Limits on Volume Changes in the Mushroom Bodies of the Honey Bee Brain

Susan E. Fahrbach,[1,2] Sarah M. Farris,* Joseph P. Sullivan,† G.E. Robinson[1,2]

[1] Department of Entomology, University of Illinois at Urbana–Champaign, 505 S. Goodwin Ave., Urbana, Illinois 61801

[2] Neuroscience Program, University of Illinois at Urbana–Champaign, Urbana, Illinois 61801

Received 10 January 2003; accepted 15 April 2003

ABSTRACT: The behavioral maturation of adult worker honey bees is influenced by a rising titer of juvenile hormone (JH), and is temporally correlated with an increase in the volume of the neuropil of the mushroom bodies, a brain region involved in learning and memory. We explored the stability of this neuropil expansion and its possible dependence on JH. We studied the volume of the mushroom bodies in adult bees deprived of JH by surgical removal of the source glands, the corpora allata. We also asked if the neuropil expansion detected in foragers persists when bees no longer engage in foraging, either because of the onset of winter or because colony social structure was experimentally manipulated to cause some bees to revert from foraging to tending brood (nursing). Results show that adult exposure to JH is not necessary for growth of the mushroom body neuropil, and that the volume of the mushroom body neuropil in adult bees is not reduced if foraging stops. These results are interpreted in the context of a qualitative model that posits that mushroom body neuropil volume enlargement in the honey bee has both *experience-independent* and *experience-dependent* components. © 2003 Wiley Periodicals, Inc. J Neurobiol 00: 000–000, 2003

***Keywords: Apis mellifera*; corpora pedunculata; juvenile hormone; Kenyon cells**

INTRODUCTION

The mushroom bodies are major insect brain centers for learning and memory (Mizunami et al., 1993; Hammer and Menzel, 1995; Strausfeld et al., 1995, 1998; Davis, 1996; Menzel and Muller, 1996; Capaldi et al., 1999). This structure has two distinct compartments: a set of intrinsic neurons referred to as Kenyon cells, and associated neuropils formed by the processes of the Kenyon cells (Mobbs, 1982, 1984; Kenyon, 1896; Mizunami et al., 1993; Farris et al., 1999). As in many regions of the vertebrate brain, there is structural plasticity in the mushroom bodies of several insect species, presumably because functional circuits change in expectation of, or in response to, certain stimuli (Withers et al., 1993; Durst et al., 1994; Heisenberg et al., 1995; Gronenberg et al., 1996; Lomassese et al., 2000; Farris et al., 2001). Such volume changes provide a summed signal of changes that occur in many neurons, and this measure is utilized both as an index of plasticity and as a precursor to cellular studies. Questions regarding the control of mushroom body plasticity will produce answers specific to insects, but questions regarding the function of regional brain growth have great generality, as it is by no means experimentally demon-

* *Present address:* ARL Division of Neurobiology, University of Arizona, Tucson, AZ 85721.

† *Present address:* Stritch School of Medicine, Loyola University Chicago, Maywood, IL 60153.

Correspondence to: S.E. Fahrbach (susanfah@uiuc.edu).

Contract grant sponsor: NSF Award (to S.E.F. and G.E.R.).
Contract grant sponsor: NIMH Award (to G.E.R.).
NSF REU-SITES Award (to S.E.F. and G.E.R.)

DOI 10.1002/neu.10256

strated why "bigger" learning centers are necessarily "better." In recent years studies of the hippocampus from this perspective have flourished, particularly in two major groups of food storing birds, the Paridae and the Corvidae (e.g., Clayton and Krebs, 1995; Basil et al., 1996), in kangaroo rats (Jacobs and Spencer, 1994), and in voles (Galea et al., 1999).

Adult worker honey bees undergo extensive behavioral development, beginning adulthood with tasks restricted to the hive and becoming foragers approximately 3 weeks into a typical life span of 5–6 weeks (Winston, 1987; Robinson, 1992). This age-based division of labor is associated with increases in the circulating levels of the insect developmental hormone, juvenile hormone (JH; Robinson et al., 1989; Robinson, 1992; Fahrbach, 1997). Foragers have the highest titers of JH (Robinson et al., 1989; Huang et al., 1991, 1994; Fahrbach, 1997). Treatment with JH or JH analogs causes precocious onset of foraging (reviewed in Bloch et al., 2002) while removal of the corpora allata, the glands that produce JH, causes a delay eliminated by hormone replacement (Sullivan et al., 2000). Honey bee behavioral maturation is also temporally correlated with an increase in the volume of the neuropil of the mushroom bodies. Foragers have the largest volumes of mushroom body neuropil (Withers et al., 1993; Durst et al., 1994; Farris et al., 2001).

Honey bee workers emerge as adults with mushroom bodies in which neurogenesis is completed and in which all subdivisions of the neuropil are present (Fahrbach et al., 1995c; Farris et al., 1999; Schroter and Malun, 2000). At this time, the volume occupied by the neuronal processes (neuropil) and the neuronal somata (Kenyon cell bodies) is characterized by a ratio of roughly 1:1 neuropil:Kenyon cell bodies. During the adult life of the bee, this ratio shifts, eventually attaining a value of 2:1 or higher (Withers et al., 1993). Two factors contribute to the change in this ratio: the neuropil volume increases as the volume occupied by the somata of the Kenyon cells decreases.

There is no evidence of any change in the number of Kenyon cells during adult life in the honey bee, either an increase or a decrease (Fahrbach et al., 1995c; Ganeshina et al., 2000). This suggests that the Kenyon cell somata must become more densely packed or shrink during adult life. Because, as is typical in insect nervous systems, there is a segregation of neuronal somata and neuropil (axosomatic synapses, e.g., do not occur in the bee brain), the meaning of a change in the volume occupied by the Kenyon cell somata is unknown. Changes in neuropil volume, however, are more susceptible to interpretation. All of the synapses of the mushroom bodies are found in these regions, and changes here must represent change in some feature of the Kenyon cell processes or their synaptic inputs, or both.

