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Juvenile hormone titers, juvenile hormone biosynthesis, ovarian development and social environment in *Bombus terrestris*

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Abstract

The effects of the social environment and age on juvenile hormone (JH) and reproduction were investigated by measuring ovarian development, hemolymph levels of JH III, and rates of JH biosynthesis from the same individual bumble bees (*Bombus terrestris)*. Differences in social environment were associated with differences in rates of JH biosynthesis, JH titer and ovarian development. Young queenless workers had a higher rate of JH biosynthesis, JH titer and ovarian development than queenright (QR) workers of similar age. Dominant workers in QR colonies had a higher rate of JH biosynthesis, JH titer and ovarian development than low ranked workers of similar size. There was a positive correlation between JH titer and ovarian development, but no correlation between rate of JH biosynthesis and ovarian development or between JH biosynthesis and JH titer. Both JH titer and rate of JH biosynthesis increased with age from emergence to 3 days of age, but 6-day-old workers, egg-laying workers, and actively reproducing queens had high JH titers and highly developed ovaries but low rates of JH biosynthesis. These results show that reproduction in *B. terrestris* is strongly affected by the social environment and the influence of the environment on reproduction is mediated by JH. Our data also indicate that the rate of JH biosynthesis measured in vitro is not a reliable indicator of JH titer or ovarian development in *B. terrestris*; possible reasons are discussed.

Keywords: Hymenoptera; Juvenile hormone; Corpora allata; Oogenesis; Dominance

1. Introduction

Juvenile hormone (JH) plays an important and diverse role in reproduction in many insect species by regulating vitellogenesis in the fat body, vitellogenin uptake by the ovaries (reviewed by Tobe and Stay, 1985; Koeppe et al., 1985; Nijhout, 1994; Davey, 1996), and oviposition behavior (Cayre et al., 1994). Results of previous studies suggest that JH is involved in the regulation of ovarian development in primitively social Hymenoptera like polistine wasps (Röseler, 1991; Barth et al., 1975) and halictine bees (Bell, 1973). In the highly social honey bee *Apis mellifera* JH is apparently involved in the regulation of division of labor and has no clear role in the regulation of reproduction (reviewed in Robinson, 1992; Robinson and Vargo, 1997). There is evidence to suggest that JH is involved in the regulation of ovary development in bumble bees as well. Queenless *Bombus terrestris* workers have faster ovarian development, higher rates of JH biosynthesis in vitro and higher JH hemolymph titers than similarly aged queenright (QR) workers (Röseler, 1977; Röseler and Röseler, 1978; Larrere and Couillaud, 1993). Differences in JH biosynthesis rates were confirmed by Bloch et al. (1996), who for the first time validated the radiochemical assay (RCA) for *B. terrestris*. However, measurements of JH titer in this species have thus far only been made with the semiquantitative *Galleria* assay (Röseler, 1977), so it is not known whether differences in rates of JH biosynthesis reflect differences in hemolymph titers. Although JH biosynthesis rates in vitro are generally assumed to reflect in vivo

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synthesis rates and JH titers (reviewed in Tobe and Stay, 1985; Feyereisen, 1985; Koeppe et al., 1985; Nijhout, 1994), the relationship between these variables is not clear. Measurements of both JH titer and rate of biosynthesis using modern techniques have been made only in a few insect species (Renucci et al., 1990; Huang and Robinson, 1995).

Worker reproduction in social insects is strongly affected by the social environment. In most species worker reproduction is inhibited by the presence of a queen, and occurs only after queen removal or death. In some species, including *B. terrestris*, worker reproduction can occur in the presence of a queen, but only during specific stages of colony development (reviewed in Wilson, 1971; Michener, 1974; Fletcher and Ross, 1985; Bourke, 1988; Spradbery, 1991; Heinze et al., 1994). *B. terrestris* provides an excellent model for studying the relationships between the social environment and hormonal regulation of reproduction and behavior. In normally developing colonies, workers that are reproductively suppressed can become reproductively active during the last stage of colony development (the competition phase, 'CPh', Duchateau and Velthuis, 1988). Moreover, previous studies showed that changes in the social environment are associated with changes in rates of JH biosynthesis and in ovarian development (Röseler, 1977; Röseler and Röseler, 1978; Bloch et al., 1996; Bloch and Hefetz, 1999). The purpose of this study is to explore two issues: (1) the effects of different social environments on JH titers, and (2) the relationships between JH titer, JH biosynthesis rate and ovarian development. These were studied by measuring these three variables from the same individuals reared under different social environments.

2. Materials and methods

2.1. Bees

Colonies of *B. terrestris* were obtained from Bio-Bee Sde-Eliahu Industry, Bet Shean, Israel, a few days after the first worker in each colony had eclosed. Colonies contained a queen, between one and ten workers, and brood at different stages of development. They were reared in Styrofoam nesting boxes (18 cm×27 cm×12 cm) with a cardboard bottom in an environmental chamber at a temperature of 29±2°C and constant darkness, except for a few minutes of light during feeding or experimental manipulations. Sugar syrup (Bee Happy, obtained from Bio-Bee Sde-Eliahu Industry) and fresh pollen (collected by honey bees) were supplied to all colonies ad libitum. Observations were performed under dim red light through a glass lid placed on top of the nest box.

