The Journal of Experimental Biology 207, 3281-3288
Published by The Company of Biologists 2004
doi:10.1242/jeb.01151

Phenotypic deconstruction reveals involvement of manganese transporter *malvolio* in honey bee division of labor

Yehuda Ben-Shahar[1,*], Nichole L. Dudek[1,†] and Gene E. Robinson[1,2]

[1]*Department of Entomology* and [2]*Neuroscience Program, University of Illinois at Urbana-Champaign, 320 Morrill Hall, 505 S. Goodwin Avenue, Urbana, IL 61801, USA*

*Author for correspondence at present address: Howard Hughes Medical Institute, 500 EMRB, University of Iowa College of Medicine, Iowa City, IA 52242, USA (e-mail: yehuda-ben-shahar@uiowa.edu)

†Present address: Department of Pharmacology, Loyola University Medical Center, 2160 South First Avenue, Maywood, IL 60153, USA

Accepted 21 June 2004

Summary

Molecular analysis of a complex behavioral phenotype is facilitated by dissecting it into simpler behavioral components. Using this approach, we present evidence implicating increased manganese transport by the *malvolio* (*mvl*) gene into brain cells as one factor that influences age-related division of labor in honey bee colonies. We studied *mvl* because manganese affects sucrose responsiveness in *Drosophila melanogaster*, and sucrose responsiveness is related to division of labor in honey bee colonies. Honey bee foragers are more responsive to sucrose in the laboratory than are younger nurse bees, and pollen foragers are more responsive to sucrose than nectar foragers. Levels of *mvl* mRNA in the brain and manganese in the head were higher in pollen foragers compared with nurses, with nectar foragers intermediate. Manganese treatment increased honey bee sucrose responsiveness and caused precocious foraging. Manganese levels showed a similar pattern to *mvl* mRNA but manganese treatment did not increase pollen foraging. These results suggest that, while there are molecular pathways common to sucrose responsiveness and division of labor, linkages between a complex behavior and some of its simpler behavioral components are not obligatory. Together with previous findings, these results support the idea that some feeding-related genes in *Drosophila* have been used in social evolution to regulate division of labor.

Key words: *mvl*, *Apis mellifera*, foraging, sucrose response threshold, Hymenoptera.

Introduction

Honey bees exhibit an age-related division of labor (Robinson, 2002). Bees perform several different behaviors in the hive during the first 2–3 weeks of adult life, including brood care (‘nursing’), and then shift to foraging mostly for nectar and pollen outside the hive for the remainder of their 5–7-week life. Like other forms of behavioral maturation such as social dominance and sexual behavior in vertebrates (Becker et al., 1992), the regulation of the transition from working in the hive to foraging in honey bees involves changes in brain chemistry, brain structure, endocrine activity and gene expression (Robinson, 2002). The transition to foraging is also dependent on the environment and can be accelerated, delayed or even reversed depending on the needs of the colony (Robinson, 2002). Microarray analysis has revealed that many genes in the brain show changes in expression levels in association with this behavioral transition (Whitfield et al., 2003), suggesting that numerous molecular pathways might be involved in the regulation of division of labor. It is important to explore methods that might help focus on pathways that are more relevant than others.

One way to facilitate the molecular analysis of a complex behavioral phenotype such as honey bee division of labor is to dissect it into simpler behavioral components. For example, understanding the molecular mechanisms underlying schizophrenia and other mental illnesses is simplified by attempting to identify symptoms that are thought to represent components of the disease that can each be studied independently; each of these symptoms is an ‘endophenotype’ of the whole disease (Leboyer et al., 1998). Using such an approach, we report on experiments that implicate increased manganese transport by *malvolio* (*mvl*) into brain cells as one factor influencing division of labor in honey bee colonies. *mvl* was discovered in *Drosophila melanogaster* when screening for genes that affect responsiveness to sucrose (Rodrigues et al., 1995); it was later found to encode a manganese transmembrane transporter (Orgad et al., 1998; Supek et al., 1996). Flies with mutations at the *mvl* locus showed reduced responsiveness to sucrose, a deficit that was fully rescued by oral treatment with manganese (Orgad et al., 1998). Manganese toxicity is known to negatively affect neural function, but the role of manganese in natural neural and behavioral plasticity is poorly understood (Takeda, 2003; Takeda et al., 2002, 2003; Verity, 1999).

We studied *mvl* and manganese transport for two reasons. First, *mvl* and manganese influence responsiveness to sucrose in *Drosophila*, and changes in sucrose responsiveness appear to be a component of honey bee division of labor. In honey bees, there are genotypic and phenotypic correlations between variation in sucrose responsiveness and two aspects of division of labor, the age at onset of foraging and the tendency to forage for either pollen or nectar (Pankiw and Page, 1999). Responsiveness to sucrose increases with age and is highest in foragers. In addition, foragers that specialize on collecting pollen show higher sucrose responsiveness than do nectar foragers. Bees selected for increased pollen collection also show increased sucrose responsiveness and an earlier age at onset of foraging (Page et al., 1998). African honey bees (*Apis mellifera scutellata* L.) also show increased pollen collection, increased responsiveness to sucrose and an earlier age at onset of foraging relative to bees derived from a mixture of subspecies that originated in Europe (Pankiw, 2003). The causal relationships between sucrose responsiveness and these two aspects of division of labor are not understood, but the evidence for their association is extensive.

The second reason we studied *mvl* is because an ortholog of another gene involved in *Drosophila* feeding behavior, *foraging* (*Amfor*), is also involved in honey bee behavioral maturation (Ben-Shahar et al., 2002a,b). Although the two genes have no known functional relationship, we wished to explore further the idea (Ben-Shahar et al., 2002b) that some feeding-related genes in *Drosophila* have been used in social evolution to regulate division of labor. Our goal was to determine whether another gene that influences *Drosophila* feeding behavior is also involved in honey bee division of labor, albeit in association with a different behavioral component, sucrose responsiveness. *mvl* has no known genetic or molecular connection to PKG (cGMP-dependent protein kinase)-related pathways.

