worker-destined larvae (WL4). 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 indicates 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 (≥ 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. QL4 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. QL4 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 autoradiographs signals of one phenotype (Xₐ), divided by the sum of the optical densities of signals of both phenotypes (Xₐ + Xₐ).  
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 (Q₁ and W₁), in last-instar queen- and worker-destined larvae (Q₄ and W₄), in adult colony queens and non-reproductive adult workers (Qadult and Wadult) and in reproductive and non-reproductive adult workers (Wexp and Wnon-exp).  

<table>
<thead>
<tr>
<th>Clone number</th>
<th>GenBank acc. no. (dbEST Id.)</th>
<th>Sequence similarity (GenBank acc. no.; E-value)</th>
<th>Functional group</th>
<th>Larval phenotypes (Q₁ versus Q₄)</th>
<th>Adult phenotypes (Wexp versus Wnon-exp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W₁, 79</td>
<td>DN048368 (27769598)</td>
<td>Larval cuticle protein† (O02387; E = 3×10⁻¹¹)</td>
<td>Structural protein</td>
<td>0.68</td>
<td>Q₁ versus Q₄ 0.09 versus W₁</td>
</tr>
<tr>
<td>Q₄, 20</td>
<td>DN048376 (27769606)</td>
<td>Hexamerin‡ (AY601637; E = 5×10⁻⁵²)</td>
<td>Storage protein</td>
<td>Band 1: 0.02</td>
<td>Q₄ versus Q₁ 0.21 versus W₄</td>
</tr>
<tr>
<td>Q₁, 39</td>
<td>DN048371 (27769601)</td>
<td>Unknown sequence</td>
<td>Band 2: 0.24</td>
<td></td>
<td>Q₁ versus Q₄ 0.24 versus W₁</td>
</tr>
<tr>
<td>Q₁, 93</td>
<td>DN048374 (27769604)</td>
<td>Unknown sequence</td>
<td>Band 2: 0.86</td>
<td></td>
<td>Q₁ versus Q₄ 0.86 versus W₁</td>
</tr>
<tr>
<td>W₁, 21</td>
<td>DN048379 (27769609)</td>
<td>60-S ribosomal protein (AY072286; E = 5×10⁻¹⁴)</td>
<td>Protein synthesis</td>
<td>Band 1: 0.97</td>
<td>W₁ versus Q₁ 0.90 versus W₁</td>
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<tr>
<td>W₁, 28</td>
<td>DN048380 (27769610)</td>
<td>Chymotrypsin‡</td>
<td>Enzyme</td>
<td>Band 2: 0.80</td>
<td>W₁ versus W₁ 0.35 versus W₁</td>
</tr>
<tr>
<td>W₁, 67</td>
<td>DN048381 (27769611)</td>
<td>Cytochrome oxidase I† (AY181169; E = 2×10⁻³²)</td>
<td>Electron transport</td>
<td>0.94</td>
<td>W₁ versus W₁ 0.33 versus W₁</td>
</tr>
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<td>W₁, 23</td>
<td>DN048378 (27769608)</td>
<td>Hypothetical protein (AAE010645; E = 7×10⁻¹¹)</td>
<td>Unknown</td>
<td>0.89</td>
<td>W₁ versus W₁ 0.00 versus W₁</td>
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<td>W₁, 68</td>
<td>DN048382 (27769612)</td>
<td>Peroxiredoxin</td>
<td>Enzyme</td>
<td>0.92</td>
<td>W₁ versus W₁ 0.69 versus W₁</td>
</tr>
<tr>
<td>Q₄, 34</td>
<td>DN048377 (27769607)</td>
<td>Fatty acetyl CoA-desaturase (AF417841; E = 5×10⁻⁴⁴)</td>
<td>Enzyme</td>
<td>0.87</td>
<td>W₁ versus W₁ 0.45 versus W₁</td>
</tr>
<tr>
<td>Q₄, 18</td>
<td>DN048375 (27769605)</td>
<td>ATP synthase beta subunit (AY580209; E = 2×10⁻⁵³)</td>
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<td>0.92</td>
<td>W₁ versus W₁ 0.29 versus W₁</td>
</tr>
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<td>Unknown sequence</td>
<td>Enzyme</td>
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<td>W₁ versus W₁ 0.14 versus W₁</td>
</tr>
<tr>
<td>Q₁, 42</td>
<td>DN048370 (27769600)</td>
<td>Imaginal disk growth factor (Q9W303; E = 3×10⁻³⁶)</td>
<td>Unknown</td>
<td>0.20</td>
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</tr>
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<td>DN048369 (27769599)</td>
<td>Super oxide dismutase 1 (AY4624198; E = 4×10⁻²⁶)</td>
<td>Enzyme</td>
<td>0.14</td>
<td>W₁ versus W₁ 0.00 versus W₁</td>
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<td>Q₁, 40</td>
<td>DN048372 (27769602)</td>
<td>CG 5520 protein (AE003763; E = 3×10⁻⁴⁵)</td>
<td>Signal transduction</td>
<td>0.46</td>
<td>W₁ versus W₁ 0.46 versus W₁</td>
</tr>
<tr>
<td>Q₁, 43</td>
<td>DN048373 (27769603)</td>
<td>CG31605-PA (AE003619; E = 1×10⁻¹³)</td>
<td>Immunity</td>
<td></td>
<td>W₁ versus W₁ 0.14 versus W₁</td>
</tr>
</tbody>
</table>

early-instar worker-destined larvae (WL1) and last-instar (and showed expression at approximately similar levels in all queen-destined larvae and last-instar worker-destined larvae, multiple bands in early-instar queen-destined larvae (QL1), worker-destined larvae (WL4), but showed no expression in A. mellifera queen- and worker-destined larvae of the honeybee, previously known to be differentially expressed between ATP-synthase beta subunit and Cytochrome Oxidase I)

associated with the presence of reproduction alone. The only gene suggesting membership of the worker caste only. The only gene 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 QL4 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 2b). 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*; Rösel 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.

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**REFERENCES**


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