2.2. Measurement of JH biosynthesis in vitro

JH biosynthesis by corpora allata (CA) incubated in vitro was measured by radiochemical assay (RCA) (Pratt and Tobe, 1974; Tobe and Pratt, 1974) specifically validated for adult *B. terrestris* (Bloch et al., 1996). Briefly, the head was removed and immersed in bee saline (ionic ratio of 1.5Na:1K, as found in *B. terrestris* worker hemolymph, see Bloch et al., 1996). Under a dissecting microscope (×50), the corpora allata–corpora cardiaca complex (hereafter referred to as the CA) was removed through an opening in the ventral part of the head and cleaned of adhering tissue. The CA from each bee was then transferred into a separate borosilicate tube (6 mm×50 mm) containing 25 μl methionine-free bee medium. No more than 2 h elapsed from the first dissection to the initiation of the incubation. The incubation was started by adding 25 μl bee medium containing 500 μM L-[^{3}H-methyl]methionine (NEN, specific activity 200 mCi/mmol) to give a final methionine concentration of 250 μM. Incubation lasted for 3 h at 39°C. JH was extracted from the culture medium after removing the CA by adding 100 μl double distilled water and 150 μl isooctane (Sigma-Aldrich, HPLC grade) (Feyereisen and Tobe, 1981; Bloch et al., 1996).

2.3. Measurement of hemolymph JH titer

Bees were immobilized with plasticine under a dissecting microscope and hemolymph samples collected (1–14 μl from workers, 10–33 μl from queens) with a small glass capillary drawn to a fine point that was inserted into the aorta through the dorsal neck membrane. Hemolymph was transferred to a piece of parafilm and immediately quantified (to the nearest 0.1 μl) with a calibrated microcapillary tube. Each hemolymph sample was diluted into 0.5 ml acetonitrile and stored at −80°C until analyzed. All glassware was baked at 500°C for 3.5 h prior to use to eliminate JH contamination. All solvents were HPLC grade (purchased from EM Science, Fisher Scientific, or J.T. Baxter Chemical Co).

A chiral-specific radioimmunoassay (Hunnicutt et al., 1989) was used to measure the JH III titer. The detection limit of the RIA was about 10 pg 10 *R* JH III per sample. Intra-assay coefficient of variation was 8.5% and inter-assay coefficient of variance was 13.4%. A detailed description of this RIA can be found in previous studies on honey bees (Huang et al., 1994; Huang and Robinson, 1995). Previous results (Goodman et al., 1993; Huang et al., 1994; Huang and Robinson, 1995) indicate that values from this RIA agree with two other RIAs, both validated with gas chromatography/ mass spectroscopy (de Kort et al., 1985; Goodman et al., 1990). For this study, the RIA was validated for adult *B. terrestris* using a method similar to that used for honey bees (Huang et al., 1994) and burying beetles (Trumbo et al., 1995).

Briefly, small amounts (approximately 7000 DPM) of [10-H^3 (N)]-JH III (NEN, 629 Gbq/mmol) were added to hexane extracts of hemolymph samples (n=6). Approximately half of each sample was fractionated by normal phase high performance liquid chromatography (HPLC) (Alltech Econosil, 5 mm, 4.6 mm×250 mm silica column; 10% diethyl ether in hexane, 1 ml/min). Previous studies have shown that this procedure separates JH from most lipids (cholesterol, cholesterol-oleate, methyl-oleate, oleic acid and triolein; see Huang et al., 1994). One-minute fractions were collected, dried, and resuspended in 20 μl ethanol. An aliquot of each fraction was analyzed for radioactivity to determine the recovery of JH III (68.4±10.2%, n=6). Another aliquot was analyzed for immunoreactivity using RIA. Similar aliquots of the unfractionated half of each sample were also analyzed for radioactivity and immunoreactivity. Because in this experiment we used tritium-labeled JH III both for calculating recovery and for measuring unlabeled JH in the hemolymph, the percent binding in the RIA was adjusted by calculating a new, maximum binding. This was done by assuming that the molecules of additional radioactive JH bind to the JH-antibody in the same proportion as do the radioactive JH molecules in the JH–antibody mixture. The amount of JH in each sample was estimated using the new adjusted percent binding value. The amount of immunoreactivity detected in the HPLC fractions of each extract was similar to the amount present in the unfractionated material (108±17%, n=6). Most of this activity (86±14%, n=6) was detected in a single peak that coeluted with [^{3}H]-JH III (Fig. 1). No other fractions had JH immunoreactivity higher than background levels (less than 10 pg per tube).

