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Physiological correlates of division of labor among similarly aged honey bees

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Abstract. Hormone analyses and exocrine gland measurements were made to probe for physiological correlates of division of labor among similarly aged adult worker honey bees (*Apis mellifera* L.). Middle-age bees (ca. 2 weeks old) performing different tasks showed significant differences in both juvenile hormone (JH) biosynthesis rates and hemolymph titers; guards and undertakers had high JH, and wax producers and food storers, low JH. Guards and undertakers had similar hormone levels to foragers, even though they were 10 days younger than foragers. No differences in JH were detected among young bees (1-week-old queen attendants and nurses) or older bees (3–4 week-old pollen foragers, non-pollen foragers, and soldiers). Hypopharyngeal gland size was inversely correlated with worker age and rate of JH biosynthesis, but soldiers had significantly larger hypopharyngeal glands than did foragers, despite their similar age and JH level. Results from soldiers indicate that exocrine gland development is not always linked with age-related behavior and endocrine development; they also support the recent claim that soldiers constitute a group of older bees that are distinct from foragers. Hormonal analyses indicate that the current model of JH's role in honey bee division of labor needs to be expanded because high levels of JH are associated with several other tasks besides foraging. JH may be involved in the regulation of division of labor among similarly aged workers in addition to its role in age-related division of labor.

Key words: Corpora allata – Division of labor – Hypopharyngeal glands – Juvenile hormone – Social insects

Introduction

Division of labor among workers in insect societies is related to differences in age ("age polyethism") or morphology (limited to a minority of ant species and nearly all termites) (reviewed by Wilson 1971; Oster and Wilson 1978). It also is becoming increasingly clear that individual differences in task specialization, independent of worker age or morphology, play a significant role in determining division of labor (reviewed by Oster and Wilson 1978; Jeanne 1987). Progress has been made studying the physiological basis of age polyethism (reviewed by Robinson 1992; see also Huang and Robinson 1992; O'Donnell and Jeanne 1993), but little is known about physiological mechanisms underlying division of labor among similarly aged workers.

Variation in task performance among similarly aged workers is common in colonies of the honey bee, *Apis mellifera* (reviewed by Seeley 1985; Winston 1987). Bees about 2 to 3 weeks old, for example, perform a variety of tasks such as building comb, receiving nectar from incoming foragers and storing it in combs, guarding the nest entrance, or removing corpses from the nest ("undertaker" behavior). Only a small percentage of a colony's workers act as guards (Moore et al. 1987) or undertakers (Sakagami 1953; Visscher 1983); most bees apparently never perform either of these tasks. There also are differences in task performance among older bees, with some workers specializing on collecting nectar, pollen, propolis, or water (reviewed by Winston 1987), or on defending the colony (Breed et al. 1990). Highly specialized individuals such as guards and undertakers are thought to contribute to colony fitness by working more efficiently than less specialized workers (Oster and Wilson 1978). Interindividual variation in task performance results, in part, from genotypic differences among workers (reviewed by Page and Robinson 1991), but physiological mechanisms are unknown.

The first objective of this study was to determine whether juvenile hormone (JH) might play a role in the regulation of division of labor among similarly aged

Abbreviations: JH, Juvenile hormone; RIA, radioimmunoassay; CA, corpora allata; HPLC, high performance liquid chromatography; TLC, thin layer chromatography

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workers by determining whether there are differences in JH among bees of similar ages performing different tasks. JH plays a major role in regulating age polyethism in honey bee workers (reviewed by Robinson 1992). Hemolymph titers of JH increase as bees age (Fluri et al. 1982; Robinson et al. 1987). Low titers are associated with the performance of in-hive tasks such as brood care during the first one to three weeks of the bee's adult life, whereas higher titers near the end of the third week are associated with foraging. Treatment with JH (Jaycox 1976), JH mimic (Jaycox et al. 1974), or JH analog (Robinson 1985, 1987a; Sasagawa et al. 1989) induces precocious foraging. Similar results from hormone treatments recently have been obtained for *Polybia* wasps (O'Donnell and Jeanne 1993). Differences in JH titers have been detected among similarly aged honey bees experimentally induced to perform either brood care or foraging (Robinson et al. 1989) but these two tasks do not co-occur temporally under natural conditions.

The second objective of this study was to examine a possible exocrine gland correlate of division of labor among similarly aged workers, the development of the hypopharyngeal glands. Exocrine gland activity and behavioral development are tightly linked in honey bees. Hypopharyngeal glands in young bees produce proteinaceous material that is fed to larvae, the queen (reviewed by Winston 1987), and other adult workers (Crailsheim 1991, 1992). Young bees engaged in brood and queen care generally have large hypopharyngeal glands (Soudek 1927; King 1933) with high rates of protein synthesis (Brouwers 1982, 1983; Huang 1990). Foragers have smaller, less active, glands that produce invertase (to convert nectar into honey) (Simpson 1960). Furthermore, JH treatments cause a decrease in hypopharyngeal gland size (Rutz et al. 1974; Jaycox et al. 1974; Jaycox 1976). Analysis of hypopharyngeal glands among similarly aged bees performing different tasks may thus provide insights into the relationship between endocrine, exocrine gland, and behavioral development.