The structural plasticity of the honey bee mushroom bodies is linked to an age-independent aspect of behavioral maturation because an increase in neuropil volume occurs earlier in bees induced to forage precociously (Withers et al., 1993; Durst et al., 1994; Farris et al., 2001). Yet, some neuropil growth also occurs in bees reared in social isolation in total darkness (Fahrbach et al., 1998) and in bees prevented from flying outside of the hive (Withers et al., 1995). Recent Golgi studies of the bee mushroom bodies demonstrate that there are at least two components to this growth (Farris et al. 2001). The first is an increase in volume that begins when the neuropils first form during the pupal stage and that continues into the adult stage. This phase thus begins long before the onset of foraging. The second component reflects foraging experience, with more experienced foragers having the largest volumes of mushroom body neuropil as well as greatly enhanced branching and outgrowth of Kenyon cell dendrites (Farris et al., 2001).

The meaning of this convergence of brain structural plasticity, behavioral maturation, and changes in JH titer in the honey bee is unclear. We have speculated that the selective expansion of the mushroom body neuropil is related to the need for foragers to learn visual landmarks (Capaldi et al., 1999), but have not previously explored whether the observed volume changes persist in the absence of foraging and the landmark learning that accompanies foraging. The studies reported here were designed to explore the *stability* of the neuropil expansion and its dependence on JH. We asked if neuropil expansion persists when bees no longer engage in foraging, either because of the onset of winter or because the colony social structure was manipulated to cause some bees to revert from foraging to inside-hive tasks such as caring for larvae (nursing; Bloch & Robinson, 2001). Winter and behavioral reversion are associated with a drop in JH titer and a decrease in rate of JH biosynthesis (Fluri et al. 1977; Huang and Robinson, 1995; Fahrbach, 1997). These behavioral states are of interest because, if JH titer and continued foraging activity are required to maintain the larger volume of mushroom body neuropil, we can predict that the mushroom body neuropil in these groups will have volumes similar to those of younger bees rather than to foragers.

We also asked if mushroom body neuropil expansion occurs in bees that have had the corpora allata surgically removed on the first day of adult life (Sullivan et al., 2000). This tests for an effect of JH on

both early and later phases of mushroom body neuropil expansion. Some of these data were previously published in abstract form (Fahrbach et al., 1995a; Farris et al., 1995).

MATERIALS AND METHODS

Honey Bees

All experiments were performed on adult worker honey bees obtained from colonies at the Bee Research Facility of the University of Illinois at Urbana–Champaign. Colonies were maintained according to standard commercial beekeeping practices. Colonies for specific experiments were derived either from naturally mated queens or instrumentally inseminated queens (Laidlaw, 1977), as described below. Newly emerged adult worker bees were obtained from honeycomb frames of pupae removed from their hive and placed overnight in an incubator maintained at 33°C, 95% relative humidity. One-day-old bees were marked on the dorsal thorax with a dot of enamel paint or a colored number tag, according to the needs of the experiment, prior to being returned to their colony.

Behavior Observations

Observations of flight behavior were made at the hive entrance. Bees returning to the hive entrance with conspicuous pollen loads in their pollen baskets were designated as foragers (Winston, 1987). Observations of nursing behavior were made after opening the hive. Nurse bees were identified as workers that insert their heads into brood cells (Robinson, 1987). These bees were collected off the comb immediately after being observed performing this behavior. Bees for neuroanatomical studies were collected individually using a modified portable vacuum cleaner (Robinson and Page, 1988). Individual experiments are described in detail below.

Histology and Volume Determination

Brains were dissected from the head capsule in a drop of bee saline (Huang et al., 1991). Brains were fixed overnight in alcoholic Bouin's fixative (Presnell and Schreibman, 1997). They were then dehydrated in ethanol, cleared in toluene, and embedded in Paraplast (Oxford Labware, St. Louis, MO) for sectioning and staining. Each brain was cut into 10 μm thick sections. A complete set of frontal plane serial sections from each brain was mounted on chromalum gelatin-coated slides, then stained using Luxol fast blue (Solvent Blue 38, Sigma S3382) and cresyl violet (Sigma C 1791) in a modification of the standard Klüver-Barrera technique (Klüver and Barrera, 1953; Fahrbach et al., 1995b). Stained slides were dehydrated in ethanol, cleared in Hemo-De (Fisher), and coverslipped with Cytoseal (Stephens Scientific).

The volume of the neuropil of the mushroom bodies was estimated by application of the Cavalieri method (Gundersen et al., 1988). Volume estimates included the medial and lateral calyces, peduncle, alpha lobe, and beta lobe [Fig. 1(A) and (B)]. Sections were viewed at a magnification of 300× on a Zeiss microscope and drawn using a camera lucida. Slides were always coded, so that the person doing the drawing did not know the experimental group. Depending on the study, one of the first four or first six sections after the first appearance of the mushroom bodies was chosen using a random number table. From this starting point, every fourth (or sixth) section thereafter was drawn. This procedure meets the requirement of the Cavalieri method for systematic random sampling. As in previous studies (Withers et al., 1993, 1995; Fahrbach et al., 1995b), it was determined that estimates made from every sixth section differed by less than 5% from estimates made using drawings of every section.

Cross-sectional areas were determined by counting the number of points on a transparent 20-mm grid that fell within the traced boundaries of the neuropil. Point counts were converted to volume estimates for each hemisphere, taking into account sampling frequency, magnification, grid size, and section thickness. The efficacy of the Cavalieri method for volume estimation is well documented (Gundersen and Jensen, 1987; Gundersen et al., 1988; Møller et al., 1990; West, 1993). Honey bee workers within a colony are monomorphic, so that corrections for body size are not necessary. Subcompartments recognizable within the calycal neuropil of the honey bee mushroom bodies reflect the distribution of different sets of afferents (e.g., from the antennal lobes or the optic lobes) to subpopulations of Kenyon cells: because present appreciation of the heterogeneity of the Kenyon cell population is likely incomplete, we report volume estimates for the total volume of this neuropil, for which boundaries can be drawn without ambiguity in brains prepared using routine histology. Previous studies suggest that changes in overall volume reflect changes in the subcompartments (Withers et al., 1995).

