Comparisons of Juvenile Hormone Hemolymph and Octopamine Brain Titers in Honey Bees (Hymenoptera: Apidae) Selected for High and Low Pollen Hoarding

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ABSTRACT We measured circulating levels of juvenile hormone III (JH) and brain region levels of dopamine, serotonin, and octopamine in honey bees, *Apis mellifera* L., from artificially selected high and low pollen-hoarding strains that show differences in their rate of behavioral development. One-day-old bees from the high pollen-hoarding strain had significantly higher JH titers than 1-d-old bees from a low pollen-hoarding strain. Conversely, there were no differences in JH levels in 12-d-old preforager bees from the high and low strains. Brain region levels of all three amines increased with age, but there were no differences between high and low pollen-hoarding bees in any of the three amines in any region of the brain. These results are consistent with the hypothesis that endocrine events occurring early in adulthood influence honey bee behavioral development.

KEY WORDS *Apis mellifera*, dopamine, serotonin, division of labor, behavioral development

COLONIES OF ADVANCED SOCIAL insects, such as the honey bee, *Apis mellifera* L., display an age-related division of labor among workers (Wilson 1971) that is based upon a stereotyped pattern of worker behavioral development. Young worker honey bees work in the hive for the first 2–3 wk of their adult lives, specializing on tasks such as feeding larvae, storing food, and maintaining the nest, and then make a transition to foraging outside the hive for the remainder of their 5- to 7-wk life span (Winston 1987, Robinson 1992). There also is a division of labor among foragers: individuals tend to specialize on collecting pollen, nectar, water, propolis, or scouting for new food sources. There are both environmental and genetic determinants of this type of behavioral specialization in honey bee colonies.

Environmental and genetic determinants of behavioral specialization in honey bee colonies have been particularly well studied with respect to pollen and nectar foraging. The allocation of effort to pollen and nectar foraging is dependent, in part, upon colony needs. Foragers can determine their colony's need for pollen and nectar independently, either through direct assessment of colony stores, indirect assessment via social interactions within the colony, or perhaps both (Camazine 1993, Seeley 1996, Pankiw et al. 1998, Dreller et al. 1999). This leads to adjustments in the amount of nectar and pollen brought back, via changes in behavioral specialization, activity levels, and load size (Fewell and Winston 1992; Fewell and Page 1993, 2000; Pankiw and Page 2001).

Specialization on pollen and nectar foraging also is strongly influenced by genetic factors (Calderone and Page 1988, 1991; Robinson and Page 1989; Hunt et al. 1995; Page and Fondrk 1995; Page et al. 1995). For example, bees have been selected for high and low pollen-hoarding behavior, initially by Hellmich et al. (1985) and again by Page and Fondrk (1995). Page and Fondrk (1995) carried out two-way selection for quantities of stored pollen resulting in the production of high and low pollen-hoarding strains of bees. The high pollen-hoarding strain stores an average of 6 times more pollen than the low pollen-hoarding strain. The total numbers of foragers do not differ between the high and low strains, but the proportion of foragers collecting pollen is significantly higher in the high strain. At least three major quantitative trait loci (QTL) have been associated with variation in foraging specialization between the high and low pollen-hoarding strains, and confirmed in wild-type colonies (Hunt et al. 1995, Page et al. 2000). Identification of specific genes and pathways associated with these QTL has not yet occurred.

The behavioral effects of selection for high and low pollen-hoarding behavior were not limited to foraging specialization. Bees from the high pollen-hoarding strains of Hellmich et al. (1985) and Page and Fondrk (1995) showed accelerated behavioral development, becoming foragers at significantly younger ages than bees from the low pollen-hoarding strain (Calderone

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and Page 1988, 1991; Robinson and Page 1989; Pankiw and Page 2001). Bees from the high pollen-hoarding strain of Page and Fondrk (1995) also show relatively low response thresholds to sucrose, and this difference is already detectable at very young ages, long before the onset of foraging (Page et al. 1998, Pankiw and Page 1999). Recent findings demonstrated phenotypic correlations among these three traits in unselected bees of African and European descent (Pankiw and Page 2000, Pankiw 2003), suggesting that some mechanisms that affect variation in foraging specialization also may affect variation in sucrose responsiveness and age at onset of foraging.

