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Caste Determination in *Bombus terrestris*: Differences in Development and Rates of JH Biosynthesis between Queen and Worker Larvae

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To study the possible role of juvenile hormone in caste determination in *Bombus terrestris*, we measured development and rates of juvenile hormone biosynthesis *in vitro* in larvae destined to develop into either workers or queens. Larvae of both castes developed through four instars and had the same growth rates. However, the duration of the instars was longer for queen larvae, and their head width at the third and fourth instars was significantly larger. After validating the well-known radiochemical assay of JH for bumble bee larvae, we show that worker larvae corpora allata exhibited a constant and low rate of JH biosynthesis, never more than 5 pmol JH/h/pair. Queen larvae, in contrast, had two peaks of JH biosynthesis: a small one during the first instar, which has previously been correlated with caste determination; and a large peak, previously undetected, above 40 pmol JH/h/pair, during the second and third instars. We suggest that caste determination in this species is mediated by JH and that the duration of larval instars is a key factor. The possibility that the queen influences caste determination via an effect on instar duration is also discussed. **© 1997 Elsevier Science Ltd. All rights reserved**

Bombus terrestris Larval development Caste determination JH

INTRODUCTION

One of the key elements structuring insect societies is caste determination: the process whereby a totipotent genotype results in either a worker or queen phenotype. Knowledge of the mechanisms underlying caste determination is thus very important to our understanding of how insect colonies have evolved and function. In the few species that have been studied to date, juvenile hormone (JH) has been found to play a major role in caste determination (reviewed by Brian, 1980; Wheeler, 1986). In the honey bee *Apis mellifera*, larvae destined to become queens ('queen larvae') have a higher titer of JH in whole body extracts (Rembold, 1987b; Rembold *et al.*, 1992) and elevated rates of JH biosynthesis by the corpora allata (CA) (Rachinsky and Hartfelder, 1990) as compared to larvae destined to be workers ('worker larvae'). In addition, several studies have shown that JH treatment, independent of nutrition, can induce the development of queens from female larvae (Wirtz and Beetsma, 1972; Rembold *et al.*, 1974; Asencot and Lensky, 1984). Similar differences between queen and worker larvae in JH titers of whole body extracts in *Scaptotrigona postica* (Hartfelder and Rembold, 1991), and JH titer in the hemolymph of *Bombus terrestris* and *B. hypnorum* (Strambi *et al.*, 1984) suggest that JH plays a comparable role in these species. JH is also involved in caste determination in the ants *Myrmica rubra* (Brian, 1974) and *Pheidole bicarinata* (Wheeler and Nijhout, 1983).

Studies of *Bombus* species have provided evidence for different types of caste determination systems (Plowright and Jay, 1977; Plowright and Pendrel, 1977; Pomeroy and Plowright, 1982 for North American species;

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Röseler, 1970, 1976, 1977; Röseler and Röseler, 1974 for the European species). In the subgenus *Pyrobombus* (the European species *B. hypnorum* and the North American *B. rufocinctus* and *B. ternarius*), queen or worker determination occurs during the last larval instar and depends primarily on food quantity. Supplying additional food or treating larvae with JH at the end of the last instar prolongs larval development and results in queen determination (Röseler, 1976). In the subgenus *Bombus* (*B. terrestris* and *B. terricola*), queen or worker determination apparently occurs earlier in larval life, during the first instar, between the third and fourth days (Röseler, 1970; Plowright and Pendrel, 1977). In *B. terrestris*, additional food or treatment with JH at the end of the last instar does not affect caste determination (Röseler, 1976).

Caste determination occurs in a complex social context. It is generally thought that adult queens inhibit the rearing of new queens by influencing the brood rearing behavior of workers. For example, in honey bee colonies only in the absence of a queen do workers feed larvae a rich enough mixture of glandular secretions to cause higher levels of CA activity and the consequent development of new queens (Winston, 1987). In honey bees the regulation of this worker or queen rearing behavior is mediated by queen pheromones (Winston *et al.*, 1990; Engels *et al.*, 1993). In *B. terrestris* colonies, it has been suggested by Röseler (1970) and Duchateau (1989) that queens also produce a pheromone that inhibits the development of larvae into queens. This pheromone may act indirectly by causing changes in worker behavior, as in honey bees, or it may act directly on the larvae themselves. Since bumble bee colonies are much smaller than honey bee colonies, such direct queen effects are theoretically possible.