We tested the hypothesis that honey bee behavioral maturation is associated with an increase in brain *mvl* expression, with foragers having higher levels of *mvl* mRNA and manganese than nurses. We used manganese treatment experiments to gain insight into whether *mvl* activity can result in increased sucrose responsiveness, precocious foraging and increased pollen foraging. We also performed similar experiments with cGMP (cyclic guanosine monophosphate), a previously identified activator of foraging behavior (Ben-Shahar et al., 2002b), because it was not known whether this treatment also affects sucrose responsiveness and pollen foraging. A fly genotype with higher PKG activity shows increased sucrose responsiveness (Scheiner et al., 2004), but treatment experiments with cGMP have not been performed.

Materials and methods

Honey bees

All bees (*Apis mellifera* L.) were maintained according to standard beekeeping techniques at the University of Illinois Bee Research Facility. One-day-old bees were used to set up experimental colonies and as subjects for treatments. They were obtained by removing honeycomb frames containing pupae from large field colonies (derived from naturally mated queens) and placing them in an incubator (33°C, 95% humidity). Bees that emerged over a 24-h period were also marked with a spot of paint (Testor's PLA) on the thorax and used as described below.

Bees used to measure brain mRNA and head manganese levels were collected from either triple-cohort colonies (Ben-Shahar and Robinson, 2001) or single-cohort colonies. Triple-cohort colonies were used to study typical patterns of behavioral development, with foragers older than nurse bees. They were established by sequentially introducing three cohorts of 800–1000 one-day-old bees to a small hive at one-week intervals. Each colony was also given two frames of honeycomb for food storage and brood rearing and an unrelated, naturally mated, queen. Nurse bees were identified as one-week-old bees that inserted their heads into honeycomb cells containing larvae, and foragers as bees older than three weeks of age returning to the hive with either clearly visible pollen loads on their hind legs or distended abdomens (bearing either nectar or water).

Single-cohort colonies were used to uncouple behavioral status and chronological age. They were established with one cohort of 800–1000 one-day-old bees; because these colonies initially contain no old bees, some colony members initiate foraging as much as two weeks earlier than usual, enabling us to sample precocious foragers and normal age nurses, all 5–9 days of age.

Treatments

Groups of 50 one-day-old bees were placed in a 6×12×18 cm wooden cage placed in an incubator (33°C, 95% relative humidity) for 4 days. Bees were treated orally with a 50% sucrose solution containing either 20 mmol l^{-1} $MnCl_2$, 100 mmol l^{-1} $ZnCl_2$, 20 mmol l^{-1} $MnCl_2$ + 100 mmol l^{-1} $ZnCl_2$, 500 mmol l^{-1} 8-Br-cGMP or sucrose alone as a control (all compounds from Sigma, St Louis, MO, USA). Zinc treatments were used because zinc is a known antagonist of *malvolio* and antagonizes the behavioral effects of manganese in *Drosophila*, possibly by inhibiting its uptake by *malvolio* (Orgad et al., 1998). 8-Br-cGMP was previously shown (Ben-Shahar et al., 2002b) to cause precocious foraging, but effects on sucrose responsiveness were not examined. Oral treatment was used for two reasons. First, this was the method used to rescue the *mvl* mutant effect in *Drosophila* (Orgad et al., 1998). Second, this non-invasive method works well for treating bees that are placed in colonies in the field to determine effects on age at onset of foraging (Ben-Shahar et al., 2002b). Solutions were made fresh daily.

Sucrose responsiveness

After 4 days of treatment, caged bees from each treatment group were cold-anesthetized and placed in individual restrainers for use in a sucrose response assay (Ben-Shahar and Robinson, 2001). Bees were tested in a sequential series

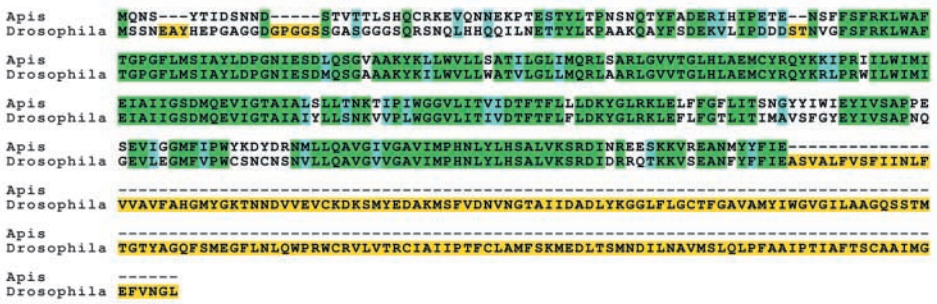

Fig. 1. Alignment of the *D. melanogaster malvolio* sequence with the putative *A. mellifera* ortholog (partial sequence). The two protein sequences are more than 80% similar for the sequence shown.

of increasing sucrose concentration: 0, 0.1, 0.3, 3, 10 and 30% (w/v). A bee extends its proboscis reflexively when the antenna is stimulated with sucrose (Page et al., 1998). We recorded the number of times each bee extended its proboscis (0–6); greater sucrose responsiveness is reflected by higher numbers of extensions. We performed four independent trials of this experiment, each with bees derived from several naturally mated queens. Bees from the different colony sources were mixed and used randomly for each treatment. Data were analyzed with a general linear model (GLM) using both treatment and trial as factors (SAS Institute, Cary, NC, USA).