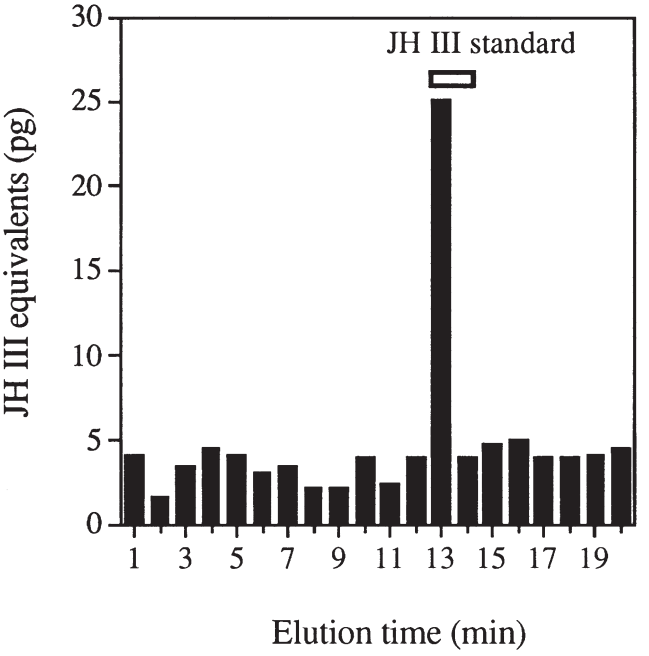

Fig. 1. Immunoreactivity of HPLC-separated adult *B. terrestris* worker hemolymph samples (n=6). Fractions were collected every minute. Immunoreactivity was determined using RIA with antibodies against JH III. For comparison, the horizontal open bar represents immunoreactivity of a JH III sample separated using a similar procedure.

2.4. Measurement of ovarian development

Bees were dissected in bee saline as described above, ovaries were removed, and the length of each terminal (basal) oocyte was measured with an ocular ruler under a dissecting microscope (×25–50 magnification). The mean length of all terminal oocytes (n=8) was used as an index of ovarian development for each bee. The number of mature oocytes with a clearly visible chorion was also recorded.

2.5. Experiment I: effect of the queen on the JH titer in young workers

Callow workers (less than 12 h after eclosion and before full coloration occurred) were collected and marked with colored numbered tags. Some of these workers were introduced into QR colonies and some were placed in groups of three in small plastic cages (95 mm×107 mm×107 mm, with a cardboard bottom) to form queenless (QL) groups. To minimize possible effects of genetic variation, callow bees from different source colonies were collected and mixed before being assigned at random to each experimental group. Measurements of JH titer, JH biosynthesis rate, and ovarian development were made from individual bees that were 0, 1, 3 or 6 days of age. Because the data for JH biosynthesis rate and ovarian development for most workers have already been published (Bloch et al., 1996), here we report only JH titers and correlations of the three variables.

2.6. Experiment II: effect of colony development on JH titers in young workers

Callow workers were introduced into intact colonies at different stages of colony development. Three stages of colony development were defined (Bloch and Hefetz, 1999): stage I, more than 2 weeks before the onset of the CPh; stage II, two weeks or less before the onset of the CPh; stage III, during the CPh. The onset of the CPh was defined as the first day in which one or more of the following events were observed:

1. oviposition behavior performed by workers;
2. two or more open egg cups constructed over 2 or more successive days;
3. only one open egg cup observed over 2 or more successive days, but with clear signs of cell destruction;
4. oophagy.

In order to maintain the same worker density, an equal number of workers was removed from the recipient colonies when the callow workers were introduced. Similarly, when the introduced workers were collected for analysis, they were replaced by an equal number of callow workers. Thus, the same colony could be used at different stages of development. In large colonies (over 50 workers) during the CPh, up to five workers were introduced without adjusting worker number. JH titer and rates of JH biosynthesis were measured in 3-day-old bees. JH biosynthesis rates for these workers have also been published already (Bloch and Hefetz, 1999); here we report only JH titer data and correlations of JH titer and JH biosynthesis rate.

2.7. Experiment III: association of social status with JH titers, rate of JH biosynthesis and ovarian development in workers

Egg-laying workers were identified under a dim red light in QR and QL colonies at the competition phase. A worker was defined as an egg layer if it was seen performing oviposition behavior, i.e. inserting her abdomen into an egg cup for approximately 2 min while moving her hind legs (Bloch and Hefetz, 1999). Previous studies indicate that oviposition behavior is performed mainly by workers with high social status (Van Honk and Hogeweg, 1981; Van Doorn and Heringa, 1986; Van Doorn, 1987). Workers of similar size that did not exhibit the behavior characteristic of dominant workers during a 5–10 min observation period were also collected from the same colony. These were assumed to be non-egg-laying workers of lower social rank. Previous observations (Bloch, unpublished data) indicate that the 5–10 min period is sufficient for assessing worker social rank, because behavioral differences between low and high ranked workers are easily distinguished (Van Honk et al., 1981; Van Doorn and Heringa, 1986). Dominance behaviors included: oviposition behavior, egg cup construction, agonistic behaviors toward other bees, egg cup destruction and oophagy. Ovarian development, rates of JH biosynthesis and JH titers were measured.

2.8. Experiment IV: comparison of JH titers and rates of JH biosynthesis in queens

JH titers and biosynthesis rates were measured in functional (actively egg laying) queens collected from colonies before or during the CPh. JH biosynthesis rates were measured in 3- and 6-day-old virgin queens. To assure that the queens were virgins, they were removed from the nest as pupae or immediately after emergence (before pigmentation) and kept in small cages without males. Ovarian development for queens was scored simply as fully developed (functional queens) or undeveloped (virgin queens).