Collections of Winter Bees with and without Flight Experience

The longest-lived honey bee workers are those that survive the winter in temperate climate zones. Winter bees may mature enough in the fall to initiate foraging, but with the onset of inclement weather they do not forage enough to expend their lives; instead, they remain within the hive, consuming stored food and contributing to colony thermoregulation (Winston, 1987). To study the mushroom bodies of winter bees, we added 200 1-day-old individuals to a typical large colony shortly before the end of the foraging season (October). The bees were from a colony derived from a naturally mated queen. One hundred of these bees were marked with a paint dot, and otherwise unmanipulated. A second set of 100 bees had a plastic disk glued to the dorsal thorax. This disk was of sufficient thickness that, when paired with placement of a mesh grid over the hive

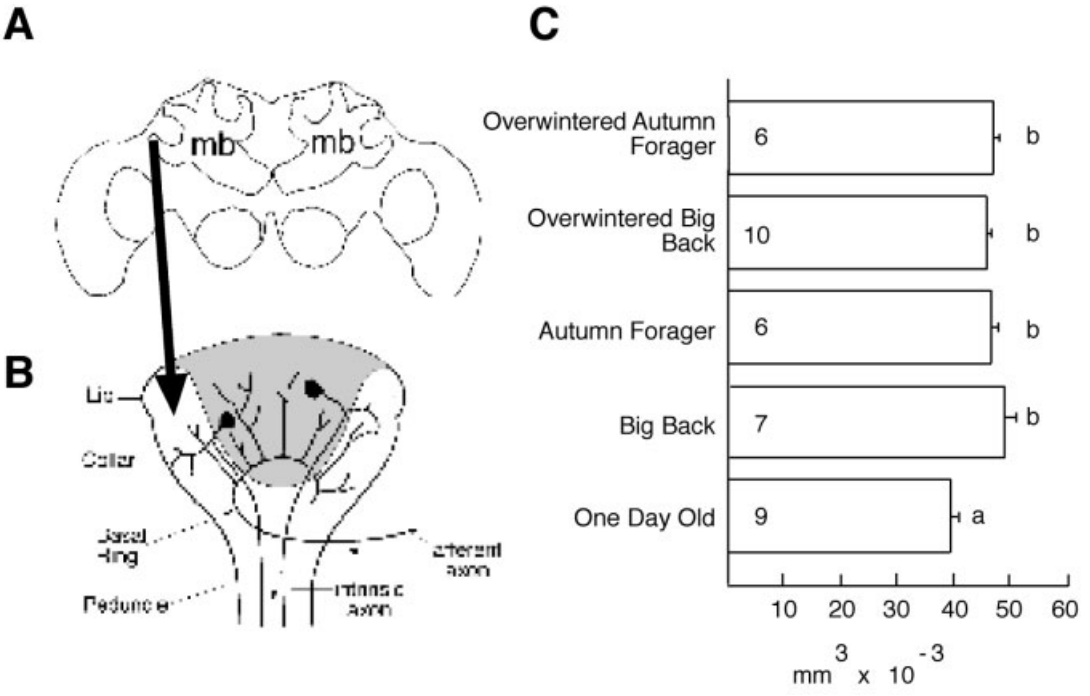

Figure 1 Stability of mushroom body neuropil volume in winter bees, with and without flight experience. (A) Schematic transverse section through the honey bee brain, showing the location of the mushroom bodies (mb) on either side of dorsal midline. Arrow indicates corresponding location in (B). (B) Expanded schematic diagram of one calyx of the honey bee mushroom bodies showing two out of several hundred thousand Kenyon cells. The lip, collar, and basal ring are regions of neuropil formed by the Kenyon cell dendrites and the terminal arborizations of afferents from the antennal and optic lobes. The axons of the Kenyon cells form the peduncle. (C) Volume of the neuropil (mean $\pm$ S.E.M.) of Autumn Foragers and Big Backs, Overwintered Foragers and Big Backs, plus 1-day-olds sampled in the autumn from the same colony. Sample sizes indicated in bars. Methods given in text. ANOVA followed by Student-Newman-Keuls *post hoc* comparisons; groups with the same letters are not significantly different than each other ($p < 0.01$).

entrance, it prevented treated bees from leaving the hive. Movement within the hive by these "big back bees" (Withers et al., 1995) is unaffected. This treatment permitted the comparison of mushroom body neuropil volume in winter bees that differed only in flight experience. A sample of 1-day-old bees was collected at the time of marking for baseline estimates of mushroom body volume.

The hive entrance was checked during periods of good weather for flight activity during the 2 weeks following introduction of marked bees. Marked bees were frequently seen leaving and entering but no big back bees were ever seen outside the hive. The hive was opened 24 days after introduction of marked bees for removal of a sample of each group for volume estimation. After this time, winter weather prevented flight. The hive was opened again the following year near the end of March, prior to the start of spring foraging, and all remaining big back and paint-marked bees were collected for analysis. At the time of this collection, marked bees were 146 days old. Groups compared in this study were: One-Day-Old; Autumn Forager; Big Back; Overwintered Big Back; and Overwintered Autumn Forager.

Collections of Bees Displaying Behavioral Reversion

We established an experimental colony derived from a colony with an adult worker population of about 50,000 bees. This colony is hereafter referred to as the "parent" colony. This colony was derived from a queen with black cuticle color instrumentally inseminated with semen from two different drones, one black and one yellow. This permitted the two resulting subfamilies of progeny to be directly discriminated by cuticle color, as black is recessive to yellow (Tucker, 1986; Robinson et al., 1994). To minimize any possible effects of genetic variation on the development of the mushroom bodies and susceptibility to the reversion stimuli, only workers from the black subfamily were used in this study.