Age at onset of foraging

After 4 days of treatment in a cage in the laboratory (see above), all surviving bees from each cage were counted (80–100% survival) and placed into a single-cohort colony, made with ~1000 one-day-old (untreated) bees and a queen. Observations at the hive entrance were made as previously described (Ben-Shahar et al., 2002b) to ensure that we observed the onset of foraging in each colony; observations then occurred for 4 h day^{-1}, 2 h in the morning and 2 h in late afternoon, times of peak foraging activity for these colonies. All bees initiating foraging during the first 7 days of observations were marked with a second spot of paint on their abdomens (so they were counted just once), and the cumulative percentage of bees that foraged (precociously) was calculated for each group. We performed six independent trials of this experiment, each with bees derived from several naturally mated queens. Differences in the proportion of bees starting to forage from each treatment group were evaluated with multiple factor survival analysis with Cox proportional hazards estimation (Ben-Shahar et al., 2002b). After concluding behavioral observations, each colony was killed (liquid nitrogen) to census the number of bees from each treatment group present. Proportions of foragers were calculated on the basis of these censuses.

Tendency to forage for nectar or pollen

In addition to the observations described in the previous paragraph, we also recorded whether each forager returned with either pollen or nectar. To analyze the effects of treatment on foraging behavior, the proportions of foragers returning with either nectar or pollen were analyzed with PROC GENMOD (SAS Institute), with colony and treatment as factors. Since manganese was the only treatment that affected response threshold to sugar we also used the Contrast function under PROC GENMOD to test the more specific hypothesis that the effect of manganese on pollen foraging is different from all other treatments.

Cloning of Amvl

We identified two ESTs (expressed sequence tags) from a honey bee brain EST project (http://titan.biotec.uiuc.edu/bee/honeybee_project.htm) annotated as orthologs of *Drosophila mvl* (GenBank accession no. AY526611). After additional sequencing of these cDNAs, the putative protein sequence of the honey bee *mvl* ortholog (*Amvl*) showed more than 80% similarity to *Drosophila mvl* (Fig. 1).

Real-time quantitative RT-PCR

Procedures and statistical analysis were as previously described (Ben-Shahar et al., 2002a). Sequences for *mvl*-specific primers and TaqMan® probe are given in Table 1. In all experiments, we collected bees from the different behavioral groups – nurses, pollen foragers and nectar foragers – according to established methods (Ben-Shahar et al., 2002a). *Amvl* expression was normalized to an RNA loading control ‘housekeeping’ gene, the honey bee *rp49* gene, as previously described (Ben-Shahar et al., 2002a). Data were analyzed by two-way analysis of variance (ANOVA) with behavior and trial as factors. Data were also analyzed with pair-wise *post hoc* tests using a Bonferroni adjustment. Bees in each trial were collected from independent colonies that were either single-cohort or triple-cohort colonies (Ben-Shahar et al., 2002a), which were

Table 1. *Real-time quantitative RT-PCR reagents for* Apis malvolio *(*Amvl*)*

Primer/probe	Sequence
Forward primer	CCTTGGTATAAAGATTATGACAGGAATATG
Reverse primer	CAAGAGCACTGTGAAGATACAAGTTATG
Dual-labeled probe	FAM6-CAAGCTGTAGGAATTGTTGGTGCAGTTATAATGC-TAMRA

each established with bees of mixed genetic backgrounds from different source colonies. We used a relative measure of mRNA fold differences, with each colony analyzed as an independent experiment. Hence, it was impossible to compare expression levels between colonies (or colony types) on an absolute basis.

Manganese quantification

Manganese concentrations in bee heads were measured using non-destructive neutron activation analysis (Landsberger, 1994). Single bee heads, weighing 2–3 mg, were placed individually in polyethylene vials and irradiated in the TRIGA® (Training, Research, Isotopes, General Atomics) research reactor at a thermal neutron flux of 4×10^{12} neutrons cm^{-2} s^{-1} for 10 min at a power level of 950 kW. The neutron reaction $^{55}Mn(n,\gamma)^{56}Mn$ was used for the analysis employing the 846.7 keV gamma-ray with its 2.56 h half-life. To avoid any spectral interference from the 843.3 keV gamma ray belonging to ^{28}Mg and its 9.45 min half-life, and to increase sensitivity by allowing other short-lived isotopes to die away, a decay time of 2–4 h was used. To avoid any possible manganese contamination from the original irradiated vial, all samples were transferred into inert vials after irradiation. Calibration was done using a National Institute of Standards and Technology certified biological reference material, NIST 1575 Tomato Leaves with a manganese value of 675±15 p.p.m. For quality control, NIST 1575 Pine Needles were analyzed for manganese. Our results of 203±5 p.p.m., 236±5 p.p.m. and 230±5 p.p.m. are in agreement with the certified value of 238±7 p.p.m. Differences in manganese levels were evaluated with ANOVA with a *post hoc* test and a Bonferroni adjustment.

In-situ *hybridization*

Procedures and conditions were as previously described (Ben-Shahar et al., 2002b). Briefly, freshly dissected brains were immediately freeze-mounted on dry ice with anterior side (identified by antennal lobes) up and transferred to the cryostat (Bright Inst. Co., Huntingdon, UK; –20°C). Brains were sectioned (12 μm) and dry-mounted on glass slides. Hybridization was performed in 50% formamide buffer with a digoxygenin-labeled anti-sense RNA probe (Roche, Basel, Switzerland) at 60°C. Sense probe was used as control. Expression patterns were studied in brains from three nurses and two pollen and nectar foragers of typical ages. The cloned *Apis mvl* was used as a template for *in vitro* transcription of riboprobes (827 bp long).