Materials and methods

Bees. Four colonies, each with a population of ~40,000 workers, were used. The following manipulations were made to increase the likelihood of observing individuals of known age performing rare tasks such as guarding and undertaking. We reduced the size of each colony by transferring ~20,000 workers of all ages, 3–4 combs of young, unsealed brood, and the queen to a different one-story Langstroth hive and moving it to a location >7 km away from the original site. Then all (5–6) combs of older, sealed brood from each original colony were transferred to an incubator (33°C). Workers that emerged from these combs over each subsequent 24-h period were marked on the dorsal surface of the thorax with a spot of paint (Testor's PLA) and reintroduced to their reduced, natal, colony. In Colony 1, 1388 bees were marked over a 5-week period, but we later had difficulty finding marked guards and undertakers (see Table 1); therefore in Colonies 2–4 we marked ~6,000 bees per colony, at a rate of ~1000 bees per day. Colonies were otherwise maintained according to standard techniques at the University of Illinois Bee Research Facility, Urbana, Illinois. They were typical of North American populations of *Apis mellifera* [a mix of predominantly European subspecies (Phillips 1915; Pellett 1938)].

Behavioral sampling. We sampled "young", "middle-age", and "old" honey bee workers performing different tasks (see Table 1). Young workers were sampled when they were about one week old, either tending brood ("nurse bees") or the queen ("queen attendants"). Both tasks are usually performed by bees during the first two weeks of adult life (reviewed by Winston 1987). Nurse bees were identified as workers with heads in cells containing larvae (Sakagami 1953; Robinson 1987a). Queen attendants were identified as bees either surrounding the queen in a retinue or antennating or licking the queen as she moved among them (Butler 1973; Seeley 1979). There is no evidence that young workers specialize on queen care (van der Blom 1992). These groups were selected for comparison with middle-age and old bees that are known to specialize on particular tasks.

Middle-age workers were sampled when they were about two to three weeks old, either as wax producers, food storers, guards, or undertakers. These tasks are usually performed by bees older than nurse bees and queen attendants, but younger than foragers (reviewed by Winston 1987). Potential wax producers were first identified as bees hanging in "festoons" in a hole cut in a comb the

Table 1. Age of bees sampled in each behavioral group. Bees were from 3 age categories: young (queen attendants and nurses), middle-age (wax producers, food storers, guards, and undertakers), and old (soldiers, pollen foragers, and non-pollen foragers). Numbers in parentheses indicate sample sizes of bees used to determine rates of JH biosynthesis (Colony 1–3) or hemolymph JH titers (Colony 4). Within each colony, values followed by different letters are significantly different from one another ($P < 0.05$, ANOVA and Tukey's Studentized Range Test). Between-colony analyses of age were not conducted. For Colony 1, the unknown ages of guards and undertakers and the small sample size for soldiers indicated below are because a relatively smaller number of marked bees was introduced into this colony and recovered in these groups. Hormonal and excrocrine gland analyses for these groups were nevertheless performed on 10 bees/group, using behaviorally defined bees of unknown age. All other bees in this study were of known behavior and age

Behavioral group	Mean Age (±SE), days			
	Colony 1	Colony 2	Colony 3	Colony 4
Queen attendants	9.6±1.4 (10) a	8.0±0.0 (15) a	8.0±0.0 (18) a	9.0±0.7 (10) a
Nurses	8.0±0.3 (10) a	8.0±0.0 (15) a	8.0±0.0 (18) a	11.3±0.3 (10) b
Wax producers	19.0±0.7 (10) b	14.6±0.3 (30) b	14.0±0.3 (19) b	14.0±0.3 (10) c
Food storers	19.0±0.7 (10) b	14.6±0.3 (30) b	13.8±0.3 (20) b	14.0±0.3 (10) c
Guards	unknown (10)	15.8±0.4 (38) b	13.9±0.3 (20) b	14.0±0.3 (10) c
Undertakers	unknown (10)	15.0±0.3 (29) b	14.1±0.3 (20) b	15.2±0.3 (10) c
Soldiers	29.2±0.7 (5) d	26.7±0.3 (20) c	19.9±0.4 (20) c	24.0±0.6 (10) d
Pollen foragers	29.5±0.5 (10) d	27.5±0.3 (34) c	20.1±0.3 (20) c	25.5±0.3 (10) d
Non-pollen foragers	29.5±0.5 (10) d	27.5±0.4 (20) c	20.7±0.4 (20) c	25.7±0.3 (10) d

previous day (Colony 1), or as bees building cells on partially built combs (Colonies 2–4). They were classified as wax producers only if they also had large wax scales in their wax glands (examined at 30 × power under an Olympus SZH dissecting microscope). Food storers were identified as bees with their heads in cells of nectar or honey on the most peripheral combs. A large proportion of these bees also had wax scales (as in Muller and Hepburn 1992), but only bees without wax scales were classified as food storers. Food storers with wax scales were excluded to increase the possible differences between food storers and wax producers. Guards were identified as bees at the hive entrance exhibiting one or more of the following behavior patterns: approaching and examining other bees, standing with front legs off the substrate, or standing with wings held away from the body (Moore et al. 1987). In Colony 2 only, most guards were located just inside the hive entrance rather than on the ramp in front of the entrance. To facilitate identification of guards in this colony, we held a freeze-killed worker from a foreign colony in forceps at the entrance and collected bees that responded with guard-like postures or aggressive behavior (rapidly approaching, biting, or attempted stinging). Undertakers were identified as bees carrying dead workers out of the hive (Visscher 1983). To induce this behavior experimentally we introduced 50 recently (<72 h) freeze-killed workers to the bottom rear of each hive, away from the entrance (Visscher 1983; Robinson and Page 1988). Guarding (Moore et al. 1987) and corpse removal (Sakagami 1953; Visscher 1983) are "specialized" tasks performed by only a small fraction of a colony's workers. In contrast, there is no evidence for specialization for food storage (Seeley 1989) or comb building (Muller and Hepburn 1992).