2.9. Statistical analyses

Measurements of ovarian development and JH titers were not normally distributed, so logarithmic transformed data were used for linear regression analyses. In all other statistical tests, non-parametric tests were used to compare ovarian development and JH titers: Kruskal–Wallis for analysis of variance and Mann–Whitney for unpaired comparisons between two groups (Sokal and Rohlf, 1995).

Relationships between rates of JH biosynthesis, JH titers and ovarian development were examined with linear regression analysis based on the a priori assumptions that JH titer and ovarian development depend on rates of JH biosynthesis and that ovarian development depends on JH titer. These paired relationships were examined both within and between experimental groups. Only data obtained from the same bees were used to examine these relationships.

To examine whether differences in JH titers among egg-laying and non-egg-laying bees were associated with differences in social status or with differences in ovarian development (Experiment III), we calculated the regression of ovarian development on JH titers and then used this relationship to remove the variation in JH titer explained by variation in ovarian development. The relationship between ovarian development and JH titer was estimated as:

$$\text{Log (JH titer)} = 2.26 + 0.7\ \text{Log (terminal oocyte length)} \quad (1)$$

(see Fig. 5 below). This regression line was used to obtained an expected JH titer value for each bee based on its oocyte length (Y adjusted; Sokal and Rohlf, 1995) using the equation:

$$Y\ \text{adjusted} = \text{mean}\ Y + d_{y \cdot x} \quad (2)$$

Mean Y is the value obtained when positioning the mean x value in the regression line and $d_{y \cdot x}$ is the deviation of the observed value from the regression line (calculated by subtracting the expected JH titer from the observed JH titer). This method could not be used to examine whether differences in rates of JH biosynthesis among egg-laying and non-egg-laying bees were associated with differences in social status or with differences in ovarian development, because regression analysis suggested that rates of JH biosynthesis and ovarian development were independent (see Section 3). Instead, we only compared rates of JH biosynthesis in egg-laying and non-egg-laying workers that had similar ovarian development. Means±standard errors are reported throughout this paper.

3. Results

3.1. Experiment I: effect of the queen on the JH titer in young workers

Workers exhibited an age-dependent increase in JH titer from emergence to 6 days of age (Fig. 2). However, the pattern of increase in JH titer was different from the pattern of increase in JH biosynthesis rate (Fig. 2). The JH titer increased more rapidly in QL workers. There were no significant differences in JH titers between 1-day-old QL or QR workers, but QL workers had significantly higher JH titers than QR workers by day 3 (Mann–Whitney test, P=0.013, n=14). A similar trend was evident at day 6 but the difference was not significant, probably due to the high variation among QL workers (Mann–Whitney test, P=0.08, n=26).

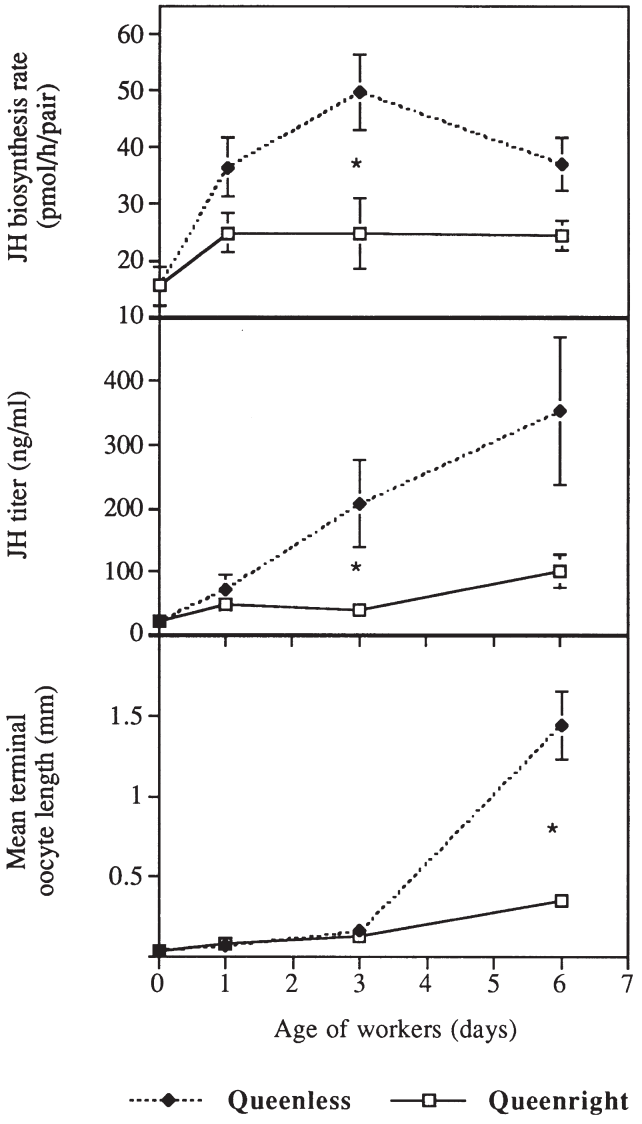

Fig. 2. Rates of JH biosynthesis, JH titer, and ovarian development for young workers reared in queenless and queenright conditions. Each point represents the mean (±SE) of 7–24 individuals. Most of the data for JH biosynthesis rates and ovarian development were taken from Bloch et al. (1996). *P<0.05.