At the start of the experiment, the entrance to the parent colony was blocked with a screen so that foragers could be collected. Bees returning with conspicuous pollen loads or with abdomens distended with nectar were vacuumed directly into a smaller beehive. This hive contained one frame of honey and pollen, one frame of larvae, and a mated

queen. Approximately 1300 black foragers were collected in one afternoon and used to create a colony that contained only older bees ("old bee colony"). The parent colony was then moved to a distant location and the old bee colony put in its place. Reverted nurses (bees in the old-bee colony that switched from foraging to nursing behavior) and persistent foragers (bees in the old bee colony that continued foraging) were observed and collected for brain analysis over a 5-day period immediately following colony establishment. Foragers were collected at the entrance as they returned with pollen loads, after which the colony was opened to collect reverted nurses. Reverted nurses were collected only if they had tattered wings, a widely accepted indicator of extensive prior flight experience (Breed et al., 1990). Three additional groups (1-day-old bees, nurses, and foragers) were collected for comparative brain volume analysis from the parent colony. These bees were collected 2 weeks prior to the creation of the old bee colony.

Collection of Bees without Corpora Allata

Bees were allatectomized within 2 h of adult emergence as previously described (Sullivan et al., 2000). Each allatectomy was completed in 1–2 min. Bees were returned immediately to a cage in a 33°C incubator with water, food, and a piece of honeycomb. Sham-operated bees were treated identically, except that the corpora allata (CA) were moved gently but not removed. Bees in the intact control group were anesthetized for 3 min, but otherwise not manipulated. Brains were sampled from allatectomized bees housed under two conditions: a colony of 20,000 to 30,000 bees in a glass-walled observation hive, and a single cohort colony (Robinson et al., 1989) initially composed of 2000 1-day-old bees. All surgically treated bees remained in an incubator for 12 h before being tagged and placed into either the observation hive or the single cohort colony. At this time, a dose of the JH analog methoprene (200 μL dissolved in 5 μL of acetone) was applied to the dorsal abdomen of a subset of the allatectomized bees placed in the single cohort colony only (methoprene was a gift of Sandoz Agro, Inc., Palo Alto, CA). Other bees were untreated, or treated with the acetone vehicle only. Detailed daily behavioral observations of allatectomized bees were made to determine the effect of lack of JH on behavioral maturation; these results have been reported elsewhere (Sullivan et al., 2000). Brains were collected both from allatectomized foragers and nonforagers, as well as from sham-operated and intact controls. One-day-old bees from the same source colony also were collected. To ensure that allatectomized bees were unquestionably foragers, a higher standard of behavioral identification was used in this study only. Bees designated as foragers had to be observed doing one of the following: taking at least two sequential flights on a single day, each lasting longer than 15 min and resulting in return with a load of pollen each time; a flight lasting longer than 15 min each day on 2 consecutive days, also returning with a load of pollen each time; or performing a waggle dance within the hive. This procedure identifies foragers with near certainty.

Radioimmunoassay of Juvenile Hormone

A radioimmunoassay (RIA) specifically validated for adult honey bees (see Hunnicutt et al., 1989; Huang et al., 1994) was used to verify the efficacy of the allatectomies and confirm that bees that reverted from foraging to nursing had low circulating JH, as previously reported (Huang and Robinson, 1996). JH extraction and RIA were performed following a previously published detailed protocol (Huang et al., 1994; Sullivan et al., 2000). The antiserum used is specific for JHIII, the only form of JH present in honey bees (Hagenguth and Rembold, 1978). Bees were anesthetized on ice for several minutes prior to blood sampling from either the abdomen or the neck. Brains were then removed as described above. Blood samples for RIA analysis were taken from nurses and foragers from the parent colony, and from reverted nurses and persistent foragers from the old bee colony. Samples were taken from parent colony nurses and foragers 2 weeks prior to the establishment of the old bee colony. Samples were taken from reverted nurses and persistent foragers 2, 3, and 5 days after establishment of the old bee colony.

RIA results for a subsample of the group of allatectomized bees studied here were reported by Sullivan et al. (2000) (Table 1; Observation Hive Colony and Single Cohort Colony 1). JH titers of allatectomized bees, with one exception, were indistinguishable from zero while intact control and sham-operated foragers had high JH titers (range: 175–584 ng/mL) typical of foragers. Only a single bee subjected to allatectomy had a high JH titer; inspection of the brain and associated neuroendocrine complexes after histological processing indicated the presence of a corpus allatum remnant in this bee, and she was removed from the study. No fragments of the corpora allata were observed in the remaining allatectomized bees. The adjacent corpora cardiaca endocrine glands were undamaged in every case.

Statistical Analysis

One-way ANOVA and Student-Newman-Keuls *post hoc* tests were used for all experiments (SuperANOVA, Abacus Concepts), with the exception of the allatectomy study for which the Tukey test was used (Zar, 1996). This test was selected for this study because it can accommodate the variation in sample size between the treatment groups present in our data; it is also a conservative test, less likely to reject the null hypothesis falsely than other *post hoc* tests. An unpaired t test (Statview II, Abacus Concepts) was performed on JH RIA data from persistent foragers and reverted nurses. For this analysis, JH titers from all bees sampled (not just those used for brain analysis) were included.

Table 1 Effects of Removal of the Corpora Allata (Juvenile Hormone-Producing Glands) on the Expansion of the Neuropil of the Mushroom Bodies in Honey Bees

		Observation Hive Colony		Single Cohort Colony
Group	*n*	Neuropil Volume[1,2,3]	*n*	Neuropil Volume
1 Day Old	4	36.0 ± 3.2 a	—	—
Control	10	46.9 ± 4.6 b	9	49.8 ± 2.0 a
Sham	7	45.5 ± 5.6 b	8	48.5 ± 3.3 a
CA − Foragers	5	42.5 ± 2.3 ab	15	46.6 ± 2.5 a
CA − Nonforagers	12	46.3 ± 4.2 b	—	—
M CA-	—	—	14	46.9 ± 2.4 a

[1] Data are expressed as means ± S.E.M. Bees were collected as foragers except 1-Day-Old and CA- Nonforager group.

[2] $mm^3 \times 10^{-3}$.

[3] Means followed by the same letter are not significantly different. Observation Hive Colony $F(4, 33) = 3.24$; $p < 0.05$; Single-Cohort Colony $F(3. 42) = 2.28$; $p = 0.09$. *Post hoc* comparisons were made using Tukey's HSD.

Abbreviations: CA − allatectomized; M CA − allatectomized and treated with methoprene; see text for full description of groups.