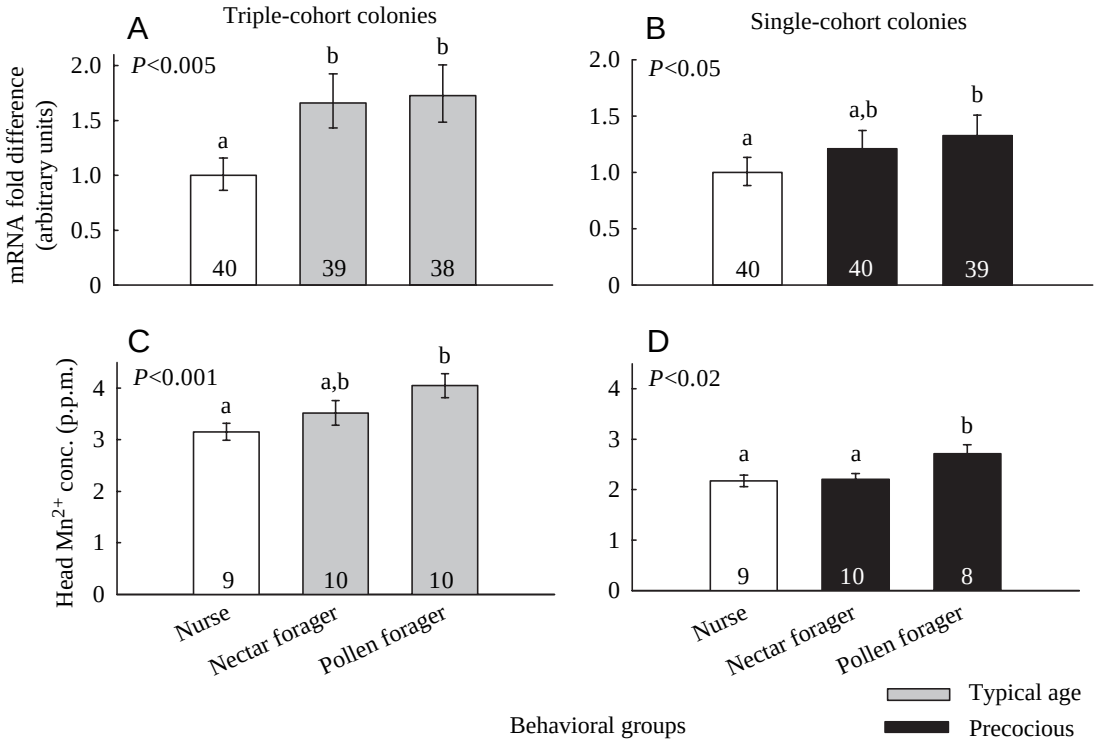

Fig. 2. Behavioral development affects *Amvl* brain expression and manganese levels. (A) qRT-PCR analysis of *Amvl* expression in individual brains of nurses and foragers from triple-cohort colonies (in which bees display age-appropriate behavior; nurses were 7 days old; foragers were >21 days old). (B) qRT-PCR analysis of *Amvl* expression in individual brains of nurses and foragers from single-cohort colonies (in which some bees display precocious behavior; nurses and foragers 7–9 days old). (C) Manganese levels in individual heads of nurses and foragers from a triple-cohort colony (ages as in A). (D) Manganese levels in individual heads of nurses and precocious foragers from a single-cohort colony (ages as in B). Graphs represent means ± S.E.M. (converted to the same arbitrary scale as the mean) from ANOVA-adjusted pooled data of four independent colonies. Different letters above bars represent groups that were significantly different by the ANOVA Bonferroni *post hoc* analysis ($P<0.05$). Numbers in bars represent sample size.

Results

mvl *expression and manganese levels in nurses and foragers*

Both nectar and pollen foragers had higher levels of *Amvl* brain mRNA than did nurse bees in triple-cohort colonies. (Fig. 2A; 2-way ANOVA; behavior, $P<0.005$; colony, $P<0.001$; behavior by colony, $P<0.001$). In single-cohort colonies, only (precocious) pollen foragers had significantly higher levels of *Amvl* brain mRNA than nurse bees (Fig. 2B; 2-way ANOVA; behavior, $P<0.003$; colony, $P<0.001$; behavior by colony, NS). Precocious nectar foragers showed intermediate expression levels (Bonferroni *post hoc* test; $P<0.05$). Results from single-cohort colonies indicate that *Amvl* upregulation in the brain was mostly related to behavior rather than chronological age. A possible effect of age in determining foraging type is suggested by the inconsistent differences in *Amvl* brain mRNA between nectar and pollen foragers.

Foragers had higher levels of manganese in their heads than did nurse bees (Fig. 2C). Bonferroni *post hoc* analysis ($P<0.05$) indicated that manganese levels in pollen foragers were higher than nurses, with nectar foragers exhibiting intermediate levels. Levels of manganese in bees from single-cohort colonies also varied significantly ($P<0.02$) with behavior. Bonferroni *post hoc* analysis indicated that manganese levels in pollen foragers were higher than in nurses, with no difference between nectar foragers and nurses.

In situ hybridization analysis revealed that *Amvl* is widely expressed in the honey bee brain (Fig. 3). High expression levels were observed in the antennal lobes and the subesophageal ganglion. In contrast to *Amfor*, a previously identified gene affecting foraging behavior (Ben-Shahar et al., 2002b), *Amvl* was not highly expressed in the mushroom bodies. There were no obvious spatial differences between nurses and foragers in expression patterns. It is thus likely that the foraging-related increase detected with qRT-PCR was mainly the result of increased expression in the same cells rather than additional neurons expressing this gene.

