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Differences in performance on a reversal learning test and division of labor in honey bee colonies

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Abstract We studied the association between honey bee (*Apis mellifera*) division of labor and performance on an olfactory reversal-learning test. Manipulations of colony age structure and flight experience were used to test whether differences in performance are associated with age, current behavioral state, or flight experience. Nurse bees showed significantly faster rates of extinction to a learned odor than did foragers. This difference was associated primarily with differences in behavioral state, rather than age; it was seen when comparing nurses and foragers from typical colonies and normal-age nurses and precocious foragers from single-cohort colonies. Differences in extinction rate were not related to differences in flight experience; there was no difference between foragers and foraging-age bees denied flight experience. These results suggest that changes in learning and memory occur in association with division of labor. We speculate on the possible functional significance of the difference in extinction rate between nurses and foragers.

Key words Proboscis extension reflex · Associative learning · Social insects

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Introduction

One challenge in cognitive ethology is to use laboratory assays to reveal variation in learning and memory abilities that is ecologically relevant. Honey bees, like many other social insects, show age-related division of labor that is based on a pattern of individual behavioral development (reviewed by Robinson 1992). Young bees attend the brood (nurse) and queen until they are about 7 days old, and then perform other hive tasks such as comb building or food handling. At about 3 weeks of age, bees show the biggest transition in behavior by starting to forage for food outside their hive (Winston 1987). Because social evolution has led to strong specialization for task performance at different ages, it is possible that the specialization also involves cognitive differences.

Forager honey bees possess important skills that apparently are not needed for the performance of hive tasks. Foragers must locate floral food sources, assess their quality, communicate their location to other foragers, and navigate to and from the hive – all while coping with continually changing environmental conditions. To accomplish all these tasks, foragers use visual, olfactory, auditory, and perhaps magnetic sensory inputs (Winston 1987). Honey bees forage from many different floral sources and learn the color and odor of flowers from highly rewarding species (Menzel et al. 1993). Not surprisingly, forager bees also perform very well in a variety of associative learning assays in the laboratory (reviewed in Hammer and Menzel 1995; Menzel and Müller 1996).

Nurses and other hive bees, in contrast, spend their days in the more constant, dark environment of the beehive, where they feed on stored food. Hive bees are thought to rely mainly on olfaction to guide their more stereotyped behavior. Little is known about how learning and memory processes might be related to nursing and other hive behaviors. A few laboratory olfactory conditioning studies of hive and foraging bees have been conducted, each using somewhat different procedures (Bhagavan et al. 1994; Ray and Ferneyhough 1997, 1999;

Sigg et al. 1997; Chandra et al. 2000). Differences were found in two studies: Ray and Ferneyhough (1999) reported a difference in acquisition rates for odor-conditioned responses, and Chandra et al. (2000) differences in latent inhibition between bees that were collected from different sites in the beehive, some inside frames (presumably younger bees) and the hive entrance (presumably older bees). In addition, Pankiw and Page (1999) reported that response thresholds to sucrose decrease with increased age, which might improve motivation in an appetitive learning assay and lead to age-related improvement in honey bee performance. Such differences in response thresholds for an appetitive stimulus have been shown to affect acquisition performance in both bees and flies (Brigui et al. 1990; Fois et al. 1991; Scheiner et al. 1999).

One of the more challenging associative learning paradigms is the reversal-learning test (Bitterman 1972). In such a test the animal is trained to discriminate between two conditioned stimuli, one coupled with a reward and the other with a punishment (A+/B–). After a fixed number of odor exposures, the two odors are reversed (A–/B+). We used an olfactory based reversal-learning paradigm to test for performance differences that are associated with honey bee behavioral development. We predicted two possible ways in which behavioral development might be associated with performance. First, forager bees might perform better in every aspect of the reversal-learning paradigm, i.e., faster acquisition/discrimination in the first phase and faster reversion in the second phase. Alternatively it is possible that neither foragers nor nurses would perform consistently better, but rather differ in performance in selected aspects of the reversal-learning paradigm.