RESULTS

Stability of Mushroom Body Neuropil Volume in Winter Bees

In an analysis that included 1-day-old bees, significant variation in the volume of the mushroom body neuropil was detected among sister bees collected at the start and end of the winter [Fig. 1(C); ANOVA, $p = 0.0002$]. However, among the four groups of experimental bees there were no effects of age or flight experience on neuropil volumes. No significant differences were detected among the Big Back Bees and Autumn Foragers collected at either 24 ($N = 7$ and $N = 6$, respectively) or 146 (Overwintered Big Back, $N = 10$; Overwintered Forager, $N = 6$, respectively) days of age. All of these groups had a significantly greater mushroom body neuropil volume than 1-day-olds ($N = 9$; Student-Newman-Keuls, $p < 0.01$). JH titers and rates of JH biosynthesis are known to be consistently low in winter bees (Fluri et al. 1977; Huang and Robinson 1995); therefore, JH measurements were not made in this study.

Stability of Mushroom Body Neuropil Volume in Bees Displaying Behavioral Reversion

Foragers that reverted to nursing displayed a significant drop in JH titer relative to foragers in the parent colony (ANOVA, $p = 0.0001$, Student-Newman-Keuls, $p < 0.01$), suggesting that behavioral reversion had a strong effect on JH titers, as reported in previous studies (Huang and Robinson 1996). When compared with foragers from the parent colony, persistent foragers in the old bee colony also experienced a drop in JH (as in Huang and Robinson, 1996) However, JH titers of persistent foragers in the old bee colony remained significantly higher than those of the reverted nurses in the same colony [Fig. 2(A), unpaired t test, $p < 0.01$] throughout the 5-day collection period.

Despite these differences in JH titers there was no effect of behavioral reversion on the volume of the mushroom body neuropil [Fig. 2(B)]. No significant differences were detected among reverted nurses ($N = 10$) or persistent foragers ($N = 10$) in the old bee colony, or when these groups were compared with nurses and foragers from the parent colony ($N = 10$). Only 1-day-old bees ($N = 8$) had significantly smaller neuropil volumes than the other groups [Fig. 2(B); ANOVA, $p = 0.0001$, Student-Newman-Keuls, $p < 0.01$].

Mushroom Body Neuropil Volume in Bees without Corpora Allata

Neuropil volume growth occurred in allatectomized bees in both colonies (Table 1). For bees from the observation hive colony, the volume of the neuropil of the mushroom bodies of control and sham-operated foragers and of foraging and nonforaging allatectomized bees was significantly greater than that of 1-day-old bees, but the older groups did not differ in pairwise comparisons. For bees from the single cohort colony, the estimated volumes of the mushroom bodies were compared for four groups: intact controls, sham-operated, allatectomized bees that foraged, and allatectomized bees treated with methoprene. There were no differences among these groups.

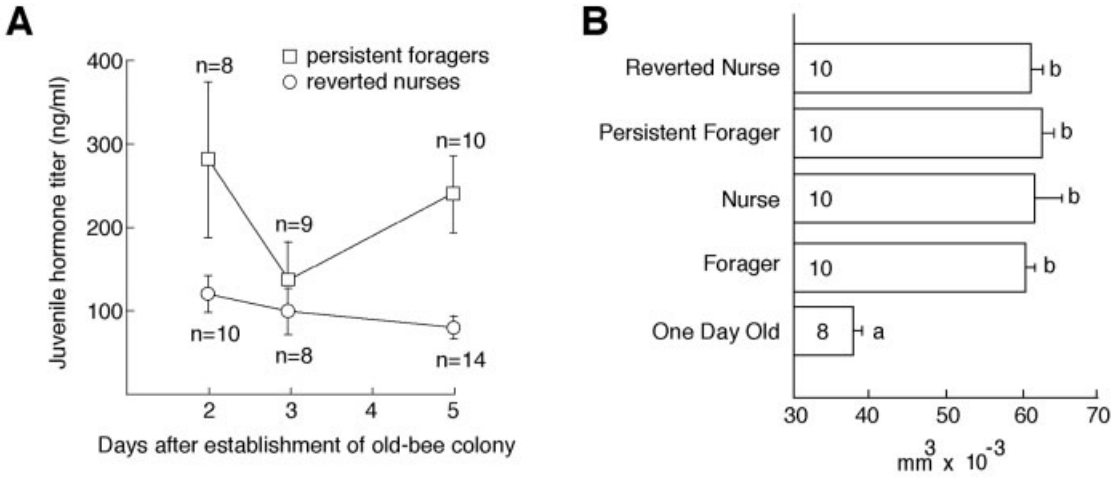

Figure 2 Change in JH titers and stability of mushroom body neuropil volume in bees displaying behavioral reversion. (A) Radioimmunoassay results (ng/mL) for JH in persistent foragers and reverted nurses in the old bee colony, by day of collection. Methods given in text. Mean ± S.E.M. is shown for each of the 3 collection days. Paired t test $p < 0.01$ for all reverted nurses versus persistent foragers. (B) Volume of the neuropil (mean ± S.E.M.) of reverted nurses and persistent foragers from the old bee colony and nurses, foragers, and 1-day-olds from the parent colony. Sample sizes indicated in bars. Methods given in text. ANOVA followed by Student-Newman-Keuls *post hoc* comparisons; groups with the same letters are not significantly different than each other.

DISCUSSION

Withers et al. (1995) treated bees prevented from foraging with JH analog (methoprene), and, upon observing an increase in mushroom body neuropil volume, suggested that adult exposure to JH was the causal mechanism. The focus of this particular study was on the specific contribution of flight experience to mushroom body growth; therefore, to isolate the potential effects of experience nonforaging bees were treated with methoprene to assure endocrine status comparable to that of foragers. The present study tested this JH hypothesis by using surgical, social, and seasonal manipulations to reduce or eliminate JH. The results from our studies show that adult exposure to JH is *unnecessary* for growth of the mushroom body neuropil during adult life, and that the growth of the mushroom body neuropil in adult bees is *not* reversed if the bee ceases to forage, whether for a few days or an entire winter. In fact, we can now state with certainty the surprising result that exposure to JH is not necessary to attain a forager-like volume of the neuropil of the mushroom bodies.