Old workers were sampled when they were older than 3 weeks of age, as pollen foragers, "non-pollen" foragers, or soldiers. Pollen foragers and non-pollen foragers were obtained by temporarily obstructing the hive entrance with a piece of 8-mesh hardware cloth. Pollen foragers were identified as bees returning to the hive with pollen loads in their corbiculae. Non-pollen foragers were identified as bees returning to the hive without pollen but with distended abdomens. Colonies 2 and 4 were sampled during a nectar dearth so many non-pollen foragers did not have distended abdomens; they were sampled only when no obvious orientation flights were taking place. Most bees sampled according to either criterion had worn wings (see Results), which suggests that bees without pollen also were foragers, most likely nectar foragers (successful or unsuccessful). Soldiers are a recently discovered group of older bees that respond to a large disturbance by flying out and attempting to sting (Breed et al. 1990). They were identified as bees that emerged from their hive after a brick was dropped on top of it and stung black leather patches that were dangled in front of the hive entrance (Breed et al. 1990). They were classified as soldiers only if they were missing their stings. Behavioral and genetic analyses have demonstrated that pollen foraging, nectar foraging, and soldier behavior are performed by different groups of similarly aged bees (Calderone and Page 1988; Robinson and Page 1989; Breed et al. 1990).

Nurses and queen attendants were collected by opening a hive and scanning combs with young larvae or visually following the movements of the queen. Middle-age bees were collected by monitoring each hive entrance daily for marked guards and undertakers, beginning when the oldest marked bees were 10 days old. Marked guards and undertakers were collected beginning the day we observed >6 of each during the first 30 min of observation. After collecting about 15 guards and undertakers, we opened the hive and sampled wax producers (N = 30) and food storers (N = 60) of comparable ages. At this time <1% of the focal bees were observed foraging, so it is highly unlikely that wax producers and food storers were foragers who happened to be located in the nest periphery. Their age (ca. two weeks old) also argues against this possibility. Guards were always collected before undertakers, and soldiers were always collected after foragers. All groups of bees were collected with a portable vacuum cleaner (Robinson and Page 1988).

After collection, bees were immediately immobilized on ice for 20 min to 3 h until their corpora allata (CA) were removed (Huang et al. 1991), or hemolymph taken (Robinson et al. 1987). Hypopharyngeal gland size was then measured, followed by scoring the degree of wing-wear (for old bees only).

Ten bees from each behavioral group usually were assayed from each colony. In cases where more than 10 bees were assayed per group, sampling and assaying were conducted on 2 (Colony 3) or 3 (Colony 2) consecutive days. The same bees were used for endocrine, exocrine gland, and wing wear analyses. A total of 496 bees were analyzed for rates of JH biosynthesis; of these, 458 were available for hypopharyngeal gland measurements. A total of 90 (different) bees were analyzed for JH titers. Table 1 summarizes the numbers and ages of all bees used in this study.

Measurement of JH biosynthesis. Rates of JH III biosynthesis were measured for individual bees from Colonies 1–3 with a radiochemical method (Pratt and Tobe 1974; Tobe and Pratt 1974) recently adapted for adult worker honey bees (Huang et al. 1991). Briefly, the CA-corpora cardiaca complex from each individual bee was removed and incubated for 3 h in 50 µl modified bee medium (Kaatz et al. 1985) containing 60 µM of L-[^{3}H-methyl]methionine (NEN, 7.4 GBq/mmol). JH produced in vitro was then extracted with 250 µl isooctane and quantified by liquid scintillation spectrometry (cocktail: BioSafe II, Research Products International; counter: Packard Tricarb 460C). Rates of JH biosynthesis are significantly correlated with hemolymph JH titers measured by radioimmunoassay (RIA) (Huang et al. 1991). JH III is the only JH homolog in honey bees (Hagenguth and Rembold 1978; Huang et al. 1991; Robinson et al. 1991). All solvents used for measurements of JH biosynthesis and titers were HPLC grade, obtained from either Fisher or J.T. Baxter Chemical Co. Glassware used was baked at 500°C for 3.5 h prior to use to minimize JH adsorption.

Measurement of JH titers. To confirm results obtained via measuring rates of JH biosynthesis, JH titers were measured for bees from a single, different colony (Colony 4). Measurements were made with a newly developed chiral-specific RIA (Hunnicutt et al. 1989; D. Hunnicutt, Y.C. Toong, D.W. Borst, unpubl.).

Hemolymph (0.73–7.8 µl) from individual bees was mixed with 500 µl acetonitrile in 13 × 100 mm glass culture tubes with Teflon-lined screw caps and stored at −20°C. Prior to RIA, JH was extracted by adding 1 ml 0.9% NaCl and 1 ml hexane to the acetonitrile-hemolymph mixture. After vigorous vortexing, samples were cooled on ice for 10 min, vortexed again and centrifuged at 2000 g for 5 min (4°C). The supernatant hexane phase, containing JH, was removed. After repeating the extraction, the pooled supernatants were dried in a vacuum centrifuge (Savant SC110).

To perform the RIA, 25 µl ethanol was added to each extract and a 2.5 µl aliquot was transferred to another tube (10 × 75 mm) containing 200 µl premixed antiserum (1:28,000) and 10,000 dpm of [10−^{3}H(N)]-JH (NEN, 629 Gbq/mmol). Incubation proceeded for 2 h at room temperature. After cooling in an ice-water mixture (0°C) for 10 min, unbound radiolabeled JH was separated from bound JH by adding dextran-coated charcoal (for 2.5 min) and centrifuging (3 min at 2000 g, 4°C). Radioactivity in the supernatant (containing radiolabeled JH bound to antiserum) was quantified by liquid scintillation spectrometry (Beckman LS6000IC).