Methods

Reversal-learning assay

The olfactory reversal-learning test was described by Bitterman (1972) and Bitterman et al. (1983). Bees were collected individually in glass vials and anesthetized by chilling on ice. Similar numbers of bees from each test group were collected on each day of training to control for possible effects of environmental variation. Once anesthetized, each bee was harnessed in a plastic tube that allowed free movement of the head and legs (Bitterman et al. 1983). Bees were then allowed to recover from the anesthesia for 30–45 min and fed to satiation (1.25 M sucrose). Training started 20–26 h after feeding. In trial 1 of Experiments 1 and 2 the pretraining protocol differed: bees were fed 0.5 µl of 1.25 M sucrose and training started 2 h after feeding. The former protocol was used more extensively because it provides more flexibility for when bees are collected and does not affect performance in this assay (B.H. Smith, unpublished work). Bees were kept at room temperature in the vicinity of the testing arena until tested.

The conditioning procedure is well established (Bitterman et al. 1983; Hammer and Menzel 1995). Sugar and salt solutions were used as unconditioned stimuli (US) and odors were used as conditioned stimuli (CS; 1-hexanol and geraniol; Sigma Chemical Co. St. Louis, Mo., USA). Each odor was delivered by a 2-ml glass syringe that contained a small piece of filter paper soaked with 1.5 µl of undiluted compound. The US was delivered by touching both antennae with a droplet of 1.5 M sucrose and then feeding the bee with 0.4 µl of the solution once she extended her proboscis. Once the bee began to extend its proboscis to odor alone, we left out the antennal component of the US and delivered the sucrose directly to the proboscis. Punishment was delivered by touching both antennae with a droplet of 3 M NaCl (Chandra et al. 2000). The discrimination phase consisted of a total of 12 forward-pairing odor exposures (conditioning trials), 6 with each odor. Each of the odors was coupled with either a reward (A+) or a punishment (B–). Odor presentations were pseudo-random (ABBABAABABBA), and the two odors were either A or B on different training days to minimize the effects of any possible innate odor preferences.

In each conditioning trial the tested bee was placed in the training arena under a weak airflow (suction of a laboratory hood) for 35 s before the odor was delivered. This was done to habituate the tested bee to the mechanical component of the odor presentation. Odor delivery was controlled by computer and was timed for 5 s. The computer signaled the experimenter to deliver either a reward or a punishment to the bee beginning 2 s after the onset of odor. Each bee received such a conditioning trial every 6–10 min (6–10 bees were trained per day, 3–5 bees from each group). Bees were kept outside the training arena between conditioning trials to prevent odor exposure. The reversal phase started 30 min after the discrimination phase ended. The reversal phase was similar to the discrimination phase but the odors were switched such that odor A was coupled with a punishment (A–), and odor B with a reward (B+). The semi-random sequence of odor presentations was done in a similar manner to the discrimination phase (BAABAB-BABAAB). Although bees were fed during the acquisition phase, the total amount of sugar fed (~3 µl) probably was not enough to cause satiation. A high level of response to the first exposure to A– in all tested groups further supports this contention. The criterion for a response was a full extension of the proboscis at the onset of odor delivery prior to touching the antennae with either the reward or punishment.

Bees

We used the European honey bee, *Apis mellifera*, which in North America is a mix of European subspecies. To reduce possible variation in performance due to polyandry-induced genetic variability (Bhagavan et al. 1994; Page 1986; Chandra et al. 2000), bees from each colony were the progeny of a single queen inseminated with semen from a single drone. All bees were maintained according to standard beekeeping techniques at the University of Illinois Bee Research Facility, Urbana, Illinois. Data were collected during two summers for typical and single-cohort colonies (1997–1998) and one summer for "big-back" colonies (1998). Bees from different genetic sources were used in different trials of each experiment.