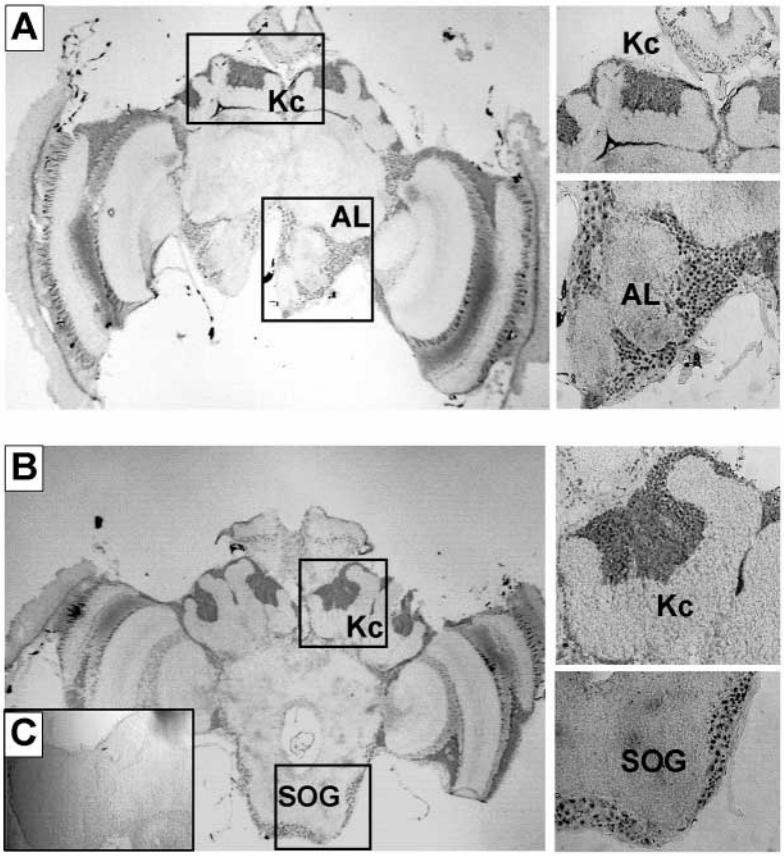

Fig. 3. *Amvl* expression in the honey bee brain. Antennal lobes (AL); Kenyon cells (KC); subesophageal ganglion (SOG). (A) Anterior coronal section, which includes the antennal lobes. Squares delineate regions shown magnified. (B) Posterior coronal section, which includes the SOG. No labeling was seen in control sections probed with a sense riboprobe (C). There were no obvious spatial differences in expression patterns between nurses and either forager type ($N=3$ brains per group); these images are from a pollen forager brain. Brains were sectioned from the anterior (AL) to the posterior end (SOG).

Effects of manganese, zinc and cGMP treatments on sucrose responsiveness

Treating bees with manganese caused a significant increase in responsiveness to sucrose (Fig. 4A). This effect was not seen in bees treated with zinc, manganese plus zinc, or cGMP (Fig. 4A). Manganese-treated bees showed a significant increase in head manganese levels (Fig. 4B), suggesting that the treatments were effective in elevating manganese levels in the brain.

Effects of manganese, zinc and cGMP treatments on age at onset of foraging

Manganese treatments caused precocious foraging in honey bee colonies. An even stronger effect was seen in bees treated with cGMP; Ben-Shahar et al. (2002b) also reported a strong effect of cGMP treatment. There was no effect of zinc or manganese plus zinc on age at onset of foraging (Fig. 5A; multifactorial survival analysis; treatment, $P<0.001$; colony, $P<0.004$). Although we started our treatment experiments with equal amounts of treated bees, a final census revealed varying amounts of bees from each group present in the colony. Bees treated with 8-Br-cGMP tend to attempt to initiate flight almost immediately upon being introduced to the colony, which may explain their somewhat smaller numbers in the final census.

Effects of manganese, zinc and cGMP treatments on the tendency to forage for nectar or pollen

There was a marginal overall effect of treatment on the proportion of pollen foragers in each colony (Fig. 5B; $P=0.047$). Manganese treatment was significantly different from all other treatments ($P<0.01$; Contrast analysis; PROC GENMOD), but there were strong colony differences ($P<0.001$) and no consistent trends within each colony.

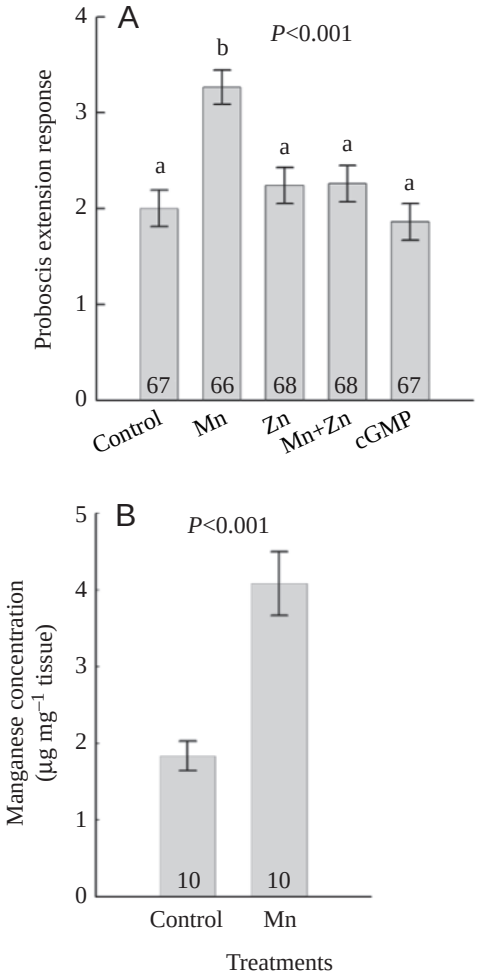

Fig. 4. Manganese treatment affects sucrose responsiveness. (A) Effects of $MnCl_2$, $ZnCl_2$ and 8-Br-cGMP treatments on sugar responsiveness using the proboscis extension assay (Ben-Shahar and Robinson, 2001). 8-Br-cGMP was used to explore the relationship between sucrose responsiveness and rate of behavioral maturation (Ben-Shahar et al., 2002b). Bees were exposed to a sequential series of increasing sucrose concentration (see Materials and methods); a higher response index indicates increased responsiveness to sucrose. (B) Effect of manganese treatment on head manganese levels. Manganese levels increased significantly in heads of treated bees relative to untreated controls (P<0.001, ANOVA), validating the treatment method. Bars represent means ± S.E.M. Numbers in bars represent sample sizes. Results of statistical analysis in text. Different letters above bars represent groups that were significantly different by ANOVA with Bonferroni *post hoc* analysis (P<0.05). Numbers in bars represent pooled data from six independent trials (A).