Although the present data rule out an absolute requirement for JH in the expansion of the mushroom body neuropil, several possible roles remain for this hormone. First, JH may act to control the *rate of development* of the mushroom bodies. A high titer may accelerate expansion of the mushroom bodies, as observed in precocious foragers, while slower growth may occur with lower titers. Such an effect would have been missed in the present study in which we only examined the brains of established foragers or matched age nonforagers. Behavioral and metabolic analyses have shown that JH acts to control the timing of the onset of foraging (Sullivan et al., 2000), possibly through regulation of the development of flight muscle (Sullivan et al., 2003). Allatectomy delays the onset of foraging, and this effect is eliminated with hormone replacement. This corroborates the general idea that JH is associated with the pacing of development in insects (Robinson and Vargo, 1997; Dingle, 2002). In crickets, JH has been shown specifically to stimulate neurogenesis in the mushroom bodies of adult females (Cayre et al., 1994), but JH effects on other aspects of Kenyon cell development in the cricket have not been reported.

One possibility is that the maximal volume of the mushroom body neuropil may be attained only in active foragers exposed to high titers of JH. At present, the evidence for this is indirect and based upon comparison of the volume of the mushroom body neuropil across independent studies. The following meta-analysis is speculative, but suggestive. In the

present study, the volume of the neuropil of the mushroom bodies increased approximately 20% in allatectomized bees. Bees reared in complete darkness in individual cages showed a similar increase, which also was not affected by exposure to the JH analog, methoprene (Fahrbach et al., 1998). Withers et al. (1995) reported a comparable significant, but also relatively low (13.8%) level of neuropil expansion in big back bees and in precocious foragers (19%). This latter group was collected at 11 days of age after 2–3 days of foraging, and hence, did not have prolonged experience of foraging in the presence of JH. Other sets of bees characterized by significant but relatively small (15 to 22%) increases in mushroom body neuropil are the Autumn Foragers, Big Backs, Overwintered Autumn Foragers, and Overwintered Big Backs described in the present report. Although the Autumn Foragers were observed flying from the hive, the relative paucity of flowers in late October and early November almost certainly resulted in a low level of foraging activity relative to that displayed by summer foragers. Results from another study (Huang and Robinson, 1995) suggest that even foragers have low JH titers at this time of year. In contrast, experienced, active foragers in the summer have the largest volumes of mushroom body neuropil both in terms of absolute values attained and in terms of percentage increase relative to 1-day-olds from the same colony. For example, established foragers sampled in the present reversion experiment had increases ranging from 50–55%, and bees with 2 weeks of foraging experience showed a 45% increase in mushroom body neuropil volume relative to 1-day-olds (Farris et al., 2001). Experienced foragers also have greatly increased branching and lengthening of Kenyon cell dendrites compared with age-matched, less experienced bees (Farris et al., 2001). Whether these differences are due to the intensity or specific nature of the foraging experience alone or foraging in conjunction with exposure to JH remains to be determined.

Once an initial expansion of the neuropil of the mushroom bodies has been achieved, is the change permanent? The present study shows that normal age foragers in both of these studies had enlarged mushroom body neuropils, and that the levels did not decline over the winter or upon resumption of nursing. Once expanded, this neuropil stays expanded. This finding simplifies our understanding of this process because we can now assume that the volume we detect at any time reflects the largest volume attained rather than a dynamic mixture of growth and regressive events.

The life history of the honey bee may seem far removed from that of longer lived vertebrates, but the processes by which the nervous system is modified during adult life are likely very similar. Studies of adult brain structural plasticity in vertebrates have also produced evidence of stability after expansion. For example, increases in cortical weight and thickness in rats following 1 month of exposure to a complex environment declined only slightly during a subsequent month of exposure to a simple environment (Katz and Davies, 1984). Increased dendritic branching in the rat occipital cortex is also maintained when the animal is moved from a complex environment to an individual cage (Camel et al., 1986). Continued housing of rats in a complex environment is not needed to retain the structural modifications that were the result of the initial exposure (Greenough and Chang, 1988). Some species of food-storing birds need to have experience storing food or performing related memory tasks before they experience volume increase in the hippocampus, but once enlarged, the hippocampus remains so even if the bird is denied further opportunity to learn the location of food items (reviewed in Capaldi et al., 1999). The experience-dependent phenomena can be contrasted with growth supported by hormonal signals. In the well-studied song system of male song birds, for example, the song nuclei wax and wane with seasonal changes in gonadal production of androgen (Nottebohm, 1981; Ball 2000; Tramontin and Brenowitz, 2000).

In summary, all adult worker bees add neuropil to the mushroom bodies during adult life. This phenomenon is seen in bees reared in constant darkness in social isolation (Fahrbach et al., 1998), in bees denied all flight experience (Withers et al., 1995), and in bees in which the glands producing JH were removed on the first day of adult life (present account). Mushroom body neuropil expansion has also been seen in queens, independent of mating or flight experience, and in drones (Fahrbach et al., 1995b, 1997). The process begins in pupae and continues through adulthood (Farris et al., 2001). This highly reproducible observation strongly suggests that there is a component of mushroom body neuropil growth in bees independent of experience. These results are consistent with reports from studies using *Drosophila* showing that there may be an experience-independent expansion of mushroom body neuropil volume in young adult fruit flies, regardless of rearing conditions (Heisenberg et al., 1995).

But the acquisition of forager status is a developmental milestone in the life of a worker bee. Foraging requires multimodal integration of sensory information, navigation abilities, the ability to learn how to manipulate flowers to obtain food efficiently and the ability to remember distinguishing characteristics of

the most rewarding species available. Once the transition to foraging occurs, conditions in the brain appear to be altered so that the process of enlarging the mushroom body neuropil is enhanced. This is evident from the results of the meta-analysis described above, showing that it is the most experienced foragers that have the largest volumes of mushroom body neuropil. This is also consistent with the association of a precocious onset of foraging with a precocious expansion of the mushroom body neuropil (Withers et al., 1993, 1995; Durst et al., 1994), a finding also reported for worker ants (Gronenberg et al., 1996).