Experiment 1: Nurses and foragers from typical colonies

Nurses and foragers were collected from typical colonies (population 20,000–40,000 bees) according to standard methods (Robinson 1987). Foragers were collected as returning bees with clearly visible pollen loads on their hind legs. Nurses were identified as bees that repeatedly inserted their heads into cells with larvae. The age of these bees was unknown but they were probably of typical age (1–2 weeks old for nurses and 3–4 weeks old for foragers). We tested bees from three unrelated colonies, one in each trial (trial 1: n = 23 and 22; trial 2: n = 15 and 15; trial 3: n = 32 and 24 nurses and foragers respectively).

Experiment 2: Normal-age nurses and precocious foragers from single-cohort colonies

Nurses and foragers differ in both current behavioral status and chronological age. Therefore, single-cohort colonies were used to uncouple the possibly confounding effects of these two factors on performance in the reversal-learning test. Each single-cohort colony was made with 800–1500 1-day-old bees (0–24 h) and an

Table 1 Performance of bees in a reversal-learning test as a function of age and behavior (experiment 1), current behavioral state (experiment 2), or flight experience (experiment 3). Results of comparisons between behavioral groups using growth curve analysis (see Methods). Results are for all trials in each experiment (*%PER* % of bees from each group that showed proboscis extension response to the presented odor, *NS* not significant with $\alpha = 0.01$)

Colony type	Trial	Δ Regression coefficient ± SE	*P*	*n*	% PER for each odor exposure											
					Odor presentations											
					Nurses						Foragers					
					1	2	3	4	5	6	1	2	3	4	5	6
Experiment 1 Typical																
A+	1	–0.30 ± 0.14	NS		4.3	65.2	91.3	91.3	95.7	95.7	13.6	36.4	90.9	100	90.9	86.4
	2				34.4	78.1	96.9	96.9	96.9	93.8	16.7	66.7	79.2	87.5	95.8	91.7
	3				0	40	80	86.7	86.7	86.7	6.7	26.7	60	73.3	80	86.7
B–	1	0.01 ± 0.07	NS	70 Nurses	39.1	8.7	26.1	13	0	0	31.6	5.3	21.1	21.1	26.3	10.5
	2				62.5	56.3	40.6	21.9	12.5	3.13	16.7	29.2	33.3	33.3	25	25
	3				40	13.3	53.3	46.7	40	20	13.3	6.7	13.3	26.7	20	33.3
A–	1	0.21 ± 0.05	< 0.0001	61 Foragers	100	78.2	82.6	34.7	13	8.7	95.5	86.4	90.9	77.3	63.6	27.3
	2				90.6	90.6	90.6	75	34.4	21.9	87.5	91.7	83.3	75	58.3	45.8