Colonies of *B. terrestris* are characterized by a change in social structure that is associated with a decrease in queen dominance and the onset of worker reproduction. This change in the social behavior of the bees is called the competition phase. The competition point (the onset of the competition phase) is correlated with queen production (Duchateau and Velthuis, 1988) suggesting that these social changes affect caste determination.

The present work explores the physiological mechanisms underlying caste determination in bumble bees. Bumble bees are particularly interesting for this purpose because queen rearing occurs under several different social conditions: not only in the absence of a queen, but also in the presence of the queen during the 'competition phase', late in the annual cycle of colony development when social order breaks down. As a first step towards elucidating the physiological mechanisms underlying caste determination in bumble bees, we describe the development of both worker and queen larvae and compare their rates of JH biosynthesis.

MATERIALS AND METHODS

Colonies

Colonies were obtained from Biological Control Industries, Kibbutz Sde-Eliyahu, 3–5 days after the first workers emerged. They were maintained in the laboratory in nest boxes (30×20×12 cm) at 28–30°C. Colonies were furnished with unlimited amounts of sugar solution and freshly collected pollen from honey bee colonies.

Preliminary studies were made on the timing of key events during colony development to determine whether our experimental conditions were comparable to those of other investigators studying *B. terrestris*. During these observations the colonies were inspected once a day and the position of all egg-cells, larvae and cocoons were recorded. The 'switch point', defined as the onset of haploid eggs laid by the queen, occurred 16±3.5 (n=17) days after the first worker emerged, similar to that reported by Duchateau and Velthuis (1988) (Mann-Whitney, p>0.05). We determined the competition point if at least one out of these three events were observed: workers were observed to lay eggs, workers were observed to destroy queen egg cells, or the co-occurrence of two open egg cells. On average the 'competition point' occurred 25±9 (n=39) days after first worker emerged, which was significantly (p<0.001) earlier than the 30.8±4.6 value reported by Duchateau and Velthuis (1988). Larval developmental time was 10.2±2.3 days for workers and 13.7±1.9 days for queens (not including a common five-day developmental time in the egg stage). Both egg and larval developmental times were longer than those reported by Röseler (1970).

Queen and worker larvae

Larvae fated to develop into workers ('worker larvae') were obtained from very young colonies that had an active, laying queen. Larvae fated to develop into queens ('queen larvae') were obtained from queenless colonies by removing the queen from the colony and selecting only the larvae that hatched after the removal of the queen (Röseler, 1970). To validate the efficacy of this method for obtaining queen larvae, we removed the queen from three colonies and allowed the larvae to develop into adults. Of the approximate 50 female larvae that hatched after queen removal (six egg-cells, an approximate eight eggs per cell), 100% of them developed into queens.

Larval development

In the genus *Bombus*, the queen lays eggs in groups of about eight inside a wax egg-cell that she builds herself (Alford, 1975; Duchateau and Velthuis, 1988; Cnaani: unpublished observations). Immediately after laying a single group (a process that can take 2–3 min) the queen seals the cell, so the larvae in each cell develop as a cohort. We used time-lapse photography to estimate larval age, by photographing each colony at 2 h intervals for the occurrence of sealed egg-cells. Thus the age of the larvae was determined with an accuracy of ±2 h.

Frequency distributions of head widths were used to determine the number of instars during development. The head capsule is the only part of the larva that has a hard cuticle and can provide a reliable measure of size (Nijhout, 1981). Head width was measured with a stereo microscope equipped with an ocular micrometer. Larvae were weighed (±0.1 mg) with an analytical balance.

Validation of the radiochemical assay

Rates of JH biosynthesis by larval CA *in vitro* were measured according to established procedures (Pratt and Tobe, 1974; Tobe and Pratt, 1974; modified by Feyereisen and Tobe, 1981). Since this assay has never before been used for *B. terrestris* larvae, it was necessary to optimize incubation conditions and validate its use according to an established protocol (Tobe and Stay, 1985). We thus tested various incubation media, incubation temperatures and methionine concentrations, determined whether JH biosynthesis rates were linear over time, and identified the radiolabeled products of the CA.

