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The critical period for caste determination in *Bombus terrestris* and its juvenile hormone correlates

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Abstract The critical period for caste determination and its juvenile hormone (JH III) correlates were studied in *Bombus terrestris*. Larvae of known age and instar were taken from young colonies, in which they would have been reared as workers, and placed into groups of queenless workers. Under these conditions the critical age for caste determination was 5 days, during the second instar. Endocrine correlates of caste determination were obtained by determining profiles of juvenile hormone titer and juvenile hormone biosynthesis, measured by chiral-specific radioimmunoassay and the in vitro radiochemical assay, respectively. By the middle of the second instar prospective queen larvae had significantly higher rates of juvenile hormone biosynthesis and juvenile hormone titer than prospective worker larvae. Based on the coincidence of timing of both the critical period and the appearance of caste-specific juvenile hormone titer, we suggest that juvenile hormone plays a role in the mechanisms that control caste determination in *B. terrestris*.

Key words *Bombus terrestris* · Critical period · Caste determination · JH · Social insects

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Abbreviations *CA* corpora allata glands · *JH* juvenile hormone · *PTTH* prothoracicotropic hormone · *QL* queenless · *QR* queenright

Introduction

One of the characteristics defining social insects is reproductive division of labor, wherein females belong to two different castes: queens or workers (Wilson 1971). In most species queens and workers differ not just in their reproductive output, but also by their physiology and morphology. In the social bee *Bombus terrestris* the two female castes differ strikingly in size and physiology, but not in other morphological characters beside body size (Michener 1974). The queen is over three times larger than even the largest workers and there is no size overlap between the two castes. This size difference is not the result of different feeding rates (Röseler and Röseler 1974; Riberio et al. 1999), food composition (Pereboom 2000), or growth rates (Cnaani et al. 1997). Rather, the molts from the second to third and from the third to fourth (and last) instar in queen larvae occur at an older age (Cnaani et al. 1997) than in worker larvae. The total feeding period of queen larvae is therefore 3 days longer, which enables queens to achieve their greater size.

It is still unknown how the timing of the penultimate molts is determined in *B. terrestris*. The endocrine events that regulate the timing of molting have been studied in depth in several species of Lepidoptera and Hemiptera. According to the generally accepted scheme, molting is an interplay between prothoracicotropic hormone (PTTH), ecdysone, and juvenile hormone (JH). While PTTH and ecdysone trigger the molt, JH directs the type of molt and the nature of the next instar. In addition, JH regulates the timing of PTTH secretion (Nijhout 1994).

Beside its role in the molting process, JH is also known to be involved in caste determination in social Hymenoptera such as *Apis mellifera* (Wirtz and Beetsma 1972; Rembold et al. 1974), *Scaptotrigona postica* (Hartfelder and Rembold 1991), *Bombus hypnorum*

(Röseler and Röseler 1974), *Pheidole bicarinata* (Wheeler and Nijhout 1983), and *Myrmica rubra* (Brian 1974). For most of these species high levels of JH during a specific critical period of larval development are required for queen induction (soldier induction in *P. bicarinata*). By measuring in vitro JH biosynthesis rates for queen and worker larvae, Cnaani et al. (1997) showed that JH is probably also involved in caste determination of *B. terrestris*. Starting from the middle of the second instar, queen larvae had higher biosynthesis rates. This difference persists during the remainder of larval development, with the largest difference in the middle of the third instar. However, early studies of caste determination in *B. terrestris* reported that the critical period for queen determination is 3.5 days during the larval first instar (Röseler 1970). This suggests that the above-mentioned elevated JH biosynthesis rates are a consequence, rather than one of the causes, of caste determination. To investigate this issue, we re-examined the critical period in larval development for caste determination in *B. terrestris*. We also quantified the hemolymph JH titer and JH biosynthesis rate during larval development, from the same individuals, in order to better evaluate the endocrine correlates of caste determination.

Materials and methods

Colony maintenance and development

Colonies were obtained from Biological Control Industries, Kibbutz Sde-Eliyahu, Israel, about 3–5 days after the first workers emerged. They were maintained in the laboratory in nest boxes (30 cm × 20 cm × 12 cm) at 28–30 °C, and furnished with unlimited amounts of sugar solution and fresh pollen collected from honeybee colonies (Duchateau and Velthuis 1988; Cnaani et al. 1997).