Because this RIA is new and has never before been used for honey bees we provide background information as follows. A standard curve based on analyses of 0, 3, 10, 30, 100, 300, 1000, 3000, and 10000 pg 50% racemic JH III (Sigma) was constructed for each assay. Parameters of the standard curve were estimated by non-linear regression (SAS Institute 1985) according to the following formula:

$$\log(\text{amount of JH}) = \frac{\log_e\left(\frac{\text{Maximum binding}}{\text{DPM bound}} - 1\right) + A}{B}$$

"Maximum binding" refers to the value obtained (in DPM) when no competing JH is present, and "DPM bound" refers to the value obtained in sample tubes containing various amounts of JH. This formula fits the standard curve data very well (mean

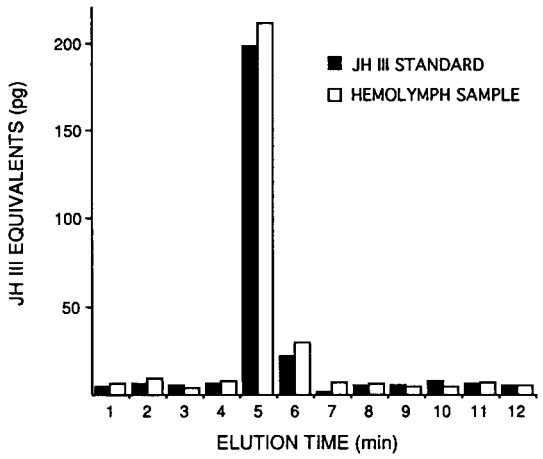

Fig. 1. Qualitative analyses of JH in hemolymph of adult worker honey bees. Immunoactivity of the different fractions following HPLC separation was estimated for a 12.5 ng JH III standard (*solid bar*), and a 22.7 µl hemolymph extract of foragers (*open bar*). Fractions were collected every 1 min, and 1/40 (standard) or 1/10 (sample) of each fraction was used to determine immunoactivity

$R2 = 0.995 \pm 0.001$, $N = 10$ curves), enabling accurate estimates of JH titer in biological samples. The JH equivalents were adjusted by multiplying by 0.5 because the racemic JH III used to generate the standard curve contains approximately 50% of each enantiomer and the antibody only recognizes the biologically active enantiomer (Hunnicutt et al. 1989; D. Hunnicutt, Y.C. Toong, D.W. Borst, unpubl.).

RIA specificity was investigated by fractionating the hexane extracts of hemolymph samples ($N = 5$) with normal-phase high performance liquid chromatography (HPLC) (5 µm, 4.6 mm × 25 cm silica column; 5 or 10% diethyl ether in hexane; 2 ml/min). One-min fractions were collected, dried, resuspended in 25 µl ethanol, and analyzed for immunoactivity by RIA as described above. For each sample, the majority ($95\% \pm 1.9\%$, SE) of the immunoactivity eluting from the column was detected as a single peak with a retention time identical to the JH III standard (Fig. 1). No other fractions had JH immunoactivity higher than background levels (~ 10 pg). Total JH immunoactivity recovered in hemolymph samples after HPLC fractionation was $91.9 \pm 11.2\%$. To determine whether this HPLC procedure separates JH from lipids, a neutral lipid standard (Sigma) containing cholesterol, cholesterol-oleate, methyl-oleate, oleic acid and triolein also was separated by HPLC and the fractions analyzed by thin layer chromatography (TLC; Silica gel, 80% hexane, 20% $CHCl_3$, 0.5% HAc) to determine the compounds present. None of the lipids co-eluted with the JH III standard, using the iodine vapor staining method (Goodman et al. 1990).

Quantitative and qualitative analyses of JH were performed by splitting hemolymph samples ($N = 10$) into two halves, subjecting one half to a purification procedure that removes lipids, and comparing the JH titers with the other half that was not purified. Radiolabeled JH (5000 dpm) was added to each sample prior to splitting to monitor JH recovery. A reverse phase C_{18} Sep-Pak cartridge (Waters) was washed with 1 ml 100% methanol and 3 ml 60% methanol. JH extracts (re-dissolved in 1 ml 60% methanol) were passed through the cartridge, followed by two washes of the sample tubes (each with 1 ml 60% methanol). The JH fraction was eluted (with 3 ml 85% methanol), dried to 1 ml with a vacuum centrifuge, and the JH re-extracted with hexane, as described above. JH titers in the two sample halves were significantly correlated with each another ($r = 0.96$, $P < 0.001$). Furthermore, the slope (0.9 ± 0.1) of the regression line was not significantly different from unity ($P > 0.4$, t-test) and the intercept (43.3 ± 29) not significantly different from zero ($P > 0.1$, t-test), indicating that the amounts of JH in the sample halves were not significantly different from one another. To verify that lipids were separated from JH with this procedure, a sample of lipids also was assayed as above; >90% of the lipids were eliminated. In addition, no lipids were detected in hemolymph samples that were not purified by this procedure, even though the samples were pooled from 3–8 bees.

As another measure of the validity of this RIA, preliminary studies were conducted to compare the results of this RIA with those obtained from the Strambi and Goodman RIA's. Measurements of JH titers for 1-day-old bees, nurses, and foragers reveal that values from this RIA were significantly correlated ($r = 0.99$, $P < 0.001$) with values obtained with either the Strambi or Goodman RIAs, both of which have been validated with GC/MS (de Kort et al. 1985; Goodman et al. 1990). More detailed comparisons among the RIA's would require splitting samples and subjecting part of each sample to a different assay, as was done for the Strambi and Goodman RIA's (Goodman et al. 1993).