To remove the CA, the head of each larva was cut with fine scissors and placed in a drop of 'bee saline' (Huang *et al.*, 1991), exposing the paired CA on both sides of the pharynx. The glands were cleaned of any adhering tissue and transferred with a fine wire loop into a drop of methionine-free incubation medium. Choice of medium was determined (see Results) by measuring rates of JH biosynthesis in CA incubated in either MEM (Sigma M-3911), Grace's (Sigma G-5649) or medium formulated for honey bees ['bee medium', formulated by Kaatz *et al.* (1985); modified by Huang *et al.* (1991)].

After performing about 10 dissections (in 1 h), we transferred the CA from each individual larva into a borosilicate tube containing 50 μl incubation medium and [methyl-^{3}H] methionine (200 mCi/mmol, NEN). The final concentration of methionine was determined by measuring rates of JH biosynthesis in CA incubated with 15, 25, 50, 100, 150 and 250 μM.

Tubes were sealed with parafilm to prevent evaporation and placed in a heated water bath. Optimal incubation temperature was determined by measuring rates of JH biosynthesis at 25, 32, 39 or 45°C. Following a 3 h incubation, the glands were removed from the tubes and the medium extracted with 100 μl distilled water and 250 μl isooctane. Finally, 200 μl from the isooctane phase were taken for counting in a scintillation counter. For the above experiments we attempted to minimize the effects of inter-individual variation between larvae by testing each of the two CA of a larva under a different condition.

To assess the linearity of JH biosynthesis, glands were incubated for 5.5 h, but were transferred to new medium every 1 or 2 h. After optimizing the conditions for the radiochemical assay (see Results), all further experiments were performed as follows: CA were incubated in 'bee medium', with 150 μM methionine, at 39°C, for 3 h.

The identity of the radiolabeled compounds biosynthesized by the CA *in vitro* was determined by HPLC. Twelve pairs of CA were incubated. Extracts of the CA and the medium were analyzed separately. Each gland pair was transferred into 50 μl distilled water, immediately extracted twice with two aliquots of isooctane (250 and 100 μl, respectively), and the combined isooctane extracts subsequently washed with 75 μl 1% NaCl (in H_2O). Four samples of gland extracts, each containing the pooled extracts of three gland pairs, were subjected to HPLC analysis. The incubation medium was extracted as above, but the medium extracts from each gland pair were analyzed individually. An aliquot (of each sample of gland or medium extract) was separated by normal phase HPLC with an Alltech Econosil SI column (5 μ, 250×4.6 mm ID) using 8% diethyl ether in hexane. Under these conditions, methyl farnesoate (MF), JH I, JH II and JH III were cleanly separated (approximate retention times: 4.8, 10.5, 11.4 and 13.3 min, respectively). The eluting material was collected for 20 min at 0.5 or 1 min intervals. The radioactivity of each of these fractions was determined in a liquid scintillation counter. This was compared with an aliquot of each sample taken before HPLC, to quantify the recovery of material analyzed. Since MF elutes close to the solvent front, its identity was further confirmed by analyzing a second aliquot of some samples with 1% diethyl ether in hexane. Under these conditions, retention time is far longer (8 min).

RESULTS

Description of larval development

Analysis of larval head widths revealed that both worker and queen larvae developed through four instars (Fig. 1 and Table 1). There was no significant difference in head width between queen and worker larvae during

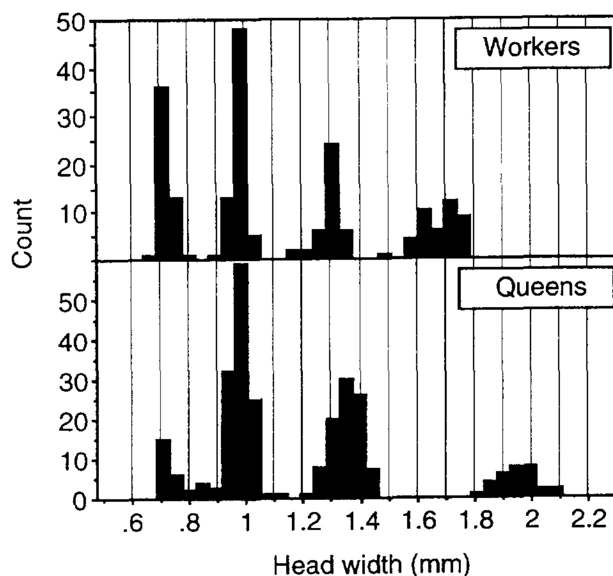

FIGURE 1. Frequency distributions of head capsule widths for queen and worker larvae.