There was much variation in the JH titers of bees in the QL groups. Within a group of three, one worker generally had a high JH titer while the other two had titers similar to those of QR workers. The mean value for 6-day-old workers with the highest titer in each group was 677.3±269.2 ng/ml (n=5), compared with 174.6±58.1 ng/ml (n=9) for the remaining bees. The variance in JH titer for 3- and 6-day-old QL bees was significantly higher than for QR bees (variance ratio 155 and 20.6, respectively; equality of variance F test, P<0.0001). A similar trend was also detected in 1-day-old bees (variance ratio 3.9, P=0.051).

3.2. Experiment II: effect of colony development on JH titers in young workers

There was no significant difference in JH titer (Kruskal–Wallis test, d.f.=2, P=0.75) among workers introduced into colonies at different stages of development (Fig. 3). However, the pattern of changes in JH titer during colony development followed a similar trend to the pattern in ovarian development (Fig. 3). Three-day-old QR workers in this experiment and in Experiment I had similar JH titers although the samples were collected during different periods (Mann–Whitney test, P=0.87, n=62).

3.3. Experiment III: association of social status with JH titers, rate of JH biosynthesis and ovarian development in workers

JH titers, JH biosynthesis rates, and ovarian development were similar for egg-laying workers from QL and QR colonies (two-way ANOVA, P=0.33, P=0.71, P=0.26, respectively). Data for workers from these two colony types were pooled for the following analyses. Egg-laying workers had significantly higher JH biosynthesis rates, JH titers, and greater ovarian development than did non-egg-laying workers (Mann–Whitney test, P=0.006, P=0.003, P<0.0001, respectively, Table 1). All egg-laying workers had developed ovaries (largest terminal oocyte length over 2.5 mm) compared with only 52.6% of the non-egg-laying workers. Eighty-two percent of the laying workers had JH titers greater than 100 ng/ml, compared with only 40% for non-egg-laying workers. Differences in JH titer, JH biosynthesis rate, and ovarian development between egg-laying and non-egg-laying workers could not be explained by body size. Head widths (an index of body size) were similar for egg-laying and non-egg-laying workers (4.39±0.1 mm and 4.53±0.08 mm, respectively, n=16 for both groups; unpaired t-test, t=1.07, P=0.29).

To assess the relationship between social rank and JH

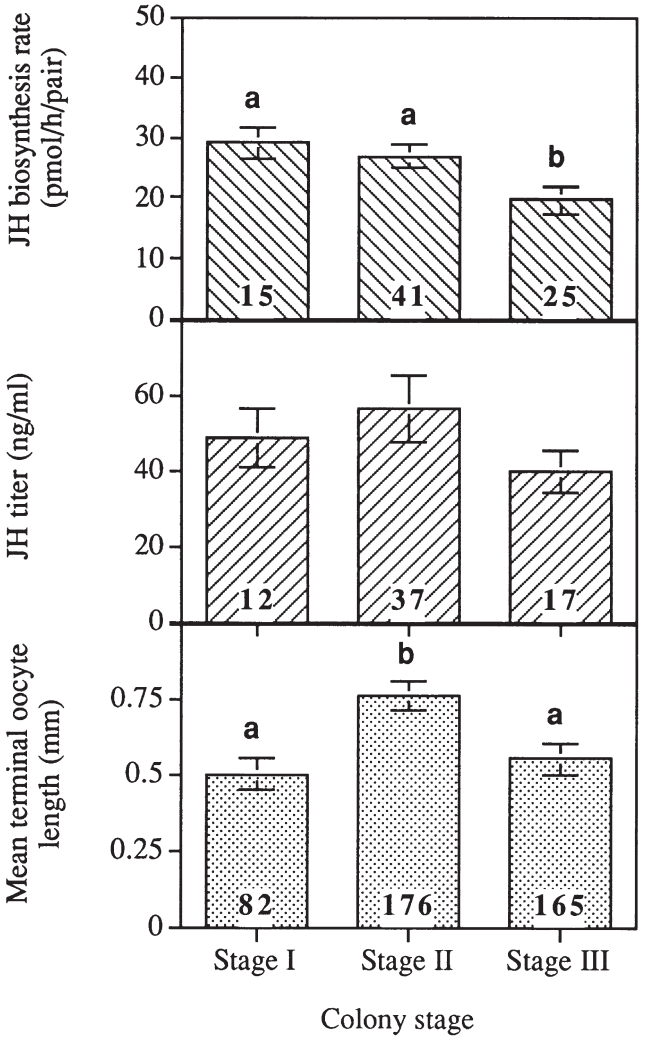

Fig. 3. Rates of JH biosynthesis and JH titer for 3-day-old workers and ovarian development for 7-day-old workers (mean±SE) introduced into queenright colonies at different stages of social development. Colony development was divided into three stages: Stage I, 2 weeks before the onset of the competition phase; Stage II, less than 2 weeks before onset of the competition phase, and Stage III, during the competition phase. Values within bars indicate sample sizes. Different letters indicate statistically significant differences between groups ($P<0.05$). Data for JH biosynthesis rates and ovarian development were taken from Bloch and Hefetz (1999).

biosynthesis, independent of ovarian development, we compared JH biosynthesis only in egg-laying and non-egg-laying workers with mean terminal oocyte lengths greater than 2.5 mm (egg-laying, 2.93±0.05 mm, n=31; non-egg-laying, 2.87±0.06 mm, n=15; Mann–Whitney test, P=0.6). Rates of JH biosynthesis did not differ for the two groups (egg-laying, 31.5±3.1 pmol/h per pair, n=31; non-egg-laying, 24.7±4.4 pmol/h per pair, n=15, d.f.=44, P=0.22).