Measurement of hypopharyngeal gland size. Hypopharyngeal gland size was measured in the same bees used to measure rates of JH biosynthesis. Glands were dissected from the head in bee saline (see Huang et al. 1991) after removing the CA. The diameters of individual gland acini were measured (at 60× magnification) as in Crailsheim and Stolberg (1989), but we measured only one representative acinus per bee. Acinus diameter was used as an index of gland size as in previous studies (King 1933; Hassanein 1952; Fergusson and Winston 1988; Crailsheim and Stolberg 1989), because the peculiar shape of the gland, with hundreds of acini attached to a central duct, precludes simple measurement of total gland size. Acinus diameter provides a valid estimate of total gland size; it is thought that the total number of acini is fixed after adult emergence, because the only structural change that occurs in the hypopharyngeal gland as the bee ages is a change in the size of individual acini (Haydak 1957). Gland size also has been estimated by classifying acinus diameter (Soudek 1927; Hassanein 1952; Maurizio 1954; Simpson et al. 1968), or by measuring gland protein content (Brouwers 1982; Huang et al. 1989) or gland weight (Fluri et al. 1982; Pickard and Kither 1983). In the only study in which these four methods were compared, estimates of gland size based on acinus diameter classification (Maurizio 1954), actual acinus diameter, or gland protein content were found to be correlated (Brouwers 1982). Gland size is a good indicator of secretory activity, as long as a normal amount of brood is present in a colony (Huang et al. 1989; Z.-Y. Huang, unpublished data).

Measurement of wing wear. To further verify the identity of soldiers, the degree of wing wear was measured for non-pollen foragers, pollen foragers, and soldiers in Colonies 2–4. This is because it has been shown that soldiers have significantly less wing wear than foragers (Breed et al. 1990). We scored bees as having either "no wing wear" if both wings showed no damage under microscopic examination, or "worn" wings if either or both wings showed any damage. Since most bee flight is associated with foraging, we assume that wing wear is a reasonable indicator of foraging experience (see Breed et al. 1990).

Statistical analyses. Differences in rates of JH biosynthesis and hypopharyngeal gland size were first analyzed by two-way analysis of variance (ANOVA), with behavioral tasks nested inside colony. Data from each colony were further analyzed by one-way ANOVA and Tukey's Studentized Range test (SAS Institute 1985). JH titers were measured to confirm the findings of JH biosynthesis analyses; therefore, we used ANOVA and a priori contrasts to verify that guards and undertakers have JH titers that are similar to foragers but higher than wax producers and food storers. Rates of JH biosynthesis, JH titers, and diameters of hypopharyngeal gland acini were transformed logarithmically before analysis when heterogeneity of variances or non-normal distribution was detected. Correlation and partial correlation analyses were performed to determine more precisely the relationship between JH and hypopharyngeal gland size. Other statistical analyses are indicated in the Results section.

Results

Rates of JH biosynthesis

Rates of JH biosynthesis differed significantly among bees performing different tasks in all 3 colonies (Colony 1: $F = 6.91$; Colony 2: $F = 10.4$; Colony 3: $F = 22.6$; Fig. 2). Tasks performed by young bees were associated with low rates, and tasks performed by old bees with high rates. Differences among colonies were not significant ($F = 0.40$, $P > 0.6$). Subsequent analyses were therefore performed on both individual colonies and all colonies pooled.

There were no significant differences in rates of JH biosynthesis between nurses and queen attendants in all three colonies and in the analysis of all colonies pooled. Similarly, there were no significant differences in rates of JH biosynthesis between pollen foragers, non-pollen foragers, and soldiers in all three colonies and in the pooled analysis.

Significant differences in rates of JH biosynthesis were detected among the middle-age bees. Guards had significantly higher rates of JH biosynthesis than did wax producers and food storers in Colonies 1 and 3. Undertakers had significantly higher rates of JH biosynthesis than did wax producers and food storers in Colonies 1 and 2. In the pooled analysis, both guards and undertakers had significantly higher rates of JH biosynthesis than did wax producers or food storers. Moreover, rates of JH biosynthesis for guards and undertakers were not significantly different than those of foragers or soldiers, even though they were about 10 days younger. Rates of JH biosynthesis for wax producers and food storers generally were not significantly different from those of queen attendants and nurses.