TABLE 1. Comparison of maximal weight and head width for the four instars of *B. terrestris* worker and queen larvae (sample size in parentheses)

Instar	Maximal weight (mg)		Head width (mm) mean±SD	
	Queen	Worker	Queen	Worker
1	7.8	6.8	0.710±0.030 (23)	0.703±0.024 (51)
2	64.0	39.5	0.963±0.038 (121)	0.959±0.026 (67)
3	256.2	95.8	1.336±0.050 (91)	1.276±0.044 (40)
4	1291.0	375.3	1.933±0.068 (31)	1.669±0.061 (41)

the first two instars (t-test, p=0.14 and 0.23, respectively). However, by the third instar, queen larvae had significantly larger head widths (t-test, $p<0.0001$). Differences were even more pronounced for the fourth instar: there was no overlap in head width between queen and worker larvae (t-test, $p<0.0001$).

Worker and queen larvae differed in the weight they attained by the end of each instar (Table 1); however, their growth rate was the same (F-test for difference between two regression coefficients, $p<0.1$, Fig. 2). This suggests that the greater mass of the queen larvae was not a consequence of more rapid growth, but rather of prolonged developmental time. Indeed, the total development time for queen larvae was significantly longer than that of worker larvae (13.7±1.9 and 10.2±2.3 days, respectively, Mann-Whitney, $p<0.0001$).

The pattern of development throughout the instars also differed between queen and worker larvae. Figure 3 depicts the proportion of larvae at each instar as a function of their age. Again, worker larvae appeared to develop more rapidly than queen larvae; at each age interval the proportion of worker larvae that achieved an advanced instar was higher than in queen larvae.

Validation of the radiochemical assay

No significant differences were found among the three incubation media evaluated (paired t-test, p>0.05 for all comparisons), so bee medium was used for all further experiments. The optimal incubation temperature was found to be 39°C. Glands incubated at this temperature synthesized significantly more JH/h than did glands at 25, 32 or 45°C (paired t-test, p=0.0015, d.f.=7; p=0.004, d.f.=6; p=0.0001, d.f.=7, respectively).

Glands incubated with 15 or 50 μM methionine had significantly lower rates of JH biosynthesis than glands incubated with 150 μM (paired t-test, p=0.007, d.f.=9 and p=0.016, d.f.=9, respectively). However no significant differences were found in rates of JH biosynthesis for glands incubated with 25, 100 and 250 μM when compared to 150 μM (paired t-test, p=0.17, d.f.=9, p=0.22, d.f.=10 and p=0.08, d.f.=8, respectively). Since glands that were incubated with 100 and 250 μM had the same JH biosynthesis rates as glands incubated with 150 μM, we used the 150 μM concentration for all further incubations.