The unexpected finding of effects of artificial selection for pollen hoarding on age at onset of foraging provides an opportunity to study physiological mechanisms of behavioral development. Several endocrine and neural mechanisms regulating age at onset of foraging have been identified (Robinson 2002). Two such factors, juvenile hormone III (JH) and octopamine, have been shown to influence behavioral development. It is possible that genetic variation in some aspect of these mechanisms plays a role in causing variation in both age at onset of foraging and foraging specialization.

There typically is an age-dependent increase in hemolymph titers of JH in adult worker honey bees, and foragers have higher levels than do bees working in the hive. Hormone treatment accelerates the onset of foraging (Bloch et al. 2002) and allatectomy delays it, a deficit that is eliminated by hormone treatment (Sullivan et al. 2000). There is a small peak of JH in 2–3-d-old bees, well before the onset of foraging, but the significance of this peak is not known (Jassim et al. 2000). However, Giray et al. (1999) showed that JH titers differ in young bees from (unselected) stocks that differ in rate of behavioral development, with higher titers associated with bees showing an earlier age at onset of foraging, suggesting that early endocrine events may influence behavioral development. Because bees from the high pollen-hoarding strain initiate foraging at a younger age, we hypothesized that they have higher JH titers before the onset of foraging.

The neurochemical octopamine also has been shown to influence honey bee behavioral development. There is an age-dependent increase in brain levels of octopamine, and foragers have higher levels than do bees working in the hive, particularly in the antennal lobes (Schulz and Robinson 1999, Wagener-Hulme et al. 1999), and octopamine treatment induces precocious foraging (Schulz and Robinson 2001). There also is genetic variation for brain levels of octopamine (Harris and Woodring 1992), but this has not been examined with respect to variation in age at onset of foraging. JH analog treatment caused octopamine levels in the antennal lobes to increase before the onset of foraging (Schulz et al. 2002a, b), suggesting that JH and octopamine interact somehow to regulate honey bee behavioral development. Octopamine also modulates sucrose responsiveness (Pankiw and Page 2003, Scheiner et al. 2003, 2004). Bees treated with octopamine showed an increase in responsiveness to sucrose. Bees more responsive to sucrose also had more activated protein kinase A (PKA) in their antennal lobes (Scheiner et al. 2003). PKA is activated by cAMP and is modulated by octopamine (Hildebrandt and Muller 1995, Blenau et al. 2000).

The purpose of this study is to investigate the potential link between early behavioral development in high pollen-hoarding bees and two physiological factors known to influence forager development; JH and octopamine. We hypothesized that bees from the high pollen-hoarding strain have higher brain levels of octopamine, particularly in the antennal lobes, before the onset of foraging. We also measured brain levels of dopamine and serotonin from the same bees. Both dopamine and serotonin do not seem to be involved in regulating age at onset of foraging (Schulz and Robinson 2001) in honey bees. However, dopamine plays a role in determining responsiveness to stimuli (Mercer and Menzel 1982) and serotonin regulates the balance of protein and carbohydrate intake in solitary insects (Cohen et al. 1988).