To examine whether JH titers were higher in egg-laying workers, independent of ovarian development, the expected JH titer was calculated (see Section 2 for description of the analysis). There were no significant difference in JH titers between egg-laying and non-egg-laying workers independent of ovarian development (unpaired t-test, t=0.3, d.f.=29, P=0.77). Using the same method, it was also found that there was no significant difference in JH titer between bees with or without mature eggs (mature eggs have a visible chorion, unpaired t-test, t=1.35, d.f.=29, P=0.19).

3.4. Experiment IV: comparison of JH titers and rates of JH biosynthesis in queens

All functional queens had developed ovaries. Most (72%) had JH titers that were above 100 ng/ml, but low rates of JH biosynthesis compared with workers (see Tables 1 and 2, and Figs. 2 and 3). All virgin queens had undeveloped ovaries, and low rates of JH biosynth-

Table 2
Rates of JH biosynthesis in vitro and JH titers in queens (mean±SE, sample size in parentheses)[a]

Queen type	JH biosynthesis rates (pmol/h per pair)	Hemolymph JH titer (ng/ml)
Pre-CPh functional queens	16.8±2.3 (16)	181.9±46.7 (14)
CPh functional queens	18.8±3.2 (21)	279.6±46.5 (22)
Virgin queens	10.8±1.65 (16)	Not determined

[a] CPh, competition phase, i.e. the phase of worker reproduction. Differences between queen types were not statistically significant ($P>0.05$, ANOVA for rates of JH biosynthesis, Mann–Whitney test for JH titers).

Table 1
Rates of JH biosynthesis in vitro, JH titers, and ovarian development (mean terminal oocyte length) in egg-laying and non-egg-laying workers (mean±SE, sample size in parentheses)[a]

Worker behavioral classification	JH biosynthesis rates (pmol/h per pair)	Hemolymph JH titer (ng/ml)	Mean terminal oocyte length (mm)
Egg-laying	34.13±3.22 (32)	394.6±81.4 (17)	2.7±0.06 (39)
Non-egg-laying	22.57±2.45 (30)	149.7±40.6 (15)	1.33±0.17 (38)

[a] The differences between egg-laying and non-egg-laying workers were significant for all three parameters ($P<0.01$, Mann–Whitney test for JH titers and ovarian development, unpaired t-test for rates of JH biosynthesis).

Table 3
Relationships between rates of JH biosynthesis in vitro, JH titers, and ovarian development[a]

	All workers	Queenright	Queenless	Between groups
JH biosynthesis–JH titer	0.04 (151)	0.007 (97)	0.075 (40)	0.22 (10)
	*	ns	ns	ns
JH biosynthesis–ovarian development	0.013 (156)	0.0001 (50)	0.12 (49)	0.07 (9)
	ns	ns	*	ns
JH titer–ovarian development	0.25 (95)	0.51 (42)	0.22 (38)	0.78 (9)
	***	***	***	***

[a] Results of linear regression analysis [r^2 (n)] based on the a priori assumptions that JH titer and ovarian development depend on JH biosynthesis rate, and that ovarian development depends on JH titer. Data for JH titers and ovarian development were analyzed after log transformation. Significance of the variance explained by linear regression: *$P<0.05$; **$P<0.01$; ***$P<0.001$; ns, not significant.

esis that were not statistically different from those of functional queens (ANOVA, P=0.09, Table 2). There was no significant difference in JH titers or rates of JH biosynthesis between functional queens from colonies before or during the CPh (Mann–Whitney test, P=0.18, Table 2).

3.5. The relationship between rates of JH biosynthesis and JH titer

JH biosynthesis rates accounted for only 4% of the variance in JH titers (Table 3, all workers). We also conducted more detailed analyses by examining the regression separately for QL and QR workers and for workers at different ages (Table 3). The regression was significant only when we restricted the analysis to 0- to 3-day-old workers, but even for these workers, variation in JH biosynthesis rate accounted for only 7% of the variation in JH titer (r^2=0.07). Within an even more homogeneous group (3-day-old QR workers, pooled data from Experiments I and II), the regression was not significant (Fig. 4, r^2=0.01, n=62, P=0.43). Likewise, the regression was not significant for functional queens (r^2=0.1, n=21, P=0.16).

