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**Hormonal and Pheromonal Regulation of
Reproduction in the Bumble Bee *Bombus terrestris***

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**HORMONAL AND PHEROMONAL REGULATION OF REPRODUCTION IN THE
BUMBLE BEE *BOMBUS TERRESTRIS*.**

Final report submitted by

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ABSTRACT

Bombus terrestris constitute important pollinators of greenhouse crops. In Israel the species utilized is, whose colonies are reared commercially. This is a primitively social species with a particular colony development. It encompasses two social phases: a eusocial phase in which the queen dominates reproduction, and a competition phase in which workers compete with the queen for the parentage of males. These workers are distinguished by accelerated ovarian development, high production of JH, and elevated levels of dopamine in the brain. Queen-worker conflict is also manifested in overt aggression among all members of the nest. High aggression is correlated with dominance status of the bees and is also correlated with octopamine levels in the brain.

After verifying that JH III is the only JH produced by the bees and validating the assay for its measurements (RCA & RIA), we used JH as an indicator of worker reproduction. Queens taken from colonies both before and after the competition phase were equally effective in inhibiting worker reproduction. Moreover, there is only a narrow window, around the competition point, in which workers may have the opportunity to initiate reproduction. Before that point they are inhibited by the dominant queen, while after that point both the queen and those workers with accelerated ovarian development exert strong inhibition on worker nestmates. Thus, "queen dominance deterioration" is not the primary cause in eliciting the queen-worker conflict.

Queens convey their presence by means of a chemical signal that is extractable in organic solvent and that is normally spread on the cuticle. Total body extract and body washes, applied on dead virgin queens, were able to inhibit the release of JH *in vitro* in queenless workers. However, none of the prominent exocrine gland investigated mimicked this function. It is possible that the source of the putative pheromone is an unknown gland, or that it emanates from an assembly of glands. Chemical analyses of the prominent glands

revealed a plethora of compounds the function of which should be further investigated. Understanding the social behavior of *B. terrestris* paves the way to facilitate colony manipulation and to adjust the colonies for specific pollination requirements.

RESEARCH OBJECTIVES

To study the factors controlling queen and worker reproduction in the bumble bee, *Bombus terrestris*, we propose the following specific objectives:

1. Determine the nature of queen pheromones that regulate worker reproduction.
2. Determine whether juvenile hormone plays a role in oogenesis.
3. Compare the regulation of workers reproduction in queenright and queenless colonies.
4. Explore the role of biogenic amines in reproductive behavior.
5. determine how pheromones, hormones (and possibly biogenic amines) are integrated to regulate reproduction.

We have met most of the objectives of the proposed research. Unfortunately we were not able to fully elucidate the putative queen pheromone that controls worker reproduction. We have developed a reliable bioassay and have completed the chemical analyses of the major exocrine glands. However, the notion that the pheromone is produced by the mandibular glands that prevailed in the literature proved wrong. We spend major efforts to assure that indeed these glands do not have the proposed function. We are on the verge of answering the question whether there is a queen pheromone that controls reproduction, or it is a general queen signal that is important in the social organization of the colony.

We have deviated from the original proposal in one subject, the process of caste determination. We have realized that this process is closely linked to the conflict between the queen and workers over reproduction. It was therefore important to learn the major physiological processes involved in caste determination.

I. GENERAL INTRODUCTION

Division of labor between queens and workers is fundamental to the organization of insect societies. The queen lays eggs, while her daughters, the workers, perform tasks related to colony growth and development and under most circumstances engage in little, if any, direct reproduction. Despite the importance of reproductive division of labor to social

insect biology, physiological mechanisms underlying the regulation of worker reproduction are not well understood, and are known for only a few species of social insects (Bell, 1973; Röseler et al., 1981; Röseler et al., 1984).

Queens inhibit worker reproduction by behavioral and pheromonal mechanisms (reviewed by Wilson, 1971). In addition, since juvenile hormone (JH) plays a major role in the regulation of reproduction in insects, it is thought that behavioral and pheromonal mechanisms act on the worker endocrine system to inhibit reproduction.