Now that it is clear that mushroom body neuropil volume enlargement in honey bees has both *experience-independent* and *experience-dependent* components, it is important to determine whether they are controlled by the same underlying molecular mechanisms.

We thank Jennifer Drew, Nick Gomes, Thomas Hallam, Cynthia Mata, Brendan O'Connell, and Jennifer Strand for excellent field assistance, Omar Jassim for assistance with the RIA, Jack Kuehn for help with beekeeping, and Tomoko Yoshimura, Kimberly Rosiak-Seo, and Alex McCampbell for help with histology.

REFERENCES

Ball GF. 2000. Neuroendocrine basis of seasonal changes in vocal behavior among songbirds. In: Hauser M, Konishi M, editors. Neural mechanisms of communication. Cambridge, MA: MIT Press, p 213–253.

Basil JA, Kamil AC, Balda RP, Fite KV. 1996. Differences in hippocampal volume among food storing corvids. Brain Behav Evol 47:156–164.

Bloch G, Robinson GE. 2001. Socially mediated plasticity in honeybee behavioural rhythms. Nature 410:1048.

Bloch G, Wheeler DE, Robinson GE. 2002. Endocrine influences on the organization of insect societies. In: Pfaff DW, Arnold AP, Etgen AM, Fahrbach SE, Rubin RT, editors. Hormones, brain, and behavior, vol. 3. San Diego, CA: Academic Press, p 195–235.

Breed MD, Robinson GE, Page RE. 1990. Division of labor during honey bee colony defense. Behav Ecol Sociobiol 27:395–401.

Camel JE, Withers GS, Greenough WT. 1986. Persistence of visual cortex dendritic alterations induced by postweaning exposure to a "superenriched" environment in rats. Behav Neurosci 100:810–813.

Capaldi EA, Robinson GE, Fahrbach SE. 1999. Neuroethology of spatial learning: the birds and the bees. Annu Rev Psychol 50:651–682.

Cayre M, Strambi C, Strambi A. 1994. Neurogenesis in an adult insect brain and its hormonal control. Nature 368: 57–59.

Clayton NS, Krebs JR. 1995. Memory in food-storing birds: from behaviour to brain. Curr Opinion Neurobiol 5:149–154.

Davis RL. 1996. Physiology and biochemistry of Drosophila learning mutants. Physiol Rev 76:299–317.

Dingle H. 2002. Hormonal mediation of insect life histories. In: Pfaff DW, Arnold AP, Etgen AM, Fahrbach SE, Rubin RT, editors. Hormones, brain, and behavior, vol. 3. San Diego, CA: Academic Press, p 237–279.

Durst C, Eichmuller S, Menzel R. 1994. Development and experience lead to increased volume of subcompartments of the honeybee mushroom body. Behav Neural Biol 62:259–263.

Fahrbach SE. 1997. Regulation of age polyethism in bees and wasps by juvenile hormone. Adv Study Behav 26: 285–316.

Fahrbach SE, Farris SM, Robinson GE. 1995a. Coincident maturation of flight behavior and the mushroom bodies in the honey bee. Soc Neurosci Abstr 21:458.

Fahrbach SE, Giray T, Robinson GE. 1995b. Volume changes in the mushroom bodies of adult honey bee queens. Neurobiol Learn Mem 63:181–191.

Fahrbach SE, Giray T, Farris SM, Robinson GE. 1997. Expansion of the neuropil of the mushroom bodies in male honey bees is coincident with initiation of flight. Neurosci Lett 236:135–138.

Fahrbach SE, Strande JL, Robinson GE. 1995c. Neurogenesis is absent in the brains of adult honey bees and does not explain behavioral neuroplasticity. Neurosci Lett 197: 145–148.

Fahrbach SE, Moore D, Capaldi EA, Farris SM, Robinson GE. 1998. Experience-expectant plasticity in the mushroom bodies of the honeybee. Learn Mem 5:115–123.

Farris SM, Fahrbach SE, Robinson GE. 1995. Effects of behavioral reversion on brain structural plasticity in the honey bee. Soc Neurosci Abstr 21:458.

Farris SM, Robinson GE, Davis RL, Fahrbach SE. 1999. Larval and pupal development of the mushroom bodies in the honey bee, Apis mellifera. J Comp Neurol 414:97–113.

Farris SM, Robinson GE, Fahrbach SE. 2001. Experience- and age-related outgrowth of intrinsic neurons in the mushroom bodies of the adult worker honeybee. J Neurosci 21:6395–6404.

Fluri P, Wille H, Grig L, Lüscher M. 1977. Juvenile hormone, vitellogenin and haemocyte composition in winter worker honeybees (Apis mellifera). Experientia 33:1240–1241.

Galea LAM, Perrot-Sinal TS, Kavaliers M, Ossenkopp KP. 1999. Relations of hippocampal volume and dentrate gyrus to gonadal hormone levels in male and female meadow voles. Brain Res 821:383–391.

Ganeshina O, Schafer S, Malun D. 2000. Proliferation and programmed cell death of neuronal precursors in the mushroom bodies of the honeybee. J Comp Neurol 417: 349–365.

Greenough WT, Chang F-LF. 1988. Plasticity of synapse structure and pattern in the cerebral cortex. In: Peters A,

Jones EG, editors, Cerebral cortex, vol. 7. New York: Plenum, p 391–440.

Gronenberg W, Heeren S, Holldöbler B. 1996. Age-dependent and task-related morphological changes in the brain and mushroom bodies of the ant Camponotus floridanus. J Exp Biol 199:2011–2019.

Gundersen HJG, Bendtsen TF, Korbo L, Marcussen N, Møller A, Nielsen K, Nyengaard JR, Pakkenberg B, Sorensen FB, Vesterby A, et al. 1988. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APMIS 96:379–394.

Gundersen HJG, Jensen EB. 1987. The efficiency of systematic sampling in stereology and its prediction. J Microsc 147:229–263.

Hagenguth H, Rembold H. 1978. Identification of juvenile hormone 3 as the only JH homolog in all developmental stages of the honey bee. Z Naturforschung 33C:847–850.

Hammer M, Menzel R. 1995. Learning and memory in the honeybee. J Neurosci 15:1617–1630.