Colony development was followed by direct daily observations. The developmental stage of the nest was determined by drawing the comb on a transparency and noting the formation of egg cells, larval development, and the number of pupae. When more exact timing of larval hatching was required, the nests were photographed every 2 h to determine the exact time of egg deposition. The onset of queen production (time of oviposition of the first egg that resulted in a queen) was determined retrospectively from the daily drawings. Colony age was calculated from the emergence of the first adult worker.

Determination of molting age and molting weight

Queen and worker larvae were sampled from queenless (QL) and queenright (QR) colonies, respectively, at known age and weight. The instar of the sampled larvae was determined by measuring their head width (Cnaani et al. 1997).

Critical larval age and instar for caste determination

The critical period is defined as the larval age after which caste determination becomes irreversible. This was determined by transplanting prospective worker larvae at different ages into QL worker groups. Since the groups were queenless, there was more likelihood that they would rear larvae as queens. Each group of 12 QL workers received one brood cell. To ensure that the transplanted larvae originated under conditions favoring worker caste development, they were taken from colonies that were younger than 5 days old when the eggs were laid. The first egg that developed into a queen was laid under our rearing conditions at an average colony age of 11 ± 3 days (range 4–20 days). Adult workers were obtained from old colonies that had already started to rear queens; they were therefore assumed to have had experience in queen rearing.

To determine the critical age for caste determination, 34 brood cells (containing an average of 7 ± 1.4 larvae/cell, a total of 243 larvae) of known age were used. The ages of the transplanted larvae ranged from 1–8 days after hatching. To determine the critical instar for caste determination, another 64 brood cells (6 ± 2 larvae/cell, 388 total, excluding the sampled larvae) were transplanted into QL conditions. We sampled two larvae from each of these cells before transplantation, allowing the remaining larvae to develop to pupation. The sampled larvae were weighed and their instar determined by measuring the width of the head capsule (Cnaani et al. 1997). Since all the eggs in a single cell are typically laid within a few minutes (Michener 1974; J. Cnaani, personal observation), the sampled larvae can be considered to be representative of their cellmates that were allowed to develop to pupation. In both experiments (determination of critical age and determination of critical instar) we monitored the percentage of larvae that developed to queens.

JH biosynthesis and titer during larval development

Biosynthesis rates and titers of JH III were measured from larvae at different ages, instars and castes. Both measures were taken from each individual larvae. Rates of JH biosynthesis by larval corpora allata glands (CA) were measured in vitro according to established procedures (Pratt and Tobe 1974; Tobe and Pratt 1974), modified for *B. terrestris* larvae (Cnaani et al. 1997). Briefly, 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 either side of the pharynx. Cleaned glands of individual larvae were transferred into borosilicate tubes containing 50 µl incubation medium with 150 µmol l^{-1} [methyl-3H] methionine (200 mCi $mmol^{-1}$, NEN). Incubations were performed at 39 °C for 3 h, after which 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 measurement of radioactivity in a scintillation counter.

Titers of JH III were measured by a chiral-specific radioimmunoassay (Hunnicutt et al. 1989) specifically validated for *B. terrestris* larvae (Cnaani et al. 2000). Measured amounts of hemolymph, collected by puncturing the aorta and drawing the accumulating drop into a calibrated capillary tube, were mixed with 5 ml of chilled acetonitrile. Samples were kept at −80 °C until further analysis.

Since worker and queen larvae differ in total development time and weight during the different instars (Cnaani et al. 1997), comparing rates of JH biosynthesis at the same age or weight would not be as informative as comparing them at similar "physiological stages" (Cnaani et al. 1997; Hartfelder et al. 2000). Briefly, each larva was first assigned to an instar and then into one of five stages within an instar, based on the minimum and maximum larval weight for each instar. For example, developmental stage 2.1 refers to larvae that were in the first weight group of the second instar (0–20% of the maximal weight of the larvae at the end of the instar). Larvae in the first instar were too small for blood sampling and were omitted from the analyses. Data on JH biosynthesis in such larvae were reported earlier (Cnaani et al. 1997).

Results

Larval developmental time

Queen and worker larvae differed in developmental time (Fig. 1). The development of queen larvae from egg

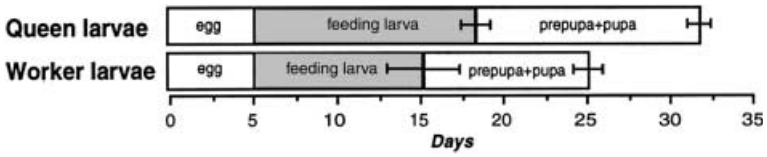

Fig. 1 Development time (days) of queen and worker larvae. *Horizontal bars* represent the SD for the egg + feeding period and for prepupa + pupa

oviposition until the end of the feeding period was 18.4 ± 1.1 days ($n = 28$), whereas that of worker larvae was 15.2 ± 2.3 days ($n = 51$; Mann Whitney $P < 0.01$). There was also a significant difference in the time from end of feeding to adult emergence, 13.4 ± 0.8 days ($n = 36$) for queens and 9.9 ± 0.9 days ($n = 96$) for workers (Mann Whitney $P < 0.01$). Variance in developmental time for workers was significantly greater than that for queens (F-test for differences in variance $P < 0.05$), but there was no difference in variance for length of prepupal and pupal stages.