Evidence supporting this model of the regulation of worker reproduction in the social insects is limited to scattered and incomplete accounts of pheromone and hormone effects in a few species. Honey bees are among the most well studied species of social insects in general, but the regulation of reproduction in this species is not well understood. The first social insect queen pheromone was identified in honey bees more than three decades ago when Butler (1959) reported that the mandibular glands are the source of a queen pheromone. "Queen substance", 9-oxo-2-decenoic acid, was thought to be the pheromone that mediated all worker-queen interactions (reviewed by Free 1987). Recent studies (Slessor et al., 1988; 1990), using more refined chemical analyses, demonstrated that four other components present in the mandibular glands also are important in regulating the behavior of the workers. However, a synthetic mixture of the full five-component blend of the queen mandibular pheromone failed to inhibit ovarian development in queenless workers (Willis et al., 1990). Thus the pheromone that inhibits reproduction in worker honey bee is yet to be discovered.

The specific identity of the gonadotropic hormone in honey bees is also unknown. Unlike most insects in which elevated levels of JH are associated with egg development and oviposition behavior, both queens and laying worker bees have low blood levels of JH. In contrast, foragers, bees that are in a non-reproductive state, have higher levels of this hormone. Levels of another major class of insect hormones, the ecdysteroids, show a reverse pattern, suggesting that they may play a role in honey bee reproduction (Robinson et al., 1991, 1992, and references therein).

Some elements of the control of reproduction are also apparent from studies of the imported fire ant, *Solenopsis invicta*. Virgin queens are reproductively inhibited in the presence of a mated queen; removing the mated queen leads to wing loss (dealation) and oogenesis. The nature of this inhibition seems to be pheromonal (Fletcher and Blum 1981, 1983), but no pheromone has been identified yet. The involvement of JH in this process is inferred from findings showing that "allatectomized" virgin queens, i.e., queens that had their corpora allata (the glands producing JH) removed, failed to dealate or develop ovaries,

even in the absence of a mated queen. Moreover, application of exogenous JH to allatectomized virgin queens led to dealation and oogenesis (Barker, 1978). Additional evidence comes from studies of the effects of mated queens on the reproductive development of other mated queens. In colonies that are polygynous (more than one queen), there is an inverse relationship between the number of queens in the colony and their fecundity. The basis for this inverse relation apparently is mutual pheromonal inhibition by queens, because freshly killed queens are effective in reducing the fecundity of living queens. However, again no pheromone has been identified. Application of methoprene, a JH analog, increases ovary development in polygynous contexts (Vargo, 1992), but there are no data on rates of JH production or blood levels of this hormone. In addition, the small size of these organisms would make such analyses relatively difficult.

In highly eusocial species of social insects, such as the honey bee and the fire ant, evidence of current conflict between workers and queens over reproduction often is subtle, and difficult to observe. This is because queen control in highly eusocial species has evolved to the point where workers are morphologically distinct from queens, often with reduced reproductive organs. As a consequence, it is a challenge to devise behavioral bioassays that can be used to investigate underlying physiological mechanisms. In contrast, the conflict between workers and queens over reproduction in primitively social species is easier to observe. There is little, if any, morphological differentiation between the two female castes, so workers are fully capable of becoming reproductive under the proper social conditions. Primitively social species are thus ideal for studying mechanisms of queen control. Bumble bees constitute such primitively social species, among which *B. terrestris* was extensively studied. In this species, apparently queen control of worker reproduction occurs by a combination of behavioral and pheromonal mechanisms. In addition, these mechanisms appear to act via JH.

Bumble bees are a large group of primitively eusocial insects that primarily inhabit the north temperate regions. Colonies are typically annual, with new colonies founded haplometrotically by overwintering inseminated queens (Michener, 1974). *Bombus terrestris* is one of the common bumble bee species that occurs in the palearctic, ranging widely from Israel in the South to Scandinavia in the North. It is also reared commercially and used extensively as a pollinating agent in Canada, Europe, and recently, the US. and Israel.