Heisenberg M, Heusipp M, Wanke C. 1995. Structural plasticity in the Drosophila brain. J Neurosci 15:1951–1960.

Huang Z-Y, Robinson GE, Tobe SS, Yagi KJ, Strambi C, Strambi A, Stay B. 1991. Hormonal regulation of behavioural development in the honey bee is based on changes in the rate of juvenile hormone biosynthesis. J Insect Physiol 37:733–741.

Huang ZY, Robinson GE, Borst DW. 1994. Physiological correlates of division of labor among similarly aged honey bees. J Comp Physiol A 174:731–739.

Huang ZY, Robinson GE. 1995. Seasonal changes in juvenile hormone titers and rates of biosynthesis in honey bees. J Comp Physiol B 165:18–28.

Huang ZY, Robinson GE. 1996. Regulation of honey bee division of labor by colony age demography. Behav Ecol Sociobiol 39:147–158.

Hunnicutt D, Toong YC, Borst DW. 1989. A chiral specific antiserum for juvenile hormone. Am Zool 29:48A.

Jacobs LF, Spencer WD. 1994. Natural space-use patterns and hippocampal size in kangaroo rats. Brain Behav Evol 44:125–132.

Katz HB, Davies CA. 1984. Effects of differential environments on the cerebral anatomy of rats as a function of previous and subsequent housing conditions. Exp Neurol 83:274–287.

Kenyon FC. 1896. The brain of the bee. A preliminary contribution to the morphology of the nervous system of the Arthropoda. J Comp Neurol 6:133–210.

Klüver H, Barrera E. 1953. A method for the combined staining of cells and fibers in the nervous system. J Neuropathol Exp Neurol 12:400–403.

Laidlaw HH. 1977. Instrumental insemination of honey bee queens. Hamilton IL: Dadant and Sons Press.

Lomassese SS, Strambi C, Strambi A, Charpin P, Augier R, Aouane A, Cayre M. 2000. Influence of environmental stimulation on neurogenesis in the adult insect brain. J. Neurobiol. 45:162–171.

Menzel R, Muller U. 1996. Learning and memory in honeybees—from behavior to neural substrates. Annu Rev Neurosci 19:379–404.

Mizunami M, Weibrecht JM, Strausfeld NJ. 1993. A new role for the insect mushroom bodies: place memory and motor control. In: Beer RD, Ritzman RE, McKenna T, editors. Biological neural networks in invertebrate neuroethology and robotics. New York: Academic Press, p 199–225.

Mobbs PG. 1982. The brain of the honeybee Apis mellifera L. The connections and spatial organization of the mushroom bodies. Philos Trans R Soc Lond B 298:309–354.

Mobbs PG. 1984. Neural networks in the mushroom bodies of the honeybee. J Insect Physiol 30:43–58.

Møller A, Strange P, Gundersen HJG. 1990. Efficient estimation of cell volume using the nucleator and disector. J Microsc 159:61–71.

Nottebohm F. 1981. A brain for all seasons: cyclical anatomical changes in song control nuclei of the canary brain. Science 21:1368–1370.

Presnell JK, Schreibman MP. 1997. Humason's animal tissue techniques, 5th ed. Baltimore: Johns Hopkins.

Robinson GE. 1987. Regulation of honey bee age polyethism by juvenile hormone. Behav Ecol Sociobiol 20: 329–338.

Robinson GE. 1992. Regulation of division of labor in insect societies. Annu Rev Entomol 37:637–665.

Robinson GE Page RE. 1988. Genetic determination of guarding and undertaking in honey-bee colonies. Nature 333:356–358.

Robinson GE, Page RE, Arensen N. 1994. Genotypic differences in brood rearing in honey bee colonies: context-specific? Behav Ecol Sociobiol 34:125–137.

Robinson GE, Page RE, Strambi C, Strambi A. 1989. Hormonal and genetic control of behavioral integration in honey bee colonies. Science 246:109–112.

Robinson GE, Vargo EL. 1997. Juvenile hormone in adult eusocial Hymenoptera—gonadotropin and behavioral pacemaker. Arch Insect Biochem Physiol 35:559–583.

Schroter U, Malun D. 2000. Formation of antennal lobe and mushroom body neuropils during metamorphosis in the honeybee, Apis mellifera. J Comp Neurol 422:229–245.

Strausfeld NJ, Buschbeck EK, Gomez RS. 1995. The arthropod mushroom body: Its functional roles, evolutionary enigmas and mistaken identities. In: Breidbach O, Kutsch W, editors. The nervous systems of invertebrates: an evolutionary and comparative approach. Basel: Birkhäuser, p 349–381.

Strausfeld NJ, Hansen L, Li YS, Gomez RS, Ito K. 1998. Evolution, discovery, and interpretations of arthropod mushroom bodies. Learn Mem 5:11–37.

Sullivan JP, Jassim O, Fahrbach SE, Robinson GE. 2000. Juvenile hormone paces behavioral development in the adult worker honey bee. Horm Behav 37:1–14.

Sullivan JP, Fahrbach SE, Harrison, JF, Capaldi EA, Fewell JH, Robinson GE. 2003. Juvenile hormone and division of labor in the honey bee: effects of allatectomy on flight behavior and metabolism. J Exp Biol 206:2287–2296.

Tramontin AD, Brenowitz EA. 2000. Seasonal plasticity in the adult brain. Trends Neurosci 23:251–258.

Tucker KW. 1986. Visible mutants. In: Rinderer TE, editor. Bee genetics and breeding. New York: Academic Press, p 57–90.

West MJ. 1993. New stereological methods for counting neurons. Neurobiol Aging 14:275–285.

Winston ML. 1987. The biology of the honey bee. Cambridge, MA: Harvard University Press.

Withers GS, Fahrbach SE, Robinson GE. 1993. Selective neuroanatomical plasticity and division of labour in the honeybee. Nature 364:238–240.

Withers GS, Fahrbach SE, Robinson GE. 1995. Effects of experience and juvenile hormone on the organization of the mushroom bodies of honey bees. J Neurobiol 26: 130–144.

Zar JH. 1996. Biostatistical analysis. Upper Saddle River, NJ: Prentice-Hall.