	3				80	86.7	73.3	40	40	0	93.3	86.7	73.3	73.3	60	26.7
B+	1	0.02 ± 0.12	NS		0	19	85.7	90.5	95.2	85.7	5.5	61.1	88.9	88.9	88.9	100
	2				9.3	50	81.3	84.4	87.5	93.8	12.5	41.7	87.5	75	83.3	83.3
	3				13.3	66.7	80	86.7	86.7	86.7	26.7	80	93.3	93.3	100	100
Experiment 2 Single-cohort																
A+	1	0.002 ± 0.1	NS		16.7	50	77.8	83.3	88.9	94.4	21.1	57.9	94.7	94.7	94.7	94.7
	2				9.1	72.7	86.4	86.4	90.9	90.9	9.1	68.2	81.8	100	86.4	86.4
	3				0	62.5	87.5	95.8	100	95.8	8.3	41.7	70.8	95.8	95.8	87.5
	4				16.7	87.5	87.5	91.7	95.8	87.5	33.3	85.7	90.5	90.5	95.2	95.2
	5				3.9	53.8	76.9	88.5	84.6	100	10	63.3	93.3	90	90	86.7
B–	1	0.21 ± 0.06	0.0005	115 Nurses	33.3	11.1	11.1	0	0	0	42.1	15.8	21.1	26.3	10.5	5.26
	2				36.4	36.4	22.7	22.7	13.6	0	36.4	36.4	22.7	22.7	13.6	0
	3				37.5	20.8	33.3	25	4.2	0	29.2	33.3	25	37.5	20.8	4.17
	4				58.3	37.5	54.2	37.5	4.2	20.8	61.9	47.6	66.7	71.4	38.1	14.3
	5				34.6	23.1	23.1	19.2	7.7	3.8	53.3	50	40	43.3	40	36.7
A–	1	0.21 ± 0.04	< 0.0001	121 Foragers	88.9	72.2	66.7	61.1	44.4	11.1	94.7	94.7	89.5	89.5	63.2	57.9
	2				100	77.3	90.9	68.2	45.5	18.2	90.9	90.9	95.5	77.3	54.5	31.8
	3				83.3	95.8	70.8	70.8	41.7	8.33	100	83.3	62.5	70.8	66.7	41.7
	4				95.8	87.5	79.2	83.3	41.7	16.7	95.2	95.2	90.5	85.7	81	61.9
	5				84.6	73.1	80.8	57.7	38.5	15.4	90	83.3	76.7	80	76.7	40
B+	1	0.24 ± 0.11	NS		0	16.7	55.6	77.8	83.3	88.9	0	63.2	100	100	100	100
	2				9.1	59.1	90.9	86.4	90.9	90.9	4.55	63.6	72.7	86.4	90.9	90.9
	3				4.17	50	70.8	83.3	91.7	100	4.17	70.8	79.2	95.8	95.8	87.5
	4				20.8	54.2	91.7	95.8	100	95.8	9.52	52.4	81	85.7	90.5	85.7
	5				11.5	53.8	76.9	88.5	88.5	88.5	53.3	73.3	86.7	86.7	93.3	100
Experiment 3 Big-back					Big-backs						Foragers					
A+	1	–0.14 ± 0.14	NS		27	73	81.1	89.2	89.2	97.3	25.8	74.2	87.1	93.5	83.9	90.3
	2				13.2	60.5	84.2	94.7	97.4	97.4	5.4	45.9	75.7	94.6	94.6	100
B–	1	0.02 ± 0.02	NS	75 Big-back	59.5	48.6	54.1	37.8	32.4	24.3	54.8	45.2	58.1	51.6	32.3	12.9
	2				42.1	34.2	44.7	39.5	34.2	7.89	45.9	24.3	40.5	48.6	29.7	24.3
A–	1	0.02 ± 0.05	NS	68 Foragers	91.9	86.5	81.1	83.8	70.3	54.1	93.5	80.6	87.1	80.6	61.3	48.4
	2				92.1	92.1	89.5	63.2	47.4	34.2	97.3	94.6	86.5	73	62.2	40.5
B+	1	0.22 ± 0.17	NS		40.5	67.6	86.5	94.6	94.6	91.9	38.7	77.4	93.5	96.8	96.8	96.8
	2				13.2	78.9	97.4	97.4	97.4	100	40.5	81.1	94.6	97.3	100	97.3