Materials and Methods

Bees. The bees used in this study were derived from the high and low pollen-hoarding strains selected by Page and Fondrk (1995) from a mixture of European races of honey bees typical of the northern California area. Bees for JH and octopamine analysis were derived from one high strain and one low strain colony, randomly selected from among the colonies in each strain. We think this is reasonable because there is high repeatability of the behavioral differences between these strains, which are now in the 17th generation of selection. One-day-old adult bees were obtained from frames of honeycomb containing old pupae that were removed from the colonies in the field and placed in an incubator at 33°C. Some 1-d-old bees were collected immediately for analysis ($n = 50$ of each strain), whereas others were marked on the thorax with a spot of paint (Testor's PLA, Testor Corporation, Rockford, IL) to indicate age and strain and placed together into an unrelated "host colony" for later sampling. The host colony was in a single story Langstroth hive and had a laying queen and presumably with a normal age distribution of brood and adult workers.

Marked bees from the high and low pollen-hoarding strains were collected when they were 12 d old. We were interested in collecting only those bees that had not yet initiated foraging because we wanted to determine whether there were genetic differences in JH and octopamine that could plausibly be linked to genetic differences in age at onset of foraging. To collect these "preforagers," the host colony was checked daily for flying marked bees. Colony entrances were observed for returning paint-marked bees for 20 min during the first 2 d after the initial introduction. Beginning on the third day, colony entrances were blocked with wire-mesh for 15-min intervals separated by at least 30 min. The entrances were blocked for a

total of 4 h per day between 0800 and 1700 hours. When the first bees from the high pollen-hoarding strain were observed to initiate foraging (they start earlier), the colony was moved to a new site (25 m away from the original site) and a trap hive placed at the original site to prevent foragers from finding the new colony location. With this method, we could thus be reasonably sure that the marked bees still in the host colony had not yet initiated foraging. Marked high and low line bees ($n = 50$ each), 12 d old, were then collected from brood combs inside the hive. Bees (both 1-d-old bees and preforagers) for high-performance liquid chromatography (HPLC) analysis were vacuumed directly from brood combs into liquid nitrogen (Wagener-Hulme et al. 1999) and stored at −80°C until brain dissection and HPLC analysis. Bees for JH analysis were collected and chilled for hemolymph sampling.

Foraging specialization was not measured in this study, but bees from the high and low pollen-hoarding strains consistently show differences in this trait (Calderone and Page 1988, Robinson and Page 1989, Calderone and Page 1991, Pankiw and Page 2001). The same source colonies used in this study demonstrated consistent differences in age at first foraging in Pankiw and Page (2001).

Hemolymph Collection and JH Radioimmunoassay. Hemolymph was collected from adult bees by piercing the intersegmental membrane between the second and third abdominal segments and collecting the hemolymph in a prebaked 5-μl microcapillary tube. At least 2 μl of hemolymph was collected, measured to the nearest 0.1 μl, and expelled into 500 μl of acetonitrile to precipitate hemolymph proteins and JH-degrading enzymes. Samples were kept at −20°C until processing by radioimmunoassay (RIA). We measured circulating JH III titers by using a chiral-specific RIA specifically validated for adult worker honey bees and described in detail previously (Huang et al. 1994, Sullivan et al. 2000). The sensitivity of this RIA is ≈5 pg of *R*-(−)-JH III/25 μl of hemolymph. Samples from both the high and low strains were analyzed in each daily assay. JH titers were determined for individual bees.

Brain Dissection and HPLC Analysis. To facilitate dissection of antennal lobes and mushroom bodies whole bee heads were partially freeze-dried for 62 min at −10°C and 300 mTorr (Schulz and Robinson 1999). Brains were removed from ≈20 bees per group and separated into three regions: the mushroom bodies (paired medial and lateral calyces and Kenyon cell somata, but no peduncle), the antennal lobes, and the remainder of the proto/deutocerebrum. Optic lobes were removed and discarded. Dissections were performed on dry ice and brains were never allowed to thaw.