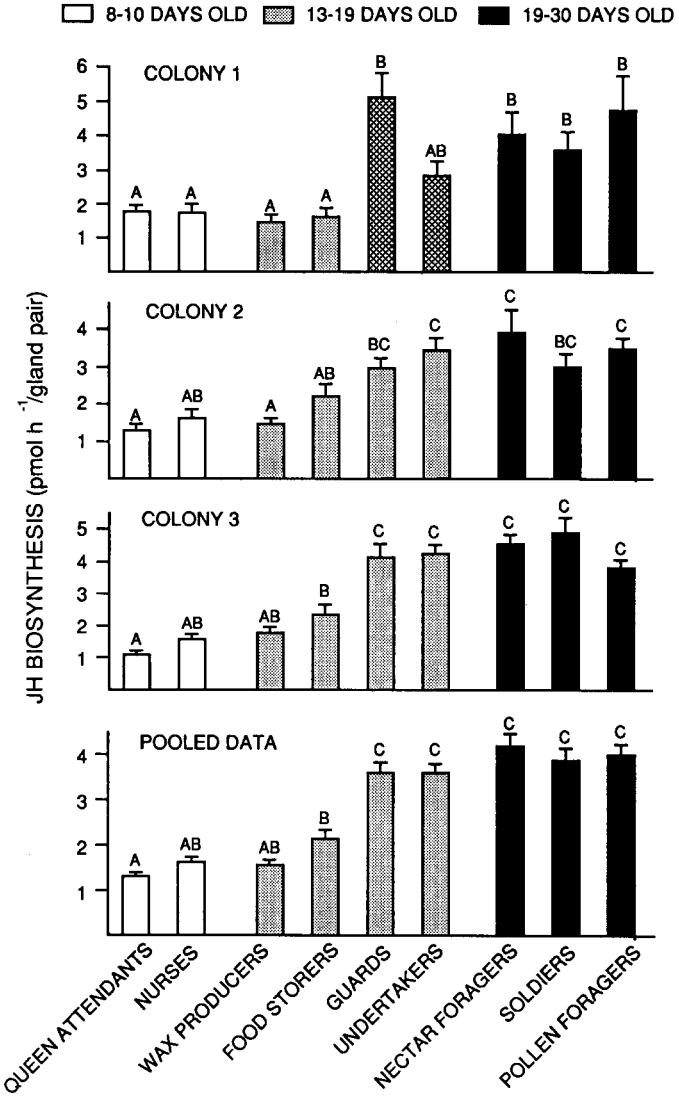

Fig. 2. Mean (+ SE) rates of JH biosynthesis for bees of known age performing different tasks. Three age groups were analyzed: young (nurses and queen attendants), middle-age (wax producers, food storers, guards, and undertakers) and old (pollen foragers, non-pollen foragers, and soldiers). For each colony (or for the pooled analysis), *bars with different letters* are significantly different from one another ($P < 0.05$, Tukey's Studentized Range Test). *Bars with the same shading* indicate similarly aged bees (See Table 1 for ages of bees). Shading is different for some bees in Colony 1 because ages of guards and undertakers are unknown

JH titers

A significant correlation between JH biosynthesis rates and hemolymph titers already has been shown for 1-day-old bees, 7–9-day-old nurses, 14–15-day-old food storers, and 21–24-day-old foragers (Huang et al. 1991). We therefore wanted to confirm whether a similar correlation also exists for similarly aged guards, undertakers, wax producers and food storers. Specifically, we wanted to confirm whether guards and undertakers have higher JH titers than do wax builders and food storers, consistent with the more extensive biosynthesis results reported above. Titer determinations from one colony are consistent with measurements of rates of JH biosynthesis (Table 2). Young bees had low titers, and old bees had high titers as in previous studies (Robinson et al 1989; Huang et al 1991). Moreover, guards and undertakers had significantly higher JH titers than did wax producers and food storers ($P < 0.001$, a priori contrast), despite their similar ages. The JH titers of guards and undertakers were not significantly different than those of foragers and soldiers ($P > 0.99$, a priori contrast), even though they were about 10 days younger. Unlike the results from JH biosynthesis analyses, nurse bees had somewhat higher JH titers than

Table 2. Mean (±SE) JH titers for bees sampled when performing various tasks ($N = 10$ individual bees/group). See Table 1 (Colony 4) for ages of bees

Behavioral Group	JH Titers (ng/ml)
Wax producers	43.2±13.6
Food storers	88.8±30.2
Guards	160.5±34.1
Undertakers	132.0±27.0
Soldiers	210.3±56.8
Non-pollen foragers	210.8±86.7
Pollen foragers	155.1±36.2

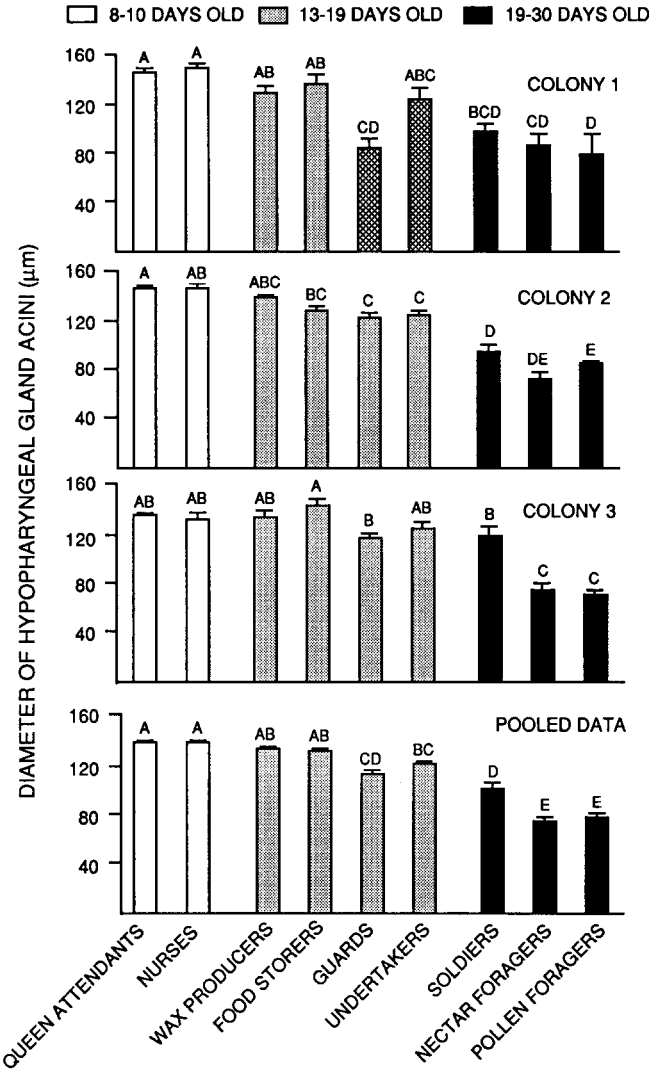

Fig. 3. Mean (+ SE) hypopharyngeal gland sizes for bees of known age performing different tasks. Glands were measured from the same bees used for JH biosynthesis measurements (Fig. 2). Worker ages and statistical analyses as in Fig. 2

did queen attendants, but this difference was not significant (62.2 ± 15.4 and 18.9 ± 8.9, respectively, $P > 0.05$, Tukey's Studentized Range test).