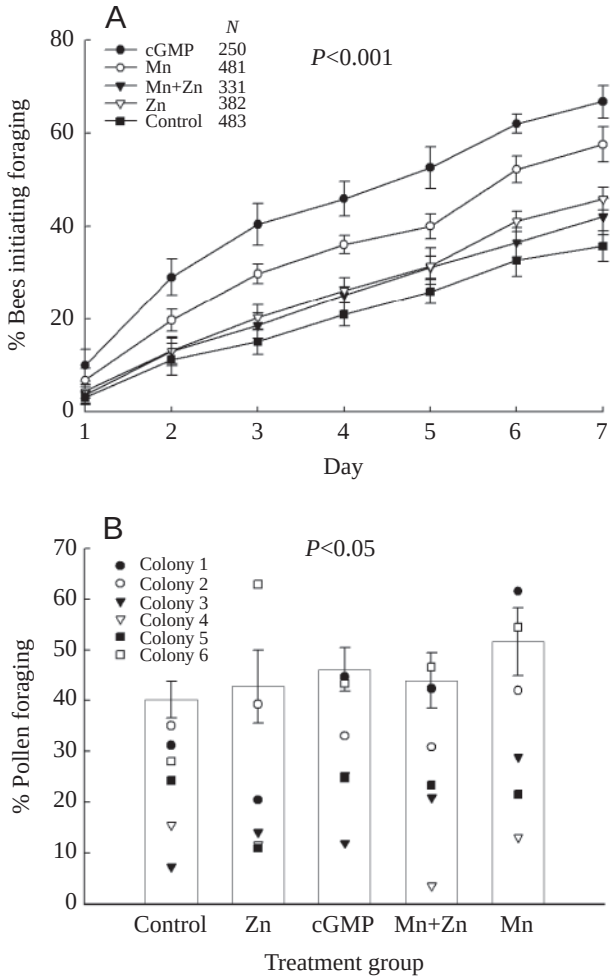

Fig. 5. Manganese treatment induces precocious foraging. (A) Effects of $MnCl_2$, $ZnCl_2$ and 8-Br-cGMP on age at onset of foraging. % initiating foraging refers to the percentage of bees from each treatment group that were observed to initiate foraging (data pooled from six individual experimental colonies; pooled numbers shown in key). (B) Effects of $MnCl_2$, $ZnCl_2$ and 8-Br-cGMP on tendency to collect pollen. Bars represent means ± S.E.M. of the percent of foragers from each colony returning with pollen. There was a significant difference among the treatment groups (P<0.05; PROC GENMOD; counts of foragers converted to percentages solely for graphical purposes), but no consistent trends were evident when examining the data for each colony (N=6; line graphs). Differences in the proportion of bees starting to forage from each treatment group were evaluated with multiple factor survival analysis with Cox proportional hazards estimation (Ben-Shahar et al., 2002b).

Discussion

Our results implicate manganese, perhaps by *mvl* transport into brain cells, as one factor that influences division of labor in honey bee colonies. Manganese (and probably iron) transport is the only known function of the proteins encoded by *malvolio* orthologs in yeast and *Drosophila* (Orgad et al., 1998; Supek et al., 1996). To our knowledge, our findings represent the first report of a link between changes in brain levels of this trace metal and naturally occurring behavioral plasticity.

Manganese deficiency results in a variety of neural deficits, perhaps mediated by AMPA and NMDA receptor functions (Takeda, 2003; Takeda et al., 2002) or other types of receptors or ion channels (Wang et al., 2003). Manganese may also function in brain metabolism as a cofactor for enzymes such as superoxide dismutases (Zelko et al., 2002). *mvl*-mediated transport is apparently not the only way manganese can enter

a cell because, in *Drosophila*, mutations of the *mvl* locus are not lethal and behavioral defects are rescued by manganese treatment (Orgad et al., 1998; Rodrigues et al., 1995). Also, some evidence suggests that manganese ions can also permeate the cell membrane *via* voltage-gated calcium channels (Nasu, 1995). However, the observation that zinc, a known antagonist of *mvl* (Orgad et al., 1998; Supek et al., 1996), attenuated the behavioral effect of manganese suggests that manganese transport *via mvl* is a primary route into brain cells, either neurons, glia or both.

Our results suggest that *mvl*-mediated manganese transport is involved in the response to a rewarding stimulus such as sucrose. Manganese is thought to function in the mammalian dopaminergic system, which plays a central role in regulating the response to various types of pleasurable stimuli (Salamone et al., 2003). Similar to the role of dopamine in mammals, octopamine is associated with the sucrose reward system in honey bees (Hammer and Menzel, 1998; Menzel et al., 1999), and this neurochemical has been implicated in both honey bee responsiveness to sucrose (Page et al., 1998; Pankiw and Page, 2003) and division of labor. Octopamine levels are higher in forager honey bees, especially in the antennal lobes (Schulz and Robinson, 1999), and octopamine treatment causes precocious foraging (Schulz and Robinson, 2001). In addition, we found *mvl* expressed in the subesophageal ganglion, and cells in this neuropil have been shown to be both octopaminergic and responsive to sucrose reward (Hammer and Menzel, 1998; Schroter and Menzel, 2003). Perhaps manganese effects in flies and bees are mediated by this neuromodulatory system.