The corpora allata synthesized JH at a constant rate

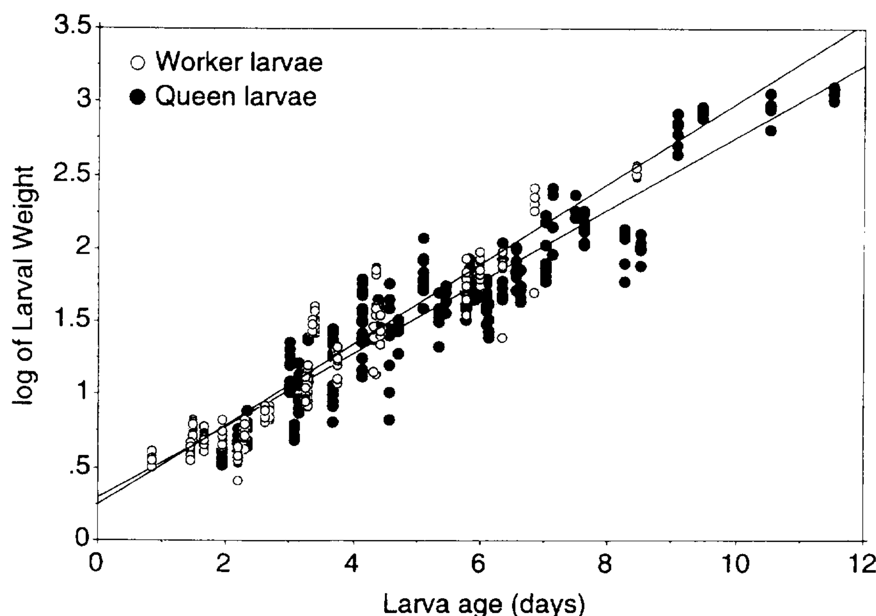

FIGURE 2. Regression analysis of log larval weight as a function of larval age. Regression equation for queen larvae, $Y=0.274X-0.95$; for worker larvae, $Y=0.275X-1.134$.

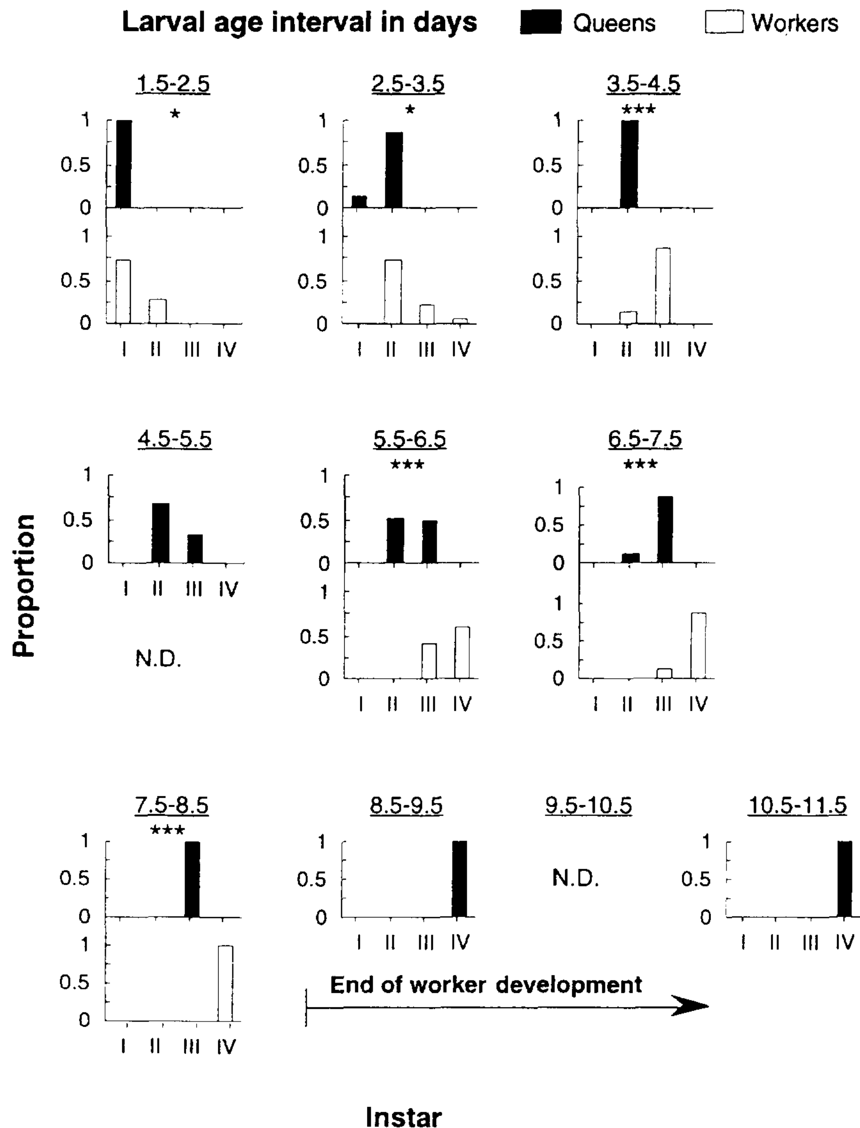

FIGURE 3. Comparison of the proportion of worker and queen larvae of *B. terrestris* at each age in each instar. Numbers above each graph represent larval age in days, starting from the age of 1.5 days, measured after egg hatching. *, $p<0.05$; **, $p<0.001$; ***, $p<0.0001$ (based on Chi-square test).

during 5.5 h of incubation (regression analysis, $r^2=0.64$, $p<0.001$ for eight pairs of glands). All incubations thereafter were conducted for 3 h, well within the linear range of synthesis.