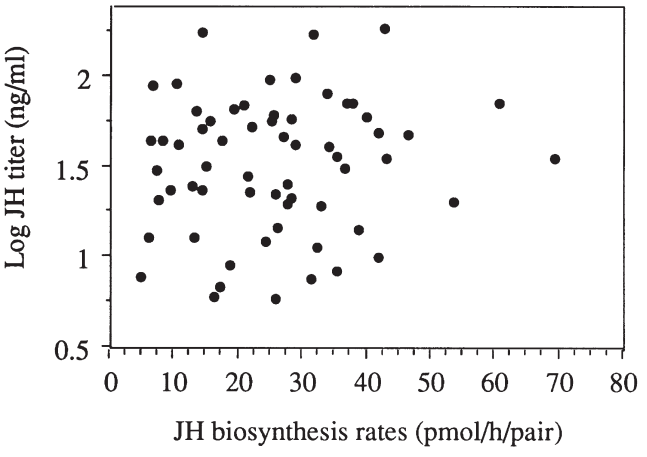

Fig. 4. The relationship between JH biosynthesis rates in vitro and JH titer in 3-day-old queenright workers. Each point represents both measurements from the same individual (n=62).

To reduce the effects of individual variation and sampling error we performed further regression analyses using the means of defined groups of bees with similar ages that developed under similar social conditions (between-group regression, Table 3). Both JH biosynthesis and JH titers increased for workers 0–3 days of age (QL and QR workers), producing a significant regression. However, the means of 6-day-old workers, egg-laying workers and non-egg-laying workers deviated from this regression line due to their high JH titer (Fig. 2). This trend was more evident in functional queens that had low JH biosynthesis rates and high JH titers.

3.6. The relationship between JH biosynthesis rates and ovarian development

The relationship between rates of JH biosynthesis and ovarian development changed under different social conditions and in different age groups. For example, the within-group regression of ovarian development on rates of JH biosynthesis was not significant in a pooled sample of all workers or in QR colonies. However, when the analysis was limited to QL workers, the regression was significant. There was no significant association between the two variables when a between-group analysis was conducted (Table 3). For example, 3-day-old QL workers showed the highest mean rates of JH biosynthesis but had undeveloped ovaries (Fig. 2), and laying workers had highly developed ovaries but moderate rates of JH biosynthesis (Table 1). Likewise, functional queens had mature eggs in their ovaries but low rates of JH biosynthesis (Table 2).

3.7. The relationship between JH titer and ovarian development

A significant linear regression was obtained between JH titer and worker ovarian development. This was observed for all workers, or for QR and QL workers

when analyzed separately (Table 3). The highest JH titers were obtained for bees with highly developed ovaries: functional queens and egg-laying workers. A comparison of the means of the different groups produced a highly significant between-group regression of ovarian development on JH titer (Fig. 5, Table 3, $Y=1.43X-3.24$, $r^2=0.78$, $n=9$).

4. Discussion

This study presents for the first time for social insects measurement of JH titers, JH biosynthesis, and ovarian development in the same individuals reared under various social conditions. Our results are consistent with previous studies demonstrating the influence of the social environment on *B. terrestris* reproduction (Duchateau and Velthuis, 1989; Röseler and Van Honk, 1990; Bloch et al., 1996; Bloch and Hefetz, 1999). They also provide important support for the hypothesis that the effects of the social environment on reproduction are mediated by JH (reviewed in Röseler and Van Honk, 1990).

The correlation between titer of JH III, the only JH homologue found in *B. terrestris* hemolymph, and ovarian development in this study, coupled with previous results showing that JH I treatment caused a dose-dependent increase in oocyte development in QR workers (Röseler, 1977), are consistent with the hypothesis that JH is a gonadotropin in bumble bees. However, to further support this hypothesis it is necessary to conduct experiments in which ovarian development is analyzed following CA removal and JH replacement.

Differences in the social environment were associated with changes in titers of JH III in *B. terrestris* hemolymph. In addition, bees of presumed different social status showed differences in JH titers. The association between social status and JH titers might, at first appearance, provide support for previous suggestions that JH is involved in the modulation of dominance behavior in bumble bees (Van Doorn, 1989; Larrere and Couillaud, 1993), in addition to its role as a gonadotropin. However, since position in a dominance hierarchy often is associated with differences in ovarian development in *B. terrestris*, these two factors can be confounded. When we statistically removed the effect of ovarian development, we found that JH titers are not correlated with high social status. This finding does not support the hypothesis that JH is involved in the modulation of dominance behavior, but is consistent with previous studies that showed that treatment with JH I did not increase worker dominance in either QL or QR conditions (Van Doorn 1987, 1989). In contrast, treatment of *Polistes* wasps with JH alone or in combination with 20-hydroxy ecdysone increased the probability of becoming dominant (Barth et al., 1975; Röseler et al., 1984).