Quantification of octopamine, serotonin, and dopamine in each brain region was performed simultaneously by HPLC with electrochemical detection as described in Schulz and Robinson (1999), except that samples were extracted in 20 μl of 0.2 M perchloric acid (Wagener-Hulme et al. 1999). Internal standards (synephrine and dihydroxybenzylamine) were used for all samples, and each run was calibrated with external standards (octopamine, dopamine, and serotonin). Brain regions were analyzed blind with respect to age and strain, and equal numbers of bees from each group in a trial were represented in each HPLC run to control for day-to-day variation. Chromatogram analyses and amine quantification were done with EZChrom Chromatography Data System version 6.8 (Scientific Software, Pleasanton, CA). Results are expressed as a concentration of amines per protein in the sample to account for any differences in dissection. Quantification of protein was performed as described in Schulz and Robinson (1999), by using a kit based on the Lowry method (Bio-Rad, Hercules, CA). Amine levels were determined for individual bees.

Statistical Analyses. Analyses were performed with StatView (SAS Institute 1998). Data for JH, octopamine, dopamine, and serotonin were tested for normality (Kolmogorov–Smirnov normality test) and when necessary, normalized by log transformation. The data were analyzed by two-way analysis of variance (ANOVA) with age (1 and 12 d of age) and strain (high and low pollen hoarding) as factors, followed by Fisher's least significant difference (LSD) post hoc analyses.

Results

There was a significant effect of strain on JH titers ($P < 0.001$, $F = 21.072$, df = 1), no effect of age ($P = 0.534$, $F = 0.389$, df = 1), and a significant strain-by-age interaction effect ($P < 0.001$, $F = 17.489$, df = 1). Age-related changes in JH titers in honey bees have been reported numerous times (Bloch et al. 2002), but these results are readily explained by examination of the data from the four groups. One-day-old bees from the high pollen-hoarding strain had significantly higher titers than 1-d-old bees from the low pollen-hoarding strain (Fig. 1). JH titers in the low pollen strain increased with age, but decreased with age in the high pollen strain (Fig. 1). As a consequence, there was no significant difference in JH titer between preforagers from the two strains.

There was a significant effect of age on levels of octopamine, serotonin, and dopamine. In the antennal lobes, mushroom bodies, and remaining deuto/protocerebra, all three amines were significantly higher in older bees than younger bees regardless of strain with one exception; there was no significant difference between 1-d-old bees and preforagers in levels of dopamine in the antennal lobes (Fig. 2). There was no strain effect for all three amines. There were no significant differences between low and high pollen-hoarding strains in levels of any of the three amines in both 1-d-old adult bees and preforagers, in the antennal lobes, mushroom bodies, or remaining deuto/protocerebra (Fig. 2).

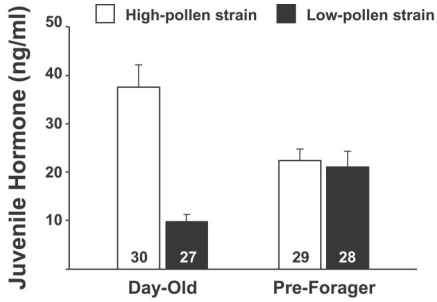

Fig. 1. Mean (+SE) hemolymph juvenile hormone titers in 1-d-old and preforager (12-d-old) honey bees from high and low pollen-hoarding strains. Sample sizes indicated in each bar.

Discussion

We hypothesized that bees from a high pollen-hoarding strain, which also have an earlier onset of foraging than bees from a low pollen-hoarding strain (Pankiw and Page 1999), will have higher hemolymph titers of JH and higher brain levels of octopamine before the onset of foraging. Our results support the hypothesis for JH (but in a surprising way), and do not support the hypothesis for octopamine.

JH titers in the low pollen strain increased with age, as is typical (Bloch et al. 2002), and the titers obtained here were consistent with previously reported results (Elekonich et al. 2001). Surprisingly, JH titers in the high pollen strain decreased with age, from day 1 to day 12. These results, and similar findings of a lack of a pronounced age-related increase reported in Pankiw et al. (1998), indicate that JH titers do not have to rise during middle age to some threshold level to initiate foraging; this means that JH does not seem to play an activational role (Elekonich and Robinson 2000). This is consistent with the observation that allatectomy delays, but does not eliminate, the onset of foraging in honey bees (Sullivan et al. 2000).