Larval age and weight at molting

Table 1 depicts the ages and weight at which 50% of the larvae molted to the next instar. There were no significant differences in the weight of worker and queen larvae at the point when 50% of them molted from the first to the second instar. However, the ages and weights at which 50% of the queen larvae molted from the second to the third instar and from the third to the fourth instar were significantly greater than for worker larvae.

Critical larval age and instar for caste determination

Figure 2 shows the critical larval age for queen determination. There is a sharp drop in the percentages of larvae that developed into queens between the ages of 5 days and 6 days. Between 80% and 100% of the younger larvae (1–4 days old) that were transferred developed into queens. Transferring the larvae at the age of 5 days resulted in 50% queen development, but there was only 20–30% queen development for older (6 days and 7 days old) transferred larvae. All 8-day-old larvae developed into workers.

Table 1 The age (days) and mass (mg) of worker and queen larvae at which 50% molted to the next instar

Molting from instar	Age (days)		Mass (mg)	
	Worker larvae	Queen larvae	Worker larvae	Queen larvae
1 to 2	2.29[a]	3.39[b]	6.51[a]	6.63[a]
2 to 3	4.52[a]	6.10[b]	31.0[a]	50.72[b]
3 to 4	6.18[a]	8.77[b]	78.39[a]	228.5[b]

[a,b] Numbers followed by different letters are significantly different (Kolgomorove-Smirnov $P < 0.01$)

The critical period for queen determination occurred during the second instar (Fig. 3). A total of 94% of the larvae that were in the first instar ($n = 43$) and 63% of the larvae that were in the second instar ($n = 156$) when transferred to QL colonies developed into queens. In eight brood cells (containing 53 larvae) one of the sampled larvae was in the second instar while the second larva was in the third instar. Among these larvae, 55% developed into queens. In contrast, in cells ($n = 13$) in which both sampled larvae were in the third instar, 91% of the larvae ($n = 69$) developed into workers. Cells containing larvae at later instars produced only worker bees.

JH biosynthesis and titer during larval development

Figure 4A shows comparative analyses of in vitro JH biosynthesis for queen and worker larvae. JH biosynthesis for queen larvae was significantly higher than for worker larvae, starting from developmental stage 2.4 through the end of the third instar. In the fourth instar, JH biosynthesis for queen larvae declined to a low level that was not different from that of worker larvae.

Measurements of JH titer in queen and worker larvae showed a slightly more complex picture (Fig. 4B). During developmental stages 2.4 and 2.5 there were significant differences between the castes (Mann Whitney $P < 0.01$). At the onset of the third instar (developmental stages 3.1 and 3.2) the differences in JH titer were not significant (Mann Whitney $P > 0.05$), but were significant again during the remainder of the third instar

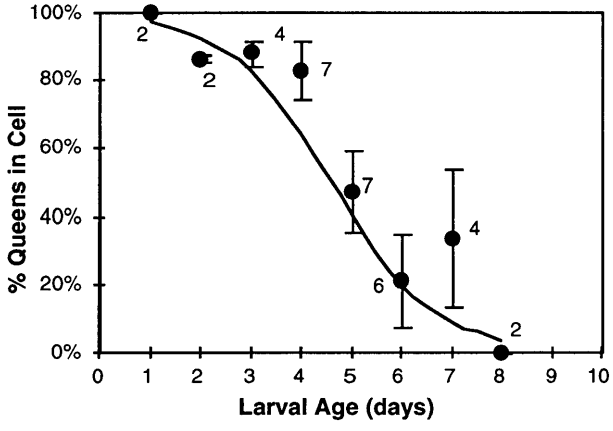

Fig. 2 Changes in the mean percentage of larvae (±SE) that develop into queens as a function of larval age when transferred from queenright (QR) to queenless (QL) conditions. *Numbers next to black circles* represent the number of repetitions (larvae cells)