unrelated queen in a small hive containing one frame of food and one empty frame for the queen to lay eggs in. One-day-old bees were obtained by removing frames with sealed brood from a typical colony and placing them in an incubator (33 °C) until adults emerged. Each 1-day-old bee was marked with a spot of paint (Testor's PLA) on the thorax prior to its introduction to the colony. The absence of older bees in these colonies induces precocious behavioral development in 5–10% of the bees (Huang and Robinson 1992). Precocious foragers are the same age as nurse bees but endocrine (Fahrbach and Robinson 1996), neuroanatomical (Withers et al. 1993), and neurochemical (Schulz and Robinson 1999) analyses indicate that they are similar in some respects to normal-age foragers. Normal-age nurses and precocious foragers were collected when they were 5–13 days old. We tested bees from five unrelated colonies, one in each trial (trial 1: n = 17 and 19; trial 2: n = 22 and 22; trial 3: n = 26 and 25; trial 4: n = 26 and 30; trial 5: n = 24 and 24 normal-age nurses and precocious foragers, respectively). Mean ages for nurses and precocious foragers were 8.97 ± 0.26 and 8.98 ± 0.26 days, respectively (t-test: $t_{115,120}$ = 1.97, NS).

Experiment 3: Big-back bees

We used the "big-back" method to test for the effects of flight experience (Withers et al. 1995). Colonies were similar to single-cohort colonies, but bees were introduced in two cohorts instead of one. The first cohort (the focal group) consisted of 120–300 1-day-old bees. Half the bees in the focal group were designated as "big-back" bees, and were marked with a plastic tag attached to the thorax (~3 mm in diameter and ~1 mm thick) that is thicker than the usual color number tags used in bee research. Bees in the other half of the focal group were marked with a paint spot on the thorax. A second cohort of 800–1000 bees was introduced to the colony 2–4 days later to increase the proportion of precocious foragers in the focal group (Page et al. 1992; Jassim et al. 2000). The entrance to the hive was covered with a metal plate with holes in it that prevented big-back bees from leaving the hive, but allowed paint-marked bees and all others to fly freely. A frame of honeycomb with drone-sized cells (bigger than worker cells) was inserted for the queen to lay eggs in; this gave big-back bees the opportunity to feed larvae despite their large tags (but nursing activity was not quantified). Returning foragers were collected as in previous experiments. Big-back bees were collected either while trying to get out of the hive or inside near the hive entrance. We tested bees from two unrelated colonies, one in each trial (trial 1: n = 38 and 37; trial 2: n = 37 and 31 big-back bees and foragers respectively). The big-back manipulation allowed us to collect bees with and without flight experience that were of the same age.

Statistical analyses

The data presented in this paper represent repeated measures of the same individuals, recorded as response/no-response (coded as 1 and 0). We applied a powerful statistical analysis well suited for these kind of data, growth curve analysis (with a logistic regression model; PROC GENMOD, SAS Institute, Cary, version 6.12), to determine whether there are differences in rates of performance between behavioral groups. Logistic growth curve analysis precisely tests the difference between several "learning curves". It retains the binary structure of the responses, both at the individual and group levels, and results in a statistical comparison of predicted curves resembling the empirical curves (Graham and Petkau 1994; Hardy and Field 1998). With this analysis, performance comparisons are made by comparing the slope and intercept of the logistic curve. Because some positive responses are apparent in all groups at the same time (second odor exposure), we set the intercept parameter to be the same for any group to be compared. We then compared slopes to test for differences. For each experiment, which always compared two groups, the model created two logistic regression lines that best fit the learning curves for each behavioral group, and then tested whether the difference between the slopes (Δ regression coefficient) was significantly different than 0. When the difference in slope is statistically significant, the difference between behavioral groups in performance rates is statistically significant.

Each experiment was performed several times with bees from different colonies. The model we used assumed that bees from different colonies might differ in rate of response (due to both genetic and environmental factors). Data were therefore "blocked" by colony to control for colony variation. The model also assumed that for each bee, performance during one odor exposure was not independent of performance in subsequent exposures. This was accomplished by using the GEE adjustment in PROC GENMOD (Littell et al. 1996). Performance for each component of the reversal-learning paradigm (A+, B–, A–, B+) was analyzed separately. Results of each regression analysis are presented as the Δ regression coefficient ± SE.

Figures 1–3 show representative regression curves from one colony per experiment for illustrative purposes. This was done because the analysis does not allow us to mathematically incorporate all curves from all colonies into one representative graph. Because of the large number of statistical tests performed, α was set to 0.01.

Results

In Experiment 1, nurses had a significantly faster rate of extinction to A– than foragers in typical colonies (Table 1, Fig. 1). There were no other significant differences.