Manganese treatment showed a strong association between responsiveness to sucrose and age at onset of foraging, but the association with foraging specialization was weaker. Our results with different colony types suggest the possibility of an interaction between bee age and foraging specialization; however, such an age effect has not yet been detected in other studies (see Pankiw and Page, 1999). Perhaps we failed to detect a stronger effect of manganese treatment on pollen foraging in typical-age foragers because we sampled too coarsely (only on the bees' first foraging trips) or because the tendency to collect nectar or pollen is influenced by a variety of colony and environmental factors (Seeley, 1995) that we could not control in this experiment. Tests under more artificial conditions might be more appropriate. Another possibility is that a more chronic treatment, extending throughout the bees' foraging career, might have better revealed effects of manganese on pollen foraging; in this study, the bees were treated for only the first four days of adulthood, prior to the initiation of their foraging career.

Our findings illustrate that molecular analysis of a complex behavioral phenotype such as honey bee division of labor is facilitated by dissecting it into simpler behavioral components. The *foraging* gene influences honey bee behavioral maturation at least in part *via* effects on phototaxis (Ben-Shahar et al., 2002a,b), and *mvl* appears to influence behavioral maturation at least in part *via* effects on responsiveness to sucrose. The results of manganese treatment support the notion that responsiveness to sucrose is related in some way to behavioral maturation in honey bees. However, the causal relationships between them are not understood. Bees that specialize in collecting pollen show increased responsiveness to sucrose in the laboratory relative to bees that specialize in collecting nectar (Pankiw and Page, 1999); it is not clear how increased responsiveness to sucrose causes pollen foraging. Either an increase in responsiveness in some way facilitates the ability of bees to leave the hive and collect food, especially pollen, or the increase in responsiveness is itself associated with another behavioral change that is more causally related to the transition to foraging behavior.

Previous findings have shown genotypic and phenotypic correlations between variation in responsiveness to sucrose and two aspects of division of labor in honey bees: the age at onset of foraging and the tendency to forage for either pollen or nectar (Pankiw and Page, 1999). Our results demonstrate that, while there are molecular pathways common to sucrose responsiveness and division of labor, linkages between them can be dissociated. cGMP treatment affected age at onset of foraging and phototaxis (Ben-Shahar et al., 2002b) but did not affect responsiveness to sucrose in the current study. We also showed that manganese treatment affected both responsiveness to sucrose and age at onset of foraging, but the association with foraging specialization was weaker. Other studies have shown that cAMP treatments increase sucrose responsiveness but do not affect age at onset of foraging (Ben-Shahar et al., 2002b; Scheiner et al., 2003), while juvenile hormone affects both (Pankiw and Page, 2003; Schulz et al., 2002). It is not known how many independent molecular pathways in the brain are involved in the regulation of honey bee behavioral maturation. The results presented here and elsewhere (Ben-Shahar et al., 2002a; Whitfield et al., 2003) suggest that there are multiple independent pathways. This is consistent with the fact that honey bee behavioral maturation involves changes in responsiveness to stimuli in various modalities, in addition to changes in other neural and physiological processes (Robinson, 2002).

There are now two genes involved in *Drosophila* feeding behavior that have been implicated in controlling the age at onset of foraging in honey bees: *malvolio* (present study) and *foraging* (Ben-Shahar et al., 2002a,b). These results support the idea that some genes that are involved in feeding-related behaviors in *Drosophila* have been used in social evolution to regulate honey bee division of labor. It is reasonable to assume that the evolution of social behavior acted, in part, on conserved mechanisms that control responses to other stimuli in the environment (Robinson and Ben-Shahar, 2002). Social cues, like other environmental cues, convey information critical for animal survival and reproduction. Genes involved in orchestrating the perception and processing of sensory information and the responses that are then triggered (Robinson and Ben-Shahar, 2002) are thus likely to figure prominently in social evolution. Further studies on *mvl* and other genes that are involved in feeding behavior in *Drosophila*

may provide important insights into the regulation of division of labor, as well as to the neural mechanisms underlying the regulation of food intake.

We thank Karen Pruiett for invaluable help with beekeeping, Carolyn Shook for help with field experiments, Kim Hughes and Amy Toth for help with statistical analysis, and Marla Sokolowski, Kim Hughes and members of the Robinson laboratory for helpful comments on earlier drafts of this manuscript. This work was supported by grants from the NIH, NSF and Burroughs–Wellcome Trust to G.E.R.

References

Becker, J. B., Breedlove, S. M. and Crews, D. (1992). *Behavioral Endocrinology*. Cambridge, MA: MIT Press.

Ben-Shahar, Y. and Robinson, G. E. (2001). Satiation differentially affects performance in a learning assay by nurse and forager honey bees. *J. Comp. Physiol. A* **187**, 891-899.

Ben-Shahar, Y., Leung, H.-T., Pak, W. L., Sokolowski, M. B. and Robinson, G. E. (2002a). cGMP-dependent changes in phototaxis: a possible role for the *foraging* gene in honey bee division of labor. *J. Exp. Biol.* **206**, 2507-2515.

Ben-Shahar, Y., Robichon, A., Sokolowski, M. B. and Robinson, G. E. (2002b). Influence of gene action across different time scales on behavior. *Science* **296**, 741-744.

Hammer, M. and Menzel, R. (1998). Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. *Learn. Mem.* **5**, 146-156.