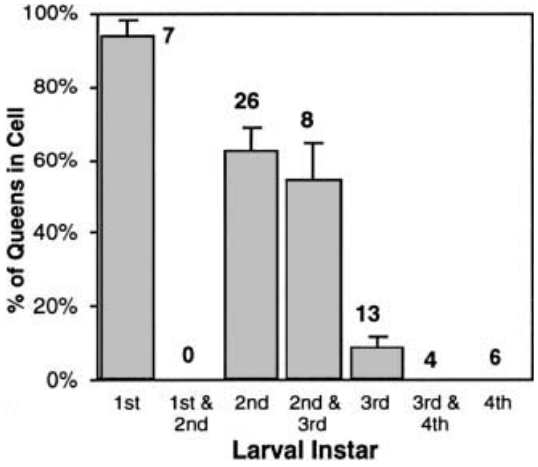

Fig. 3 Changes in the mean percentage of larvae (±SE) that develop into queens as a function of larval instar when transferred from QR to QL conditions. Notation as in Fig. 2. Zero values indicate that there were no cells in which both instars were present

(Mann Whitney $P < 0.01$ for each of the stages 3.3, 3.4, 3.5). During the fourth instar, the JH titer in queen larvae declined considerably and was significantly different from that of workers only during the first developmental stage (4.1).

Discussion

Caste differences in *B. terrestris* are expressed by differences in size, physiology and behavior (Röseler and Röseler 1974). In this study we determined that the critical period for caste determination occurs when larvae were 5 days of age, during the second instar. Röseler (1970) found a younger critical age for queen determination (3.5 days). The differences between Röseler's and our results are hard to explain due to the scanty information given about his methods, especially the method he used to calculate the critical age.

There were clear differences in JH in vitro rates of biosynthesis between queen and worker larvae as already shown by Cnaani et al. (1997). Caste differences began during the middle of the second instar (stage 2.3 or 2.4), and the rate of JH biosynthesis in queen larvae increased from that point until stage 4 of the third instar. Throughout this period rates of JH biosynthesis in queen larvae were eight to ten times higher than in worker larvae. There were also clear differences in JH titer between queen and worker larvae, mainly during the second half of the second and third instars. Worker and queen larvae however, did not differ in their JH titer at all stages.

In many studies the changes in JH titer and JH biosynthesis rate are correlated (Tobe and Stay 1985), but the differences we detected are not entirely surprising. Rachinsky and Hartfelder (1990) noted that during the last larval instar in *A. mellifera* the JH titer decreases while the rate of JH biosynthesis increases. They argued

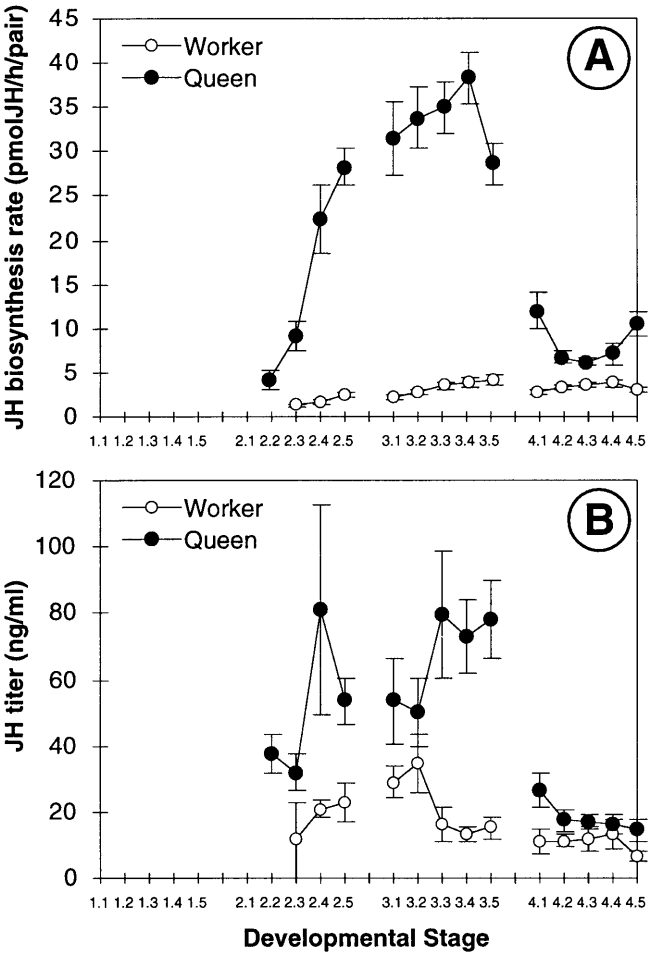

Fig. 4 Changes in mean (±SE) Juvenile hormone (JH) biosynthesis rate **A** and JH titer **B** during larval development (instar 2, 3, and 4). Developmental stages are represented by two digits. The first (2, 3, or 4) represents the instar, and the second (1–5) represents the stage inside the instar (see text for explanation)