HPLC analysis of extracts of CA and incubation media revealed that over 99% (±4.7% SE, $n=10$) of the extracted radiolabeled material was recovered from the column in two peaks. In extracts of incubation medium, most (90±5% SE, $n=6$) of the radioactive material comigrated with JH III. A small amount of the radioactivity (8±1% SE, $n=6$) coeluted with methyl farnesoate. Similarly, the major peak of radiolabeled material recovered from extracts of CA (80±4% SE, $n=4$) coeluted with JH III. Most of the remaining radioactivity (14±2% SE, $n=4$) comigrated with methyl farnesoate. Radioactive material comigrating with methyl farnesoate eluted with this standard when a lower polarity solvent (1% diethyl ether in hexane) was used, partially confirming its identity.

JH biosynthesis during larval development

Since worker and queen larvae differed in total development time and weight during the different instars, comparing rates of JH biosynthesis at the same age or weight would not be as informative as comparing them at a similar 'physiological stage'. To calculate the physiological stage, each larva was first assigned to an instar (on the basis of head width; see Fig. 1), and then into one of five stages within an instar, based on the minimum and maximum larval weight for each instar (Fig. 4). For example, larvae assigned to stage one of a particular instar had completed 0–20% of the maximal growth for that instar. For the first instar, the weight of the egg (1.5 mg) was set as the minimal instar weight. The youngest larvae in the first instar that were analyzed were stage-two larvae since stage-one larvae were too small to handle for dissection. At the fourth instar, larvae start to spin their cocoon before they stop feeding

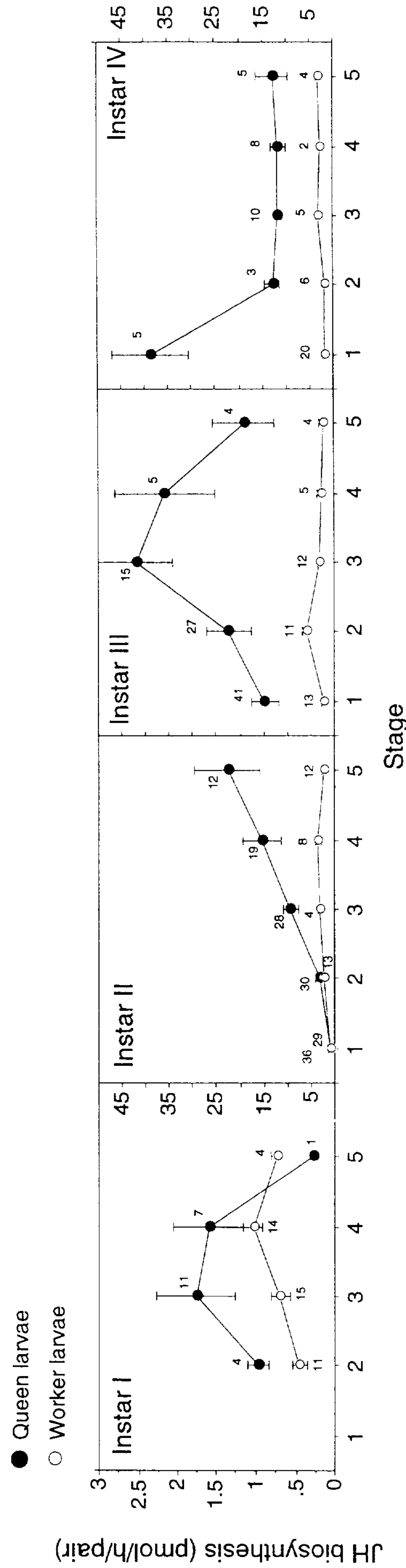

FIGURE 4. JH biosynthesis rates as a function of larval development. In each of the four instars, larvae were divided into five stages (for details see 'Results'). Each point represents the mean±SE for each developmental stage. The number near each point is the number of CA pairs analyzed.