Size of hypopharyngeal glands

Hypopharyngeal gland size differed significantly among bees performing different tasks in all 3 colonies (Fig. 3). Young bees, with lower rates of JH biosynthesis, had larger glands, and old bees, with higher rates of JH biosynthesis, had smaller glands. The size of hypopharyngeal glands decreased as the age of the bee increased ($N = 458$, $r = -0.61$, $P < 0.001$). There was a significant negative correlation between hypopharyngeal gland size and rates of JH biosynthesis ($r = -0.37$, $P < 0.001$). The correlation also was significant after the effect of worker age was removed (partial correlation, $r = -0.23$, $P < 0.01$). Differences among colonies were not significant ($F = 0.12$, $P > 0.8$). There were no significant differences in hypopharyngeal gland size between nurses and queen attendants in all three colonies and in the pooled analysis. Similarly, there were no consistent differences in hypopharyngeal gland size among wax producers, food storers, guards, and undertakers, but guards tended to have smaller glands.

Significant differences in hypopharyngeal gland sizes were detected among the older bees. Soldiers had significantly larger hypopharyngeal glands than did pollen foragers or non-pollen foragers in 2 out of 3 colonies and in the pooled analysis, despite their similar age and hormonal status. Soldiers also had significantly less wing wear than did foragers (pollen and non-pollen foragers combined) (G-test, $P < 0.01$ for all 3 colonies). The proportion of soldiers without wing wear was 70%, 80%, and 90% for Colonies 2, 3, and 4, respectively, while the proportion of foragers without wing wear was 20%, 25% and 40%, respectively. Differences in wing wear between foragers and soldiers are consistent with the results of Breed et al. (1990) and further confirm that the bees we collected were indeed a different population from that of foragers.

Discussion

This study demonstrates the first endocrine correlate of naturally occurring division of labor among workers that are similarly aged. Task-dependent variation in both JH biosynthesis rates and JH titers was found among middle-age bees. Behavioral analyses have identified guarding (Moore et al. 1987) and corpse removal (Sakagami 1953; Visscher 1983) as "specialized" tasks performed by only a fraction of a colony's workers. Our results indicate that guards and undertakers also are hormonally distinct from other middle-age bees. The observed correlations do not establish a causal relationship between a particular behavioral state and a certain level of JH. A causal relationship between high levels of JH and guarding behavior is, however, suggested by the finding that JH treatments cause bees to show earlier and more frequent guarding behavior (Sasagawa et al. 1989). Because the likelihood of becoming a guard or an undertaker is influenced by variation in worker genotype (Robinson and Page 1988), the observed hormonal differences also may have a genetic basis. However, genetic analyses of behavior were not performed in this study. Hormonal differences have been detected among genotypically distinct groups of bees showing different patterns of age-related division of labor (Robinson et al. 1989). Genotypic differences among workers are thought to influence response thresholds to task-associated stimuli (Robinson and Page 1988), but mechanisms underlying these genetic differences are unknown. Our results suggest that differences in JH might be involved.

Both nurses and queen attendants had low JH biosynthesis rates, a result consistent with previous studies of nurses (Robinson et al. 1987; 1989; Huang et al. 1991; Huang and Robinson 1992). The lack of difference between nurses and queen attendants is consistent with the

fact that there is no evidence that young workers specialize on queen care; it seems to be a transient behavior performed by nurse bees when the queen is near (van der Blom 1992; Free et al. 1992).

All old bees had high biosynthesis rates and titers of JH, consistent with previous results for foragers (Robinson et al. 1987; Huang et al. 1991; Huang and Robinson 1992). Despite the similarity in endocrine status, behavioral and genetic evidence indicates that pollen foraging, nectar foraging, and soldier behavior are performed by different groups of similarly aged bees (Calderone and Page 1988; Robinson and Page 1989; Breed et al. 1990). These behavioral differences are evidently not associated with variation in JH.

The current model of the role of JH in honey bee division of labor now can be expanded, because our results demonstrate that high levels of JH are associated with several other tasks besides foraging. The four tasks now known to be associated with high levels of JH are guarding, undertaking, soldier behavior, and foraging. Because these are the only four tasks that involve flight and other activities outside the nest, high levels of JH may lead to higher metabolic activity and/or positive phototaxis, rather than just a decrease in response thresholds for foraging behavior. JH has been suggested to play a role in the control of metabolic rate in other insect species (see Denlinger et al. 1984). The hypothesis of a connection between high levels of JH and positive phototaxis is weakened, however, by the fact that young bees briefly become positively phototactic when they take defecation/orientation flights (see Winston 1987), perhaps on a daily basis. This observation also weakens the hypothesis that high JH leads to high metabolic activity, but perhaps these brief flights are not as metabolically demanding as activities performed by undertakers, guards, foragers, and soldiers. This distinction is supported by the finding that foragers show a 50% increase in mass-specific maximal oxygen consumption relative to 8–12 day-old hive bees (Harrison 1986). Undertakers apparently also have relatively high rates of oxygen consumption (J. Coelho, personal communication). Because high JH titers are associated with four different tasks, JH may influence behavioral specialization in concert with other, unidentified, chemical signals.