Colony development in *Bombus terrestris* is characterized by several phases (Duchateau and Velthuis, 1988). During the "colony foundation" phase in early spring the queen lives solitarily and performs all the duties in the nest, including egg laying, cell

construction, brood care, and foraging. After the first workers that emerge take over all the nest duties, the queen becomes more and more restricted to egg laying. All the emerging individuals at this stage are workers, and the nest's population increases exponentially. This constitutes the "ergonomic" phase of the nest, which also can be regarded as the eusocial phase. As in highly eusocial species, the queen during this time is virtually the sole reproductive member of the society; the division of labor between queen and workers is clear, and the interactions between the two castes are cooperative, or at least not overtly hostile. As the colony's worker population increases it enters the third phase, the "competition" phase. At the "switch point" between the ergonomic and competition phases, the queen starts to lay haploid eggs, destined to develop into males. This event is associated with profound changes in colony social organization. Some of the existing and all of the newly hatched young diploid larvae are fed differently than before the switch point, and therefore develop into queens rather than workers. In addition, some "elite" workers (Doorn and Heringa, 1986) begin to show developed ovaries and lay haploid (male) eggs, despite the presence of a queen. After the events associated with the switch point occur, workers begin to show aggressive behavior towards the queen and other workers. They also destroy cells and eat eggs of other workers and the queen (Doorn, 1988). The competition phase marks the end of the colony cycle; workers are no longer produced and males and virgin queens emerge. Successfully mated queens will then overwinter as solitary individuals.

The objectives of the present research were to study the factors controlling queen and worker reproduction in the bumble bee, *Bombus terrestris*, specifically,

1. Determine whether juvenile hormone plays a role in oogenesis.
2. Compare the regulation of workers reproduction in queenright and queenless colonies.
3. Determine the nature of queen pheromones that regulate worker reproduction.
4. Explore the role of biogenic amines in reproductive behavior.

Most of the results obtained within the frame of this proposal were either published, or are in press, or were submitted for publication, or are at a final stage of manuscript preparation. We therefore constructed the report as a series of manuscripts. Some of the sections however were changed in order to include data that were not included in the original publications.

II. COLONY DEVELOPMENT UNDER LABORATORY CONDITIONS

INTRODUCTION

As mentioned in the general introduction above the various social interactions within the colony of *B. terrestris* occur at specific stages of colony development. Since all our studies were conducted with laboratory reared colonies and constituted bees from the local Israeli population, it was important to ascertain that our rearing conditions are suitable. It was also important to determine the precise timing of the social events as a function of colony age, in order to compare the biochemical and behavioral results with studies performed in other laboratories. The first task at the onset of the project was therefore to investigate the development of the *B. terrestris* colonies reared in our laboratory.

MATERIALS AND METHODS

Colonies of *B. terrestris* were obtained from the following sources: 1.) Laboratory founded from virgin queens that were mated in the laboratory and treated for hibernation prevention according to the established procedure (Röseler, 1985). 2.) Bio-Bee, Sde-Eliyahu a few days after the first workers emerged, 3.) Apiary of Yad Mordechai. In the laboratory the colonies were reared in nesting boxes (30x20 cm) supplied with cardboard bottom. Commercial honey water (Bee Happy) and fresh pollen (collected by honey bees) were supplied to all colonies. The colonies were held in a climatic room at a temperature of $29 \pm 1^\circ\text{C}$ in constant darkness. Observations and treatments were made under dim red light.

Colony development was followed by direct daily observations of young colonies, about 4 days after the first worker emerged. The developmental stage of the nest was registered by drawing the comb on a transparency while noting the formation of egg cups, emergence of larvae and their ages, and the number of pupae. When a more exact timing of larval emergence was required, time lapsed photography was used. The experimental nests were photographed every two hr. thus allowing the determination of the exact time of egg deposition. For following nest demography the bees were individually marked with a colored numbered tag that was glued on the thorax. In some of the observation nest the bees were marked according to the age group by a color paint on the thorax.

Larval development was followed by sampling the brood at selected intervals, weighed and their head capsule was measured under stereoscopic microscope equipped with a micrometer. The size of the head capsule was selected as a measure of the instar.

The switch point was estimated retrospectively after the first male emerged from the daily drawings. However we have noticed that the developmental duration of the males varied from 24 to 31 days (mean $\pm$ SE = 27.1 ± 0.4) depending on the age of the colony. Accordingly, the switch point was calculated using a regression line and the resulting equation: Developmental duration = $0.19 * \text{Colony age} + 18.88$ ($P < 0.0001$, $r^2 = 0.49$, $n = 28$).