Landsberger, S. (1994). Delayed instrumental neutron activation analysis. In *Chemical Analysis by Nuclear Methods* (ed. Z. B. Alfassi), pp. 121-142. New York: John Wiley & Sons.

Leboyer, M., Bellivier, F., Nosten-Bertrand, M., Jouvent, R., Pauls, D. and Mallet, J. (1998). Psychiatric genetics: search for phenotypes. *Trends Neurosci.* **21**, 102-105.

Menzel, R., Heyne, A., Kinzel, C., Gerber, B. and Fiala, A. (1999). Pharmacological dissociation between the reinforcing, sensitizing, and response-releasing functions of reward in honeybee classical conditioning. *Behav. Neurosci.* **113**, 744-754.

Nasu, T. (1995). Actions of manganese ions in contraction of smooth muscle. *Gen. Pharmacol. Vasc. Syst.* **26**, 945-953.

Orgad, S., Nelson, H., Segal, D. and Nelson, N. (1998). Metal ions suppress the abnormal taste behavior of the *Drosophila* mutant *malvolio*. *J. Exp. Biol.* **201**, 115-120.

Page, R. E., Jr, Erber, J. and Fondrk, M. K. (1998). The effect of genotype on response thresholds to sucrose and foraging behavior of honey bees (Apis mellifera L.). *J. Comp. Physiol. A* **182**, 489-500.

Pankiw, T. (2003). Directional change in a suite of foraging behaviors in tropical and temperate evolved honey bees (*Apis mellifera* L.). *Behav. Ecol. Sociobiol.* **54**, 458-464.

Pankiw, T. and Page, R. E., Jr (1999). The effect of genotype, age, sex, and caste on response thresholds to sucrose and foraging behavior of honey bees (*Apis mellifera* L.). *J. Comp. Physiol. A* **185**, 207-213.

Pankiw, T. and Page, R. E., Jr (2003). Effect of pheromones, hormones, and handling on sucrose response thresholds of honey bees (Apis mellifera L.). *J. Comp. Physiol. A* **189**, 675-684.

Robinson, G. E. (2002). Genomics and integrative analyses of division of labor in honeybee colonies. *Am. Nat.* **160**, S160-S172.

Robinson, G. E. and Ben-Shahar, Y. (2002). Social behavior and comparative genomics: new genes or new gene regulation? *Genes Brain Behav.* **1**, 197-203.

Rodrigues, V., Cheah, P. Y., Ray, K. and Chia, W. (1995). *malvolio*, the *Drosophila* homologue of mouse NRAMP-1 (Bcg), is expressed in macrophages and in the nervous system and is required for normal taste behaviour. *EMBO J.* **14**, 3007-3020.

Salamone, J. D., Correa, M., Mingote, S. and Weber, S. M. (2003). Nucleus accumbens dopamine and the regulation of effort in food-seeking behavior: implications for studies of natural motivation, psychiatry, and drug abuse. *J. Pharmacol. Exp. Ther.* **305**, 1-8.

Scheiner, R., Muller, U., Heimburger, S. and Erber, J. (2003). Activity of portein kinase A and gustatory responsiveness in the honey bee (*Apis mellifera* A.). *J. Comp. Physiol. A* **189**, 427-434.

Scheiner, R., Sokolowski, M. B. and Erber, J. (2004). Activity of cGMP-dependent protein kinase (PKG) affects sucrose responsiveness and habituation in *Drosophila melanogaster*. *Learn. Mem.* **11**, 303-311.

Schroter, U. and Menzel, R. (2003). A new ascending sensory tract to the calyces of the honeybee mushroom body, the subesophageal-calycal tract. *J. Comp. Neurol.* **465**, 168-178.

Schulz, D. J. and Robinson, G. E. (1999). Biogenic amines and division of labor in honey bee colonies: behaviorally related changes in the antennal lobes and age-related changes in the mushroom bodies. *J. Comp. Physiol. A* **184**, 481-488.

Schulz, D. J. and Robinson, G. E. (2001). Octopamine influences division of labor in honey bee colonies. *J. Comp. Physiol. A* **187**, 53-61.

Schulz, D. J., Sullivan, J. P. and Robinson, G. E. (2002). Juvenile hormone and octopamine in the regulation of division of labor in honey bee colonies. *Horm. Behav.* **42**, 222-231.

Seeley, T. D. (1995). *The Wisdom of the Hive: the Social Physiology of Honey Bee Colonies.* Cambridge, MA: Harvard University Press.

Supek, F., Supekova, L., Nelson, H. and Nelson, N. (1996). A yeast manganese transporter related to the macrophage protein involved in conferring resistance to mycobacteria. *Proc. Natl. Acad. Sci. USA* **93**, 5105-5110.

Takeda, A. (2003). Manganese action in brain function. *Brain Res. Rev.* **41**, 79-87.

Takeda, A., Sotogaku, N. and Oku, N. (2002). Manganese influences the levels of neurotransmitters in synapses in rat brain. *Neuroscience* **114**, 669-674.

Takeda, A., Sotogaku, N. and Oku, N. (2003). Influence of manganese on the release of neurotransmitters in rat striatum. *Brain Res.* **965**, 279-282.

Verity, M. A. (1999). Manganese neurotoxicity: a mechanistic hypothesis. *Neurotoxicology* **20**, 489-497.

Wang, J., Luthey-Schulten, Z. A. and Suslick, K. S. (2003). Is the olfactory receptor a metalloprotein? *Proc. Natl. Acad. Sci. USA* **100**, 3035-3039.

Whitfield, C. W., Cziko, A. M. and Robinson, G. E. (2003). Gene expression profiles in the brain predict behavior in individual honey bees. *Science* **302**, 296-299.

Zelko, I. N., Mariani, T. J. and Folz, R. J. (2002). Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radic. Biol. Med.* **33**, 337-349.