(unpublished observation; and see also Plowright and Jay, 1977). Queen larvae, for example, pupate at a weight of about 1300 mg, but start spinning and defecate at a weight of about 600 and 800 mg, respectively. The end of larval development was thus determined as the time of cocoon completion. Prepupae were not included in our analysis.

During the beginning of the first instar (stages two and three), queen larvae had significantly higher rates of JH biosynthesis than worker larvae (p=0.016, t-test after log transformation, for both stages). JH biosynthesis rates in queen larvae then declined to the levels exhibited by worker larvae (p=0.2) during the last two stages of the instar (stage four and five). In the first three stages of the second instar, rates of JH biosynthesis were similar for worker and queen larvae (p=0.1, 0.2 and 0.25 for stages one, two and three, respectively). From stage four onward, queen larvae had significantly higher rates of JH biosynthesis than worker larvae (p=0.03 and 0.006, respectively). JH biosynthesis rates in queen larvae reached their maximum during stage three of the third instar (42 pmol JH/h/pair), decreasing thereafter until the last part of the fourth instar, where they remained at a constant level (about 12 pmol JH/h/pair) until the end of larval development. In contrast, rates of JH biosynthesis of worker larvae remained relatively constant during the third and fourth instar (approximately 2–3 pmol JH/h/pair) and were significantly lower at each developmental period compared to queen larvae ($p<0.05$ for all the stages in the third and fourth instar).

DISCUSSION

In eusocial insects queens constitute a caste that is morphologically and physiologically distinct from workers. Caste determination, therefore, must involve marked differences in postembryonic development and hormonal balances.

The possible role of JH in caste determination was studied by several authors in several bees species. Some of the studies addressed the question by measuring JH titer in whole body extract (Rembold, 1987b; Rembold *et al.*, 1992 for *A. mellifera*; Hartfelder and Rembold, 1991 for *S. postica*) or in the hemolymph (Strambi *et al.*, 1984 for *B. terrestris* and *B. hypnorum*), while others measured JH synthesis by the corpora allata (Rachinsky and Hartfelder, 1990 for *A. mellifera*; Hartfelder, 1987 for *S. postica*). Strambi *et al.* (1984), measured JH titer in the hemolymph of *B. terrestris* and *B. hypnorum* during prepupal development. They found that although the levels and pattern of JH titer were similar in queens and workers of *B. terrestris*, JH peaked in workers earlier than in queens. They also determined that JH III is the only JH found in *B. terrestris* larvae, which was also confirmed in the present study.

Our results demonstrate that differences in rates of JH biosynthesis are associated with caste differences in *B. terrestris* larvae. Worker larvae are characterized by a relatively constant and low level of CA activity that does not exceed 5 pmol JH/h/pair. Queen larvae, in contrast, have two peaks of JH biosynthesis: a small peak during the first instar; and a second, larger, peak that exceeds 42 pmol JH/h/pair from the middle of the second instar through most of the third instar. The small peak is of special interest since it coincides with the timing of caste determination. According to Röseler (1970, 1976) caste determination takes place at the age of 3.5 days, during the first instar. This is approximately when this peak occurred, taking into account that developmental times for bees in our study were shorter than in Röseler's (in our study, larvae 3.5 days old are already in the middle of the second instar). The second large peak presumably affects the length of each of the following instars resulting in the distinctively larger size of the queen. Differences in JH titer were also found in the prepupal stage (Strambi *et al.*, 1984). The fact that the levels and patterns of hemolymph JH titer were similar in queens and workers preclude the possibility that it affects caste determination. The fact that in queens JH peaks later than in workers, probably reflects the prolonged time of postembryonic development that characterizes queen larvae.