The initiation of the competition phase, the competition point (CP), was identified by one or more of the following events: 1) the first day a worker was seen to oviposit; 2) two or more open egg cups were observed over at least two successive days. Since *B. terrestris* queens construct one egg cup at a time, the presence of 2 or more open egg cups indicates that either a worker is constructing an egg cup, or that an egg cup made by the queen was destroyed; 3) Clear signs of cup destruction was observed, indicating that queen or worker egg cups were destroyed by opponents (even when only one open egg cup was observed); 4) Observation of oophagy. All these signs are reliable indications for the initiation of an overt queen - worker conflict (Duchateau and Velthuis 1988; Doorn and Heringa 1986; Blom 1986, and the present study).

RESULTS AND DISCUSSION

Colony development

Nests showed normal development under laboratory conditions. Egg laying by the queen was frequent and amounted to 1-2 egg cells (containing 6-8 eggs) per day (Table 1). Larval developmental time differs between queens and workers. Developmental time from egg to spinning larvae in queens was 15.2 ± 2.3 (n=51) for workers and 18.4 ± 1.1 (n=28) for queens. The time from spinning to adults emergence was for workers 9.9 ± 0.9 (n=96) and for queens 13.4 ± 0.8 (n=36). Thus the overall developmental time of queens day was longer than that for workers (31.1 ± 1.6 and 25.3 ± 2.0 for queens and workers respectively). This is in sharp contrast to the case of the phylogenetically related honeybee where the queen developmental time is significantly shorter than that of workers.

Figure 1a shows the frequency distribution of head capsule width of larvae at different developmental stages and Fig.1b depicts the relation between size and larval age from egg hatching to pupation. Head capsule is the only sclerotized part in the larval body. It does not grow during the instar but increase in size in a saltatory fashion from instar to instar. It is therefore a good measure of the instar. Judging from the head capsule width, there are clearly 4 major size intervals with little intermediate forms.

Following the emergence of the first workers, the nest enters the ergonomic phase that is characterized by a sharp increase in queen oviposition and increasing numbers of workers. Figure 2 depicts the development of four colonies for which we have detailed observation and that characterizes the variation in colony development. Generally nest population reached a peak about 50 days later, thereafter worker mortality starts. The first queens emerged on the average on day 43.1 ± 0.7 , and first males on the average after 40.8 ± 0.9 days (Table 2). Total number of queens can reach anywhere from a dozen to about 60 queens. It can be seen that there is a slight overlap between the last workers that emerged and the first queen emergence. This is due to the fact that when the switch point occurs there are diploid larvae that are older than 3 days, thus committed to develop as workers. Younger larvae, on the other hand, develop into queens. It is assumed that the weakening of the queen perpetuates all these social and developmental changes.

Workers oviposition

As can be seen from Figure 2, workers start to oviposit at a certain stage of colony development. The number of ovipositing workers increase with time as is the number of worker-borne eggs. At this stage there is mutual aggression between the workers and the queen and among workers. This aggression is accompanied by oophagy and marks the onset of the competition phase.

Social events

Nest development proceeds normally until the competition phase. Table 2 describes the timing of competition and switch point and their maximal and minimal values. Under our laboratory conditions the switch point (e.g., the oviposition of haploid male eggs) occurred on the average after 14.1 ± 0.7 ($n=85$), similar to that reported by Duchateau and Velthuis (1988) (Mann-Whitney, $p>0.05$). The competition phase, under our conditions occurred significantly ($p<0.001$) earlier than reported by Duchateau and Velthuis (1988) (24.0 ± 0.7 days ($n=92$) in our colonies vis a vis the 30.8 ± 4.6 reported earlier). Although these two events are successive and once the switch point occurs, competition is bound to follow, there is no correlation between these two events (Fig. 3a). On the other hand, there is a correlation between the timings of queen development and the competition point (Fig. 3b), but not between the timing of the switch point and the emergence of first queen (Fig. 3c).