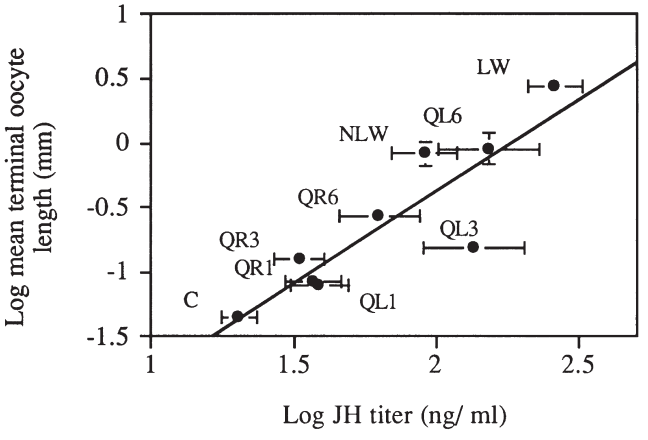

Fig. 5. The relationship between JH titer and ovarian development between means of defined groups of bees. Each point represents the mean±SE of 7–15 individuals. C, callow workers less than 12 h old; QR, queenright; QL, queenless; LW, laying workers; NLW, non-laying workers. Numbers indicate bee age (days). The solid line is the regression line for the two variables ($Y=3.2X-7.27$, $r^2=0.79$, $P<0.001$).

Differences in the social environment were also associated with differences in rates of JH biosynthesis. However, these rates were not always correlated with JH titers or with ovarian development. Correlations were better for young workers than for old workers. The relationship between rates of JH biosynthesis and JH titer has been closely examined only in a few insect species. In our study (using RIA) and in a previous study (using the *Galleria* bioassay), these two variables were not correlated when measured in the same individuals (Lanzrein et al., 1978). In two other studies where both measurements were obtained from the same individuals, these variables were correlated, but different correlations were obtained at different times (Huang and Robinson, 1995) or under different physiological conditions (Renucci et al., 1990). The lack of correlation between JH biosynthesis rate and JH titer raises two fundamental questions: (1) is the level of hormone biosynthesis rates measured in vitro a reliable estimate of hormone production in vivo? (2) Is it reasonable to expect that hormone production will always be correlated with its circulating levels?

Studies relying on measurements of JH biosynthesis by the CA in vitro have been very successful in elucidating the hormonal bases of reproduction in many insect species (reviewed in Tobe and Stay, 1985; Feyereisen, 1985; Koeppe et al., 1985; Nijhout, 1994). We suggest that there are species-specific differences, and stage-specific intraspecies differences in the regulation of the CA (Unnithan et al., 1998), that might result in in vitro activity that does not reflect changes in JH titer. There are at least five possible mechanisms, acting alone or combined, that may be responsible for a lack of correlation between rates of JH biosynthesis in vitro and hemolymph JH titers.

1. There is a time lag between the changes in JH biosynthesis rates and changes in its titer. For example, it appears that the fast increase in JH biosynthesis after 1 day of QL conditions in our study resulted in an elevation of JH titers only after 2 or 3 days (Fig. 2, see also Röseler and Van Honk, 1990; Bloch et al., 1996).
2. CA activity is influenced by neural stimuli and exposure to allatostatins and allatotropins (e.g. Gadot and Applebaum, 1985; Stay and Woodhead, 1993; Kaatz et al., 1994; Woodring and Hoffmann, 1994; Stay et al., 1997); the absence of these factors in vitro may in some cases lead to different rates of JH biosynthesis than in vivo. For example, Okuda et al. (1996) reported a negative correlation between JH biosynthesis rates in vitro and ovarian development in the locust *Locusta migratoria*, a species in which JH regulates ovarian development. This is possibly because the CA in vitro were removed from endogenous sources of inhibition (Okuda and Tanaka, 1997). Likewise, Unnithan et al. (1998) recently showed for the cockroach *Diploptera punctata* that denervation releases the CA from brain inhibition.
3. JH titers might not be determined solely by JH biosynthesis, but also by changes in JH degradation and excretion (Tobe and Stay, 1985; Hammock, 1985).
4. Insects can have high JH titers but low CA activities due to a negative feedback mechanism (for review see Feyereisen, 1985; Tobe and Stay, 1985; Khan, 1988). For example, in our study it is possible that laying workers and functional queens had low rates of JH biosynthesis because they produced only enough JH to balance degradation and excretion, but they had high JH titers due to previously high levels of JH biosynthesis.
5. JH might be produced in a pulsatile fashion, typical of many vertebrate hormones, or with a circadian rhythm (Krieger, 1979; Krieger and Aschoff, 1979; Turek and Van Cauter, 1988; Leng and Brown, 1997). Given that the radiochemical assay for measuring rates of JH biosynthesis in vitro has been so useful in developing our understanding of JH regulation of reproduction, these more recent studies suggest that it is important to study JH dynamics and the regulation of the CA in a boarder array of insect species.

Conducting such studies on *B. terrestris* would help illuminate issues and perhaps increase our understanding of social regulation of endocrine-mediated reproduction. Our study suggests that the role of JH in bumble bees is different from its role in the honey bee, a related species from the same family (Apidae). In the honey bee, JH is not known to be involved in the regulation of ovarian development (Engels et al., 1990; Robinson et al. 1991, 1992), but has been implicated in the regulation of age-dependent division of labor among workers, (reviewed in Robinson, 1992; Robinson and Vargo, 1997), including effects of the social environment on JH-mediated worker behavior (Huang and Robinson, 1992, 1996). This suggests that comparative studies on the effect of the social environment on JH in bumble bees and honey bees can be used to explore evolutionary aspects of this regulatory mechanism.

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