that during this stage, hemolymph volumes increase so rapidly that even the observed increase in JH biosynthesis rate would not be enough to result in a comparable increase in JH titer. A bumble bee queen larva shows an almost 1000-fold increase in mass during its growth from the 1.5-mg egg to the large 1300-mg prepupa (Cnaani et al. 1997). This presumably leads to a rather large increase in hemolymph volume, which makes the Rachinsky and Hartfelder (1990) suggestion plausible for bumble bees as well. Other factors that might explain differences between JH titers and rates of biosynthesis relate to the possible role of JH esterase (JHE) and JH binding proteins (Dekort and Granger 1996). These factors have not been studied in *B. terrestris*.

Our results indicate that the critical period for caste determination coincides with an increase in JH titer and biosynthesis rates. This suggests that the high JH titer during the second half of the second instar is required for queen development and is probably part of the caste determination mechanism. This is consistent with find-

ings from other social insect species where either queen larvae have higher JH titers or JH application induces queen development (Nijhout and Wheeler 1982). Röseler and Röseler (1974) failed to induce queen development by JH application to *B. terrestris* larvae, but they treated fourth-instar larvae, well after the critical period for caste determination that we have determined here. In contrast, JH treatment of first- and second-instar larvae resulted in a relatively high percentage of individuals developing into queens (Bortolloti et al. 1999).

It is still unknown how JH controls development in polymorphic insects in general, and in social insects in particular. Cnaani et al. (1997) showed that differences in body size – the only morphological difference between the castes in *B. terrestris* (Michener 1974) – are the result of differences in molt timing. Size differences are mostly due to prolongation of the instars rather than different growth rates between workers and queen larvae. We hypothesize that in *B. terrestris* JH influences caste determination by affecting the timing of the molting process. According to this hypothesis, a high JH titer at the end of the second and the third instar results in a later molt and larger adult size, i.e., a queen. In contrast, a low JH titer at the end of the second and the third instar results in an earlier molt and smaller adult size, i.e., a worker. This hypothesis is consistent with the observation that a high titer of JH in the last instar can postpone the larval-pupal molt in several species of Lepidoptera (Nijhout 1981). In addition, Wheeler and Nijhout (1983, 1984) suggested that elevated JH titers in the last larval instar are responsible for creating the size differences between workers and soldiers in the ant *Pheidole bicarinata*. Evidence for the involvement of JH in molt timing during penultimate instars, as per hypothesis, is less conclusive. Sakuri (1983) showed that JH analog treatment had no effect on molting in fourth-instar larvae of *Bombyx mori*, but Safranek (quoted by Williams 1976) found that in *Manduca sexta* the duration of all instars was prolonged as a result of chronic JH treatment. Treatment with the JH analog fenoxycarb caused a prolongation of all instars in *B. mori* larvae (Kamimura 1995).

The social determinants of caste differentiation in *B. terrestris* remain elusive. Röseler (1970) suggested that a queen pheromone is involved and presented evidence suggesting the presence of a non-volatile contact pheromone that must be actively transferred from the queen to the workers and perhaps to the larvae. Cnaani et al. (2000) further developed the queen pheromone hypothesis, suggesting a direct effect on the larvae. Regardless of what social determinants affect caste differentiation in *B. terrestris*, our evidence suggests that they must act by affecting JH biosynthesis in the larvae. In honey bees, one social factor, differential feeding of larvae, has been shown to lead to differences in JH titer between larvae destined to become workers or queens (Hartfelder and Engels 1999). In both species, however, there is still no answer to the question of how a primer signal for caste determination is transduced into a neuroendocrine signal that leads to the appropriate rate of JH biosynthesis by the corpora allata.

Nijhout and Wheeler (1982) suggested that during critical periods in larval development genes or groups of genes are turned on or off in response to changes in JH titers. Caste-dependent differences in gene expression were recently demonstrated for honey bees (Corona et al. 1999; Evans and Wheeler 1999) but their dependence on JH remains to be explored. Caste determination in social insects, including *B. terrestris,* provides an excellent opportunity to explore connections between social factors, endocrine signals, and gene expression.

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