This is the first study to measure the pattern of JH biosynthesis throughout larval development in any bee species. Previous studies of honey bees (Rachinsky and Hartfelder, 1990) and the stingless bee *Scaptotrigona postica* (Hartfelder, 1987) focused only on specific developmental stages. For both species, higher rates of JH biosynthesis are exhibited by queen larvae. In queen larvae of *S. postica* CA activity increases at the end of the feeding period and the beginning of spinning. In honey bee queen larvae JH biosynthesis increases during the transition from the fourth to fifth instar and reaches a maximum at the end of the feeding period. Another caste-specific peak in JH biosynthesis occurs in queen larvae at the end of the spinning period. In *B. terrestris* as in the above two species, queen larvae possess higher rates of JH biosynthesis than worker larvae. However, there are differences in the patterns of JH biosynthesis that possibly reflect species specificity. While in *A. mellifera* and *S. postica* JH biosynthesis rates peak at the end of the feeding period, *B. terrestris* show a decline at the same developmental stage. There are also differences between *B. terrestris* and *A. mellifera* in queen larva development. Unlike in *B. terrestris*, in honey bees there are no caste-specific differences in the duration of the first four instars, and the fifth instar is shorter in queens (Rembold, 1987a). Growth rate of queen larvae is higher and the peak of CA activity is at the end of the feeding period (Rachinsky and Hartfelder, 1990). In contrast, *B. terrestris* queen larvae develop more slowly through the instars and the overall development time of the queens is longer, but growth rates of the two castes are similar. CA biosynthetic activity peaks in the middle of the third instar, whereas in honey bees it rises only towards the end of the feeding period. These differences may reflect differences in the mechanisms of caste differentiation.

In bumble bees, caste determination may occur early in larval life as in *B. terrestris* (Röseler, 1970, 1976) and *B. terricola* (Plowright and Jay, 1968) or late in larval life as in *B. hypnorum* (Röseler, 1970). In *B. terricola*, early caste determination does not result in different growth rates of the two castes. Rather, the queen larvae have a longer feeding stage than worker larvae (Plowright and Jay, 1977). In a more recent study, Ribeiro (1994) found that *B. terrestris* worker larvae showed a more rapid increase in mass than queen larvae. Our results with *B. terrestris* are similar to those in *B. terricola* and are in contrast to those obtained by Ribeiro. Under our conditions queen larvae achieve their higher weight apparently by feeding for an additional three days. We found no difference in growth rate between the two castes, but there is a significant difference in the rate of development through the instars: worker larvae advance more quickly through the instars than do queen larvae. The differences between Ribeiro's results and ours could be due to differences in the method of obtaining queen larvae. Ribeiro obtained queen larvae from queenright colonies in the phase of queen production, while we obtained them from queenless colonies.

We have identified caste-specific differences in both rates of JH biosynthesis and instar duration, but it is not known whether the former cause the latter. Most studies of the relationship between these two factors have focused on Lepidoptera and only on the last larval instar. They commonly report that a high titer of JH can delay the pupal molt by inhibiting the secretion of PTTH (Nijhout and Williams, 1974; Nijhout, 1994). Data on larval-larval molting are very limited. Safranek (quoted in Williams, 1976) found that the duration of all the larval instars of *Manduca sexta* were prolonged as a result of chronic treatment with JH, but Sakurai (1983) reported that treating fourth-instar larvae of *Bombyx mori* with JH analog does not delay ecdysis to the fifth instar. However, both Safranek *et al.* (1980) and Sakurai (1983) found that JH has an inhibitory effect on the secretion of ecdysone from the prothoracic glands. Recently, Kamimura (1995) showed that the JH analog fenoxycarb can lengthen all five instars of *Bombyx mori* in a dose-dependent manner. These results suggest that the observed differences in JH biosynthesis rates may affect instar duration in *B. terrestris*, but the situation in Lepidoptera may be different than in Hymenoptera. It is also necessary to determine whether changes in rates of JH biosynthesis reflect changes in JH hemolymph titers in *B. terrestris* larvae.

It is generally assumed that queens play an important role in caste determination in insect colonies, but supporting data are limited. In honey bees this influence is indirect, by affecting worker brood care behavior. In *B. terrestris* Röseler (1970) reported that dominant queens can inhibit the production of young queens. The present study suggests that queens may somehow influence the duration of larval instars. Whether the queen affects the larvae directly or indirectly (via changes in worker behavior), and whether the presumed queen effects are pheromonally mediated, remain to be investigated.

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