Our JH results highlight the possibility that endocrine events occurring early in adulthood influence honey bee behavioral development. Jassim et al. (2000) detected a peak of JH in 2-d-old bees, as did Kaatz et al. (1992), and found that the magnitude of this peak was higher in colonies manipulated to induce precocious foraging. Giray et al. (1999) also detected this peak in some, but not all, cases. In addition, treat-

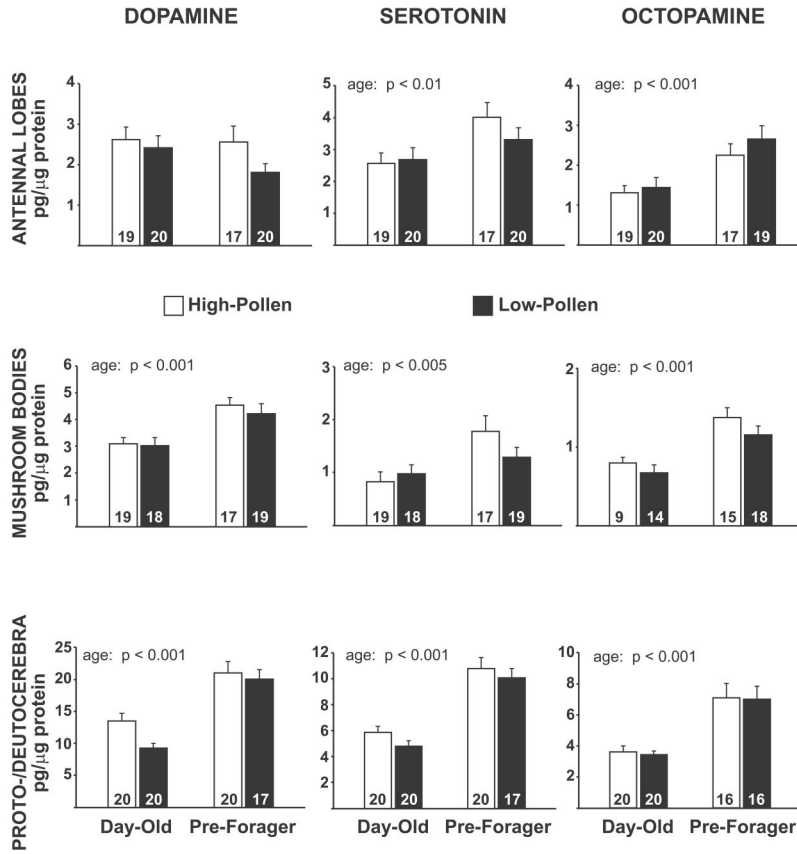

Fig. 2. Mean (+SE) concentrations of dopamine, serotonin, and octopamine in brain regions from 1-d-old and preforager (12-d-old) honey bees from high and low pollen-hoarding strains. Results were analyzed with two-way ANOVA to determine whether differences in juvenile hormone were due to age, genetic strain, or both. Only age effects were detected.

ment with JH or JH analogs on day 1 of adulthood causes precocious foraging (Bloch et al. 2002) and an increase in responsiveness to sucrose (Pankiw and Page 2003). These results suggest that the high JH titer we measured in 1-d-old bees from the high pollen-hoarding strain might in some way be related to an early onset of foraging, but these results are only correlative. Alternatively, it is also possible that, although JH is involved in the basic process of honey bee behavioral development (Bloch et al. 2002), it does not play a role in causing genotypic differences in rate of behavioral development, at least not in the high and low pollen-hoarding strains studied here. Giray et al. (1999) found no consistent association between high JH titer and faster rate of behavioral development in a comparison of different pairs of colonies from unselected strains.