In Experiment 2, normal-age nurses had a significantly faster rate of extinction to A– than precocious foragers in single-cohort colonies (Table 1, Fig. 2). Precocious foragers also had a significantly faster rate of response to B–. There were no other significant differences between normal-age nurses and precocious foragers. Although both groups averaged about 9 days of age (see methods), bees within each group ranged in age from 5–13 days old. Growth curve analysis was used to assess the effects of this variation on performance in the reversal-learning as-

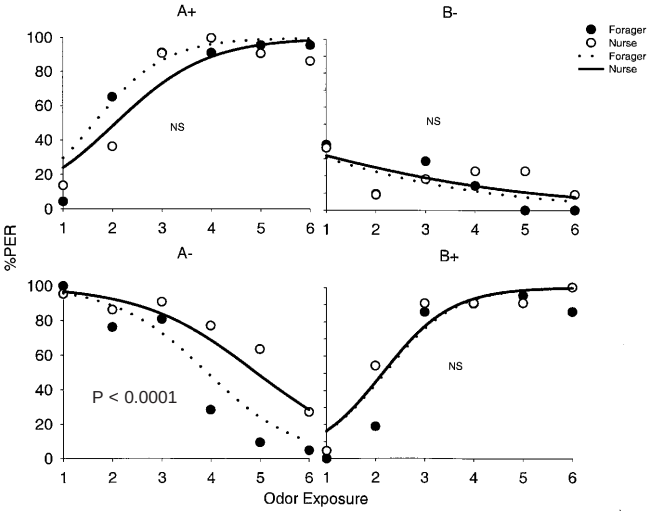

Fig. 1 Representative logistic regression curves from one typical colony (trial 2). Each data point gives the percent of bees that showed the proboscis extension response (PER) during odor exposure. Results of statistical analysis in Table 1

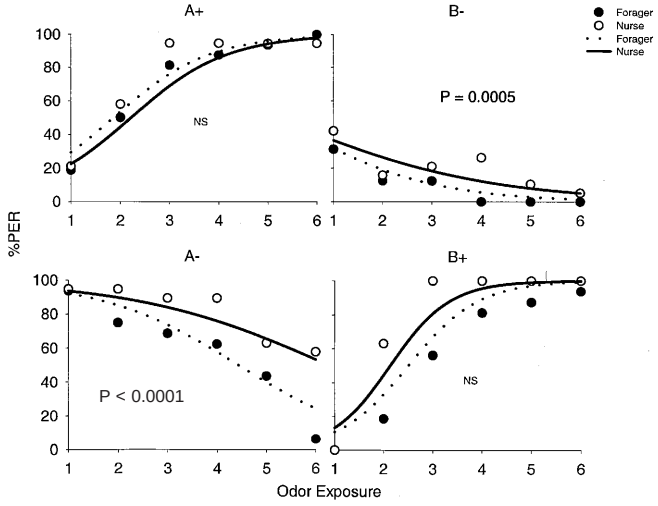

Fig. 2 Representative logistic regression curves from one single-cohort colony (trial 1). Data as in Fig. 1. Results of statistical analysis in Table 1

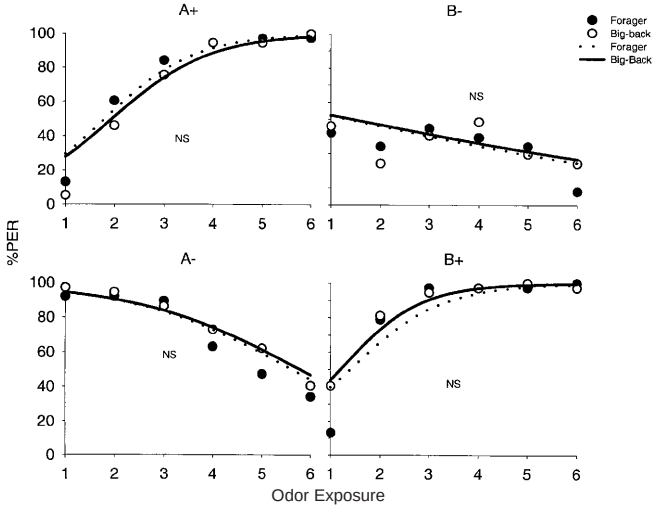

Fig. 3 Representative logistic regression curves from one big-back colony (trial 1). Data as in Fig. 1. Results of statistical analysis in Table 1

say. Bees were grouped into two sub-cohorts, 5–7 days old and 8–13 days old. There was no effect of age on performance in any component of the reversal-learning test (data not shown). In Experiment 3 there were no differences in performance between big-back bees and foragers (Table 1, Fig. 3).