Previous studies have suggested that elevated levels of JH play a role in regulating aggressive behavior in honey bees (Breed 1983). A specific effect of JH on aggressive behavior seems unlikely because undertakers have JH levels as high as guards. In addition, soldiers do not have higher levels of JH than do foragers. These results suggest that JH influences the expression of aggressive behavior indirectly, as older bees are more aggressive than younger bees. Consistent with this suggestion is the finding that JH treatments cause a premature increase in sensitivity to alarm pheromones in honey bees (Robinson 1987b).

Rates of JH biosynthesis were consistent with measurements of JH titers for all behavioral groups analyzed, including guards and undertakers. Huang et al. (1991) also have demonstrated that rates of JH biosynthesis and JH titers are correlated in honey bees. JH titers were more variable than rates of JH biosynthesis; variability in JH titers also has been seen with other RIA's (see Robinson et al. 1989; Huang et al. 1991; Goodman et al. 1993). The reason for this difference is not known. The RIA used here also yielded results that are consistent with measurements from two other JH RIA's. The assay is relatively simple to perform, making it compatible with the relatively large sample sizes required for behavioral analyses. Moreover, its sensitivity enables determination of JH titers from individual workers. These advantages are undoubtedly related to the fact that the assay is performed without sample purification. Our results validate the use of this assay without extensive sample purification for honey bees. No difference in JH was found between non-purified samples and those purified by a Sep-Pak procedure. Furthermore, the lipid content in bee hemolymph is relatively low even in samples that were not purified. Quantitative and qualitative analyses of JH confirmed the specificity of this RIA for analyses of honey bee hemolymph. The efficacy of this technique should be determined for other species, especially for those that may have a higher ratio of lipid to JH in the hemolymph than do honey bees.

It is clear from this and other studies that low rates of JH biosynthesis and low JH titers are associated with tasks performed by young bees, and high rates and titers with tasks performed by older bees (see also Fluri et al. 1982; Robinson et al. 1987, 1989; Huang et al. 1991). However, it is not known whether changes in CA activity and JH titer during behavioral development are discrete or continuous. Huang et al. (1991) reported two discrete states of CA activity: "low", for young bees (nurses) and middle-age bees (food storers) and "high", for older bees (foragers). Robinson et al. (1987) found no differences in JH titers between bees aged 5, 10, or 15 days in one experiment, which is consistent with the results of Huang et al. (1991). A continuous increase in JH titers was, however, detected in a second experiment (Robinson et al. 1987), which suggests that different intermediate titers may be associated with different tasks performed by young and middle-age bees. Our results indicate only two significantly different states of CA activity, but smaller differences may be revealed by more detailed analyses.

Hypopharyngeal gland size was correlated inversely with worker age (for bees >8 days of age), which is consistent with previous findings (King 1933; Rösch 1930; Fluri et al. 1982; Fergusson and Winston 1988). The size range of hypopharyngeal gland acini we report (75–141 μm) is similar to that of others (60–150 μm) (Brouwers 1982; Fergusson and Winston 1988; Crailsheim and Stolberg 1989). Hypopharyngeal gland size was correlated inversely with rate of JH biosynthesis, which agrees with findings that hypopharyngeal glands decrease in size either with the onset of foraging (Rösch 1925; Hassanein 1952; Haydak 1957) or after injection with high doses of JH or JH mimic (Rutz et al. 1974; Jaycox et al. 1974; Jaycox 1976). Our results, however, demonstrate that exocrine gland development is not strictly linked with age-dependent behavior or JH status. Soldiers had larger hypopharyngeal glands than did similarly aged foragers, but the two groups had indistinguishable rates of JH

biosynthesis. Guards and undertakers had marginally smaller (13%) hypopharyngeal glands than similarly aged wax producers and food storers, but much higher (93%) rates of JH biosynthesis. Guards and undertakers had larger hypopharyngeal glands than did foragers, but indistinguishable rates of JH biosynthesis. Since the relationship between rate of protein synthesis and gland size is complicated by the fact that some large glands can have low activity (Brouwers 1982; Huang et al. 1989), analyses of protein synthesis from the hypopharyngeal glands of soldiers, guards, and undertakers might be more informative.

Our results support the recent claim (Breed et al. 1990) that soldiers constitute a group of bees that are distinct from foragers. Soldiers had significantly less wing wear than did foragers, as was shown by Breed et al. (1990). In addition, we report that soldiers had significantly larger hypopharyngeal glands than did foragers, despite their similar age and JH levels. The relationship of JH to behavior is particularly intriguing for soldiers because we do not know what soldier bees do when they are not defending their colony. Soldiers either maintain high levels of JH, even when they are not exhibiting defensive behavior, or they show rapid changes in JH titer that occur just prior to, or shortly after, the expression of defensive behavior. Hormonal changes within 10–30 min in response to social interactions have been detected for vertebrates (Wingfield 1985; Wingfield and Wada 1989), but not for insects.

In summary, high levels of JH are associated not only with foraging, but also with guarding, undertaking, and soldier behavior. Hypopharyngeal gland size does not only vary with worker age and JH level, but for older bees can also vary with the specific task that is performed. The insights and questions generated by this study indicate that much can be learned about the physiology of division of labor in general by further analyzing the mechanisms of behavioral differentiation among similarly aged bees.

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