Octopamine levels in the antennal lobes increased with age, consistent with previous studies (Schulz and Robinson 1999; Schulz et al. 2002a, b), and overall levels of all three amines were consistent with previous studies as well. However, no differences were detected between the high and low pollen-hoarding strains, at either age. One interpretation of these results is that although octopamine is involved in the basic process of honey bee behavioral development (Schulz et al. 2003), it does not play a role in causing genotypic differences in rate of behavioral development. However, octopamine treatment caused precocious foraging (Schulz and Robinson 2001) and an increase in sucrose responsiveness (Pankiw and Page 2003; Scheiner et al. 2003, 2004). In the following paragraphs, we speculate on two possible scenarios that do not preclude an involvement of octopamine or other elements of the octopamine pathway in the behavioral differences displayed by the high and low pollen-hoarding strains.

One possibility for why bees from the high and low pollen-hoarding strains failed to show a difference in brain octopamine is that octopamine levels increase very close to the onset of foraging, and we sampled bees that were too young. Octopamine treatments are effective over short-term time scales (<24 h; Schulz and Robinson 2001), and the foraging-related increase in brain octopamine becomes more pronounced as bees grow older and closer to becoming foragers (Schulz et al. 2002a, b; Spivak et al. 2003). Octopamine is thought to act as a neuromodulator in this system over relatively rapid time scales, altering responses to specific task-related stimuli (Barron et al. 2002, Schulz et al. 2003). Our study would have benefited from the collection and measurement of actual foragers from these groups to determine how similar preforager levels of octopamine were to those of actual foragers.

A second possibility for why bees from the high and low pollen-hoarding strains failed to show a difference in brain octopamine is that genotypic differences exist for sensitivity to, rather than production of, octopamine. Perhaps bees from the high pollen-hoarding strain are more sensitive to octopamine in the brain, due to increased receptor density or responsiveness. Giray et al. (1999) concluded that some genotypic differences in rate of behavioral development were related to differences in sensitivity to JH, based on experiments in which bees showing faster rates of behavioral development displayed increased sensitivity to methoprene treatment. Another possibility is that octopamine signaling is more potent in the high pollen-hoarding strain, eliciting higher levels of PKA activation (Humphries et al. 2003); PKA activation increases responsiveness to sucrose (Scheiner et al. 2003).

Our results also indicate that variation in brain levels of dopamine and serotonin are not associated with behavioral variation between the high and low pollen-hoarding strains. As with octopamine these results do not completely rule out any involvement of these neurochemicals. Dopamine and serotonin do not seem to be involved in regulating age at onset of foraging (Schulz and Robinson 2001, Schulz et al. 2003) in honey bees. However, dopamine plays a role in determining responsiveness to stimuli (Mercer and Menzel 1982), and serotonin regulates the balance of protein and carbohydrate intake in solitary insects (Cohen et al. 1988).

Robust phenotypic correlations suggest that some mechanisms that affect variation in foraging specialization on nectar or pollen also may affect variation in age at onset of foraging and responsiveness to sucrose (Pankiw and Page 2000, Pankiw 2003). However, recent findings indicate that the linkages between these phenotypes can be experimentally dissociated. cGMP treatment affects age at onset of foraging (Ben-Shahar et al. 2002) but does not affect responsiveness to sucrose or foraging specialization (Ben-Shahar et al. 2004). cAMP treatment increases sucrose responsiveness but does not affect age at onset of foraging (Ben-Shahar et al. 2002, Scheiner et al. 2003). Manganese treatment affects both responsiveness to sucrose and age at onset of foraging, but the association with foraging specialization is weak (Ben-Shahar et al. 2004). These findings suggest that that age at onset of foraging and foraging specialization on nectar or pollen are regulated by a mixture of common, and distinct, mechanisms. Identifying both kinds of mechanisms will help to better understand division of labor in honey bee colonies.

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