Discussion

It would seem that a learning test that involves making an association between an odor and a sugar reward would favor foragers. Foragers make precisely such associations in nature on a regular basis, but it is not obvious how the formation of such associations figure in the lives of nurse bees. We found, however, that nurses performed as well as foragers in several components of the reversal test and in one component, extinction, nurses performed "better", i.e., showed faster rates of change in learned behavior.

We propose two interpretations for the differences between nurses and foragers in extinction. First, the slow rate of extinction to A– by foragers relative to nurses suggests that foragers are more resistant to breaking a previously rewarded association. Because the rate of extinction is thought to be dependent on the initial discrimination phase (Bitterman 1972), we speculate that this "resistance" to change is the result of a stronger effect of a previous similar positive association in foragers compared to nurses. Resistance to switching in foragers may be adaptive because it would allow more persistence during foraging (Waddington 1983). Associative learning apparently underlies honey bee "flower constancy", the tendency to forage from the same floral resource once it has proven to be good source of pollen or nectar. The second interpretation for the faster extinction rate by nurses is that it relates in some way to the in-hive life style. These two interpretations are not mutually exclusive. Fresquet (1999) investigated similar processes in the fruit fly *Drosophila melanogaster* and reported that acquisition and extinction rates for conditioned stimuli change with age; older flies were faster for acquisition but slower for extinction. Fresquet (1999) suggests that the resistance of older flies to extinction is probably due either to the effects of prior stronger acquisition or to "behavioral rigidity". Our data are consistent with the "behavioral rigidity" hypothesis because we did not observe differences in acquisition between nurses and foragers.

Precocious foragers in single-cohort colonies also differed from nurses in response to B–, showing higher initial levels of response. This difference was not seen in tests of normal-age foragers and nurses from typical colonies. Bees have been shown to first generalize from a positive (A+) to a negative (B–) odor, resulting in a higher initial rate of response to B– (Smith 1993). Once bees learn to ignore "background" stimuli (mechanical and visual), their response to B– drops to zero (Smith 1993; Hammer and Menzel 1995). Positive responses to an odor coupled with a punishment (B–) can be seen as "mistakes" from the perspective of a laboratory test. Smith (1993) however, argues that such generalization might be adaptive for foragers because it helps them ignore minor natural variation in floral sources that would otherwise interfere with foraging. There is probably an optimal level of generalization for foragers. Looking at the results of both Experiment 1 and 2, it appears that precocious foragers show a deficit in this regard relative to foragers from typical colonies that are assumed to be of normal age. Perhaps bees forced to forage precociously cannot modulate the generalization response. This may not be related to the effects of flight experience, because we showed that flight experience did not influence performance. This speculation can be tested by observing the foraging performance of normal-age and precocious foragers in the field.

We found differences in some aspects of performance on a reversal-learning assay that are associated with the division of labor. Using three different kinds of colonies, our findings extend those of Ray and Ferneyhough (1999) and Chandra et al. (2000) and indicate that differences in learning performance between different behavioral groups in the beehive can be found. However, we do not know either the proximate or ultimate reasons for the differences, nor do we understand how directly they relate to the different behavioral states that characterize division of labor. It would be interesting to compare bees of different ages performing the same behavior or bees doing a greater variety of tasks. In addition, it is not known whether the observed differences reflect cognitive differences between nurses and foragers. Differences in previous experience (Gerber et al. 1996), and sensory capabilities such as sucrose response thresholds (Page et al. 1998; Scheiner et al. 1999) also maybe involved. Given that there are structural differences in the bee brain between young bees and foragers (Withers et al. 1993; Durst et al. 1994; Sigg et al. 1997; Fahrbach and Robinson 1995) it is tempting to speculate that they are related to differences in the cognitive abilities of nurses and foragers. It is premature to conclude that this is the case, but the results of our study suggest that this is a fruitful area for further research.

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