III. VALIDATION OF THE RADIOCHEMICAL ASSAY (RCA) FOR JH

INTRODUCTION

Ovarian development in *B. terrestris* was reported to be regulated by juvenile hormone (JH) (Röseler, 1977). JH involvement in oogenesis was deduced from a correlation between rates of JH release *in vitro* by the corpora allata (CA) and ovarian development. Using the *Galleria* bioassay, it was also demonstrated that the haemolymph titer of JH mainly reflects the JH release rate (Röseler and Röseler, 1978; Röseler, 1977). It also appears that an increase in JH can overcome queen inhibition of worker reproduction, since injection of JH into queenright workers induces dose dependent ovarian development (Röseler, 1977; Doorn, 1987). Finally, JH release rates in callow workers exposed to queen mandibular gland extracts were low and similar to those of workers exposed to intact live queens (Röseler et al., 1981). Although these findings support the hypothesis that JH is a gonadotropin in *B. terrestris*, the assay used for estimating CA synthetic activity was done under suboptimal conditions (Röseler and Röseler, 1978) and the *Galleria* bioassay is only semiquantitative.

It was therefore imperative to validate the radiochemical assay before using this parameter for investigating the regulation of worker reproduction. JH is also a morphogenetic hormone, and since we were also interested in the process of queen differentiation we validated the assay for both adults and larvae.

MATERIALS AND METHODS

Validation of the RCA in adults *B. terrestris*

Haemolymph pH, osmolarity and ion composition

Haemolymph samples were collected from immobilized bees under a binocular microscope, using a small glass capillary drawn to a fine point. The tip was inserted into the aorta through the dorsal part of the head - thorax joint. Blood pH was measured using a pH paper indicator with a discrimination of 0.2 pH units. Osmolarity was measured using a vapor pressure osmometer (Wescor 5500). For the analysis of ion composition, haemolymph from 4-5 workers or 2-3 virgin queens was pooled to a final volume of 50 μ l and analyzed with a flame photometer (Corning 480).

Incubation medium

Several incubation media were evaluated. These were: bee medium, an incubation medium based on honey bee organ culture medium (Kaatz et al., 1985) and modified for honey bee CA (Huang et al., 1991); M-199 (Biological industries, Bet Haemek); MEM (Sigma M-3911); and Grace's medium (Sigma G-5649).

The media were prepared according to their original formulations with some modifications: pH was adjusted as close as possible to 6.8 (with an equimolar mixture of 1.5NaOH:1KOH, v:v), osmolality was adjusted to 520 mOsmol (with sucrose), and 20 mg/ml Ficoll (Pharmacia 17-0400-01) were added. In the bee medium, the concentration of sodium phosphate (monobasic) was reduced to 2.5 mM. All chemicals used for media preparation were tested for cell culture by Sigma Chemical Co. The media were sterilized by filtration (Filtrap, 0.20 micron).

Assay procedure

Bombus terrestris heads were immobilized under a binocular microscope and immersed in bee saline (Huang et al., 1991). Using fine forceps, a small opening was made in the ventral part of the head. Either the corpora allata or corpora cardiaca-corpora allata complex (CC-CA complex) were removed and cleaned of any adhering tissue. The glands were then transferred into borosilicate tubes (6x50mm) containing 25 µl methionine-free bee medium. No more than 2 h elapsed from the first dissection to the initiation of the incubations. Preliminary experiments revealed that JH release rates were similar for thoroughly cleaned CA and for the CC-CA complex (t-test, $P=0.993$, $df=16$). Thereafter CC-CA complexes were incubated and are referred to as CA.

Incubations followed established procedures (Pratt and Tobe, 1974; Tobe and Pratt, 1974). Briefly, the incubation was started by adding an additional 25 µl bee medium that contained L-[^3H -methyl]methionine (NEN, specific activity 175-208 mCi/mmol). The incubation period was set at 3 h following experiments showing that JH release rates are constant for at least 6 h. In this experiment CA of queenless workers were incubated and transferred to new methionine-labeled medium after 1, 2 and 3 h, and the incubation was terminated after 6 h. After removing the CA, the medium was extracted and analyzed as described below. The optimum methionine concentration was found to be 250 µM and was used in all other experiments. The optimum incubation temperature was 39°C. Following the incubation, the medium was extracted for JH following the method of Feyereisen and Tobe (1981).

For studies comparing different incubation conditions, CA were obtained from 6 day old callow workers maintained in groups of three. In all optimization and validation

experiments we only used workers with developed ovaries (mean length of the terminal oocyte > 1.5 mm). In order to reduce genetic differences between the experimental groups, callow workers were taken randomly from a pool of newly emerging workers from different colonies.

Identification of radiolabeled compounds synthesized by the corpora allata

Following incubation, the CA and culture medium were placed in separate tubes containing 100 μ l cold, double-distilled, water. Each sample was extracted twice with 250 μ l isooctane. Before analysis by HPLC, the radioactivity in an aliquot of each sample was determined with a liquid scintillation counter. This value was then compared to the radioactivity of the eluting fractions from the HPLC to determine overall recovery. Another aliquot of each extract was separated by normal phase HPLC on an Alltech Econosil SI column (5 microns, 250 mm X 4.6 mm I.D) using 10% diethyl ether in hexane (one ml/min). Under these conditions, methyl farnesoate, JH I, JH II and JH III were clearly separated (approximate retention times of 4.8, 10.5, 11.4, and 13.3 min, respectively). The eluting material was collected for 20 min in either 0.5 or 1 min fractions. The radioactivity of each fraction was determined using a liquid scintillation counter. Since methyl farnesoate elutes close to the solvent front using these conditions, its identity was further confirmed by analyzing a second aliquot of some samples with 1% diethyl ether in hexane (1 ml/min). Using these latter conditions, methyl farnesoate eluted with a retention time of about 8 min.

Validation of the radiochemical assay in *B. terrestris* larvae

Validation procedure was similar to that described for adult bees. To remove the CA, the head of each larva was cut with fine scissors and placed in a drop of "bee saline" 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. After performing about 10 dissections (in one hour), the CA from each individual larva were transferred into a borosilicate tube containing 50 μ l incubation medium and [methyl- 3 H] methionine. The parameters that were tested for the optimization of the incubation conditions were as described for adults.

RESULTS

Optimization and validation of the radiochemical assay for adult worker *B. terrestris*

In a preliminary experiment rates of JH released by glands incubated in bee medium or Grace's medium were higher than rates observed in CA incubated with M-199 (Mann Whitney, $P < 0.05$). In a complementary experiment rates of JH released by glands incubated in bee medium or Grace's medium were higher than rates observed in CA incubated with MEM medium (ANOVA, $P = 0.034$, Fisher PLSD, post hoc test, $P < 0.05$, after log transformation, Fig. 4). In contrast, no significant difference in JH release was observed for glands incubated in bee medium or in Grace's medium. The bee medium was used for later experiments but its composition was adjusted to that of the haemolymph of *B. terrestris* (Table 3).

To determine the optimum concentration of methionine CA were incubated in methionine-free bee medium supplemented with 5 different methionine concentrations from 5 to 750 μM . Fig. 5 demonstrates that JH release rates were highest in media containing 250 μM methionine (ANOVA, $P < 0.05$, Fisher PLSD post hoc test, $P < 0.05$). Therefore, bee medium containing 250 μM methionine was used in the rest of these studies.

To determine the optimum incubation temperature, CA were incubated at 5 different temperatures ranging from 24°C to 44°C. JH release rates were significantly higher (ANOVA, $p < 0.0001$, Scheffe post hoc test $p < 0.05$) for CA incubated at 39°C (Fig. 6). This temperature, although high, is within the physiological range of active bumblebees (Heinrich, 1979) and was used for the rest of the experiments.

The optimum incubation period was investigated by incubating CA for time periods of 1 to 6 h. Linearity of hormone synthesis occurred irrespective of differences in release rates: both workers with developed ovaries (and high JH release rates) and workers with undeveloped ovaries (and low JH release rates) demonstrated linear rates of JH production (linear regression analysis for workers with developed ovaries: $r^2 = 1.0$, $P = 0.0001$, for workers with undeveloped ovaries: $r^2 = 0.995$, $P = 0.003$, Fig. 7). These results demonstrate that there is no deterioration of the CA during incubation and that the incubation medium can support JH release for at least 6 h. An incubation period of 3 h was used for the rest of the experiments.

The substances produced by CA incubated *in vitro* were identified by HPLC. The CA and medium from several incubations were separately extracted and analyzed by normal phase HPLC (Fig. 8). Over 97% ($\pm 6\%$ SE, $n = 18$) of the radiolabeled material was

recovered from the HPLC column. Both the culture medium and the CA contained two radiolabeled compounds. In extracts of culture medium, 93% ($\pm 3\%$ SE, $n = 8$) of the radioactive material comigrated with JH III. A small amount of the radioactivity ($2\% \pm 0.4\%$ SE, $n = 8$) coeluted with methyl farnesoate. Similarly, the major peak of radiolabeled material ($68\% \pm 6\%$ SE, $n = 10$) from the CA extracts also coeluted with JH III. Most of the remaining radioactivity ($25\% \pm 3\%$ SE, $n = 10$) 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, further supporting their identification.

Validation of the radiochemical assay for *B. terrestris* larvae

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, $P = 0.0015$ $df = 7$; $P = 0.004$ $df = 6$; $P = 0.0001$ $df = 7$ respectively).

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

The corpora allata synthesized JH at a constant rate during 5.5h of incubation (regression analysis, $r^2 = 0.64$, $p < 0.001$ for 8 pairs of glands). All incubations thereafter were conducted for 3h, well within the linear range of synthesis.

HPLC analysis of extracts of CA and incubation media revealed that over 99% ($\pm 4.7\%$ SE, $n = 10$) of the extracted radiolabeled material was recovered from the column in two peaks. In extracts of incubation medium, most ($90\% \pm 5\%$ SE, $n = 6$) of the radioactive material comigrated with JH III. A small amount of the radioactivity ($8\% \pm 1\%$ SE, $n = 6$) coeluted with methyl farnesoate. Similarly, the major peak of radiolabeled material recovered from extracts of CA ($80\% \pm 4\%$ SE, $n = 4$) coeluted with JH III. Most of the remaining radioactivity ($14\% \pm 2\%$ 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.

DISCUSSION

Several conditions must be satisfied before the radiochemical assay can be assured of providing a reliable quantification of JH release for a particular species. These conditions include the use of a culture medium that is as similar as possible to the composition of the haemolymph, the identification of a concentration of methionine that gives maximum rates of synthesis, the determination of the optimal incubation temperature, reasonable linearity of hormone production over the incubation period, and confirmation of the radiolabeled product as JH (Weaver et al., 1980; Tobe and Stay, 1985). Our results demonstrate that these requirements are now met for *B. terrestris*. The CA released JH at a linear rate for six hours. The observed rates of JH release are high and within the range reported for other species. (Tobe and Stay, 1985). The radiochemical assay has been used previously to measure rates of JH release in CA from *B. terrestris* (Röseler and Röseler, 1978; Larrère et al., 1993), but the assay was not optimized. This may explain the relatively low rates of JH production reported in those studies. For example, Röseler and Röseler (1978) reported a mean rate of JH release of about 17 pmol/pair/h for 3 day old queenless workers and Larrère and Couillaud (1993) reported rates of about 5 pmol/pair/h for post diapause queens and for 10 day-old queenless workers. These values are considerably lower than the 50 pmol/h/pair produced by CA of 3 day old queenless workers in this study.

HPLC analysis confirmed earlier reports that JH III is the major radiolabeled product released by the CA of *B. terrestris* (Larrère et al., 1993; Röseler and Röseler, 1978). The other radiolabeled compound released by the CA, methyl farnesoate, is present in negligible amounts. Thus, it is possible to quantify the amount of JH III released during the incubation without further separation. Some of the JH III synthesized during the incubation was still present within the glands, but this was only a small amount of the total JH synthesized (about 10%). A small amount of radiolabeled JH III was also detected in CA from honey bee workers (Huang et al., 1991) and other species (Tobe and Stay, 1985). Our results are thus consistent with those showing that CA do not store JH, and newly synthesized JH is rapidly released from the CA irrespective of the overall rates of JH release.

IV. MEASUREMENTS OF JH TITER IN THE BLOOD BY CHIRAL SPECIFIC RIA

MEASUREMENT OF HEMOLYMPH JH TITERS

Bees were immobilized with plasticine under a binocular microscope. Hemolymph samples from individual bees (1 - 14 μ l from workers, 10 - 33 μ l from queens) were collected using a small glass capillary drawn to a fine point inserted into the aorta through the dorsal part of the neck membrane. The hemolymph was then placed on a piece of paraffin paper and quantified by immediate collection into a calibrated micro-capillary tube. The amount of hemolymph was measured to the nearest 0.1 μ l, and stored in 0.5 ml acetonitrile at -80°C until analyzed. All glassware that may come into contact with JH was baked at 500°C for 3.5 h prior to use to minimize JH adsorption. All solvents were HPLC grade, obtained from either EM Science, Fisher Scientific, or J.T. Baxter Chemical Co.

A chiral-specific radioimmunoassay (Hunnicut et al., 1989) was used to measure the JH III titer. The detection limit of the RIA is about 5 pg R(-) JH III per sample. Intra-assay coefficient of variation was 8.5% and inter-assay coefficient of variance was 13.4%.

This RIA has been validated for adult worker honey bees by Huang et al., (1994). 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 of which have been validated with gas chromatography / mass spectroscopy (de Kort et al., 1985; Goodman et al., 1990). We also validated the use of this assay in *B. terrestris* in the course of this study. We determined whether the radioimmunoreactivity was due to the presence of JH using a method similar to what has been used in previously published studies (Huang et al., 1994; Trumbo et al., 1995). A small amount of [³H]-JH III (approximately 7000 DPM) was added to hexane extracts of haemolymph samples. Approximately half of each extract was fractionated by normal phase high performance liquid chromatography (HPLC) (Alltech Econosil, 5 mm, 4.6 x 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 ml ethanol. An aliquot of each fraction was analyzed for radioactivity to determine the recovery of JH III after HPLC (68.4 $\pm$ 10.2 %, n = 6). Another aliquot was analyzed for immunoactivity using RIA. Similar aliquots of the unfractionated remainder of each sample were also analyzed for radioactivity and immunoreactivity. RIA results were adjusted for any

additional radioactive ligand present. After correcting for the recovery of JH III (estimated from the radioactivity present in each sample), the amount of immunoactivity detected in the HPLC fractions of each extract was similar ($108 \pm 17\%$, $n=6$) to the amount present in the unfractionated material. Most of this activity ($86 \pm 14\%$, $n=6$) was detected in a single peak that coeluted with [^3H]-JH (Fig. 9). No other fractions had JH immunoactivity higher than background levels (<20 pg/tube).

Correlation between JH production and JH titer in the blood

Using the above assay we have determined the rate of JH release by the corpora allata and its titer in the blood in the same individual, repeating the measurements for larvae and adult workers (Figure 10 A-E). In the larvae there is a good correlation between the rates of JH release and its titer in the blood. When we examined a single age cohort the correlation improved somewhat, but not significantly. Neither did the weight of larvae examined affect this correlation. We can conclude from these results that in *B. terrestris* larvae the rates of JH biosynthesis measured by an *in vitro* incubation of the corpora allata reflects the hormone titer in the blood. Thus it is reliable to use the RCA for assessing JH effect on physiological events in the larvae.

The situation in adult workers was less clear. Although the regression line was statistically significant, the correlation coefficient was rather low (Fig. 10, D). When we calculated the regression as a double log the correlation improved considerably and was significant (Fig. 10, E). These results indicate that there is a correlation between the rates of JH biosynthesis and its titer in the blood, but not in a linear fashion (see below for a description of the effects of the social environment on JH titer in the blood).

V. DEVELOPMENT AND RATES OF JH PRODUCTION IN WORKER AND QUEEN PROSPECTIVE LARVAE

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, 1987; 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; 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-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-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 workers feed the 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-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.

The present work explores the physiological mechanisms underlying caste determination in bumble bees. *B. terrestris* 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

Production of 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 ca. 50 female larvae that hatched after queen removal (6 egg cells, and ca. 8 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 width 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.

RESULTS

Description of larval development

Analysis of larval head widths revealed that both worker and queen larvae developed through 4 instars (Fig. 1a and Table 4). There was no significant difference in head width between queen and worker larvae during the first two instars (t test $P=0.14$ and $P=0.23$ 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 4); however, their growth rate was the same (F-test for difference between two regression coefficients, $p<0.1$ Figure 11). 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 12 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.

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 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. 13). For example, larvae assigned to stage 1 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 2 larvae since stage 1 larvae were too small to handle for dissection. At the fourth instar, larvae start to spin their cocoon before they stop feeding (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 2 and 3), 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 4 and 5). In the first three stages of the second instar, rates of JH biosynthesis were similar for worker and queen larvae ($P=0.1$, $P=0.2$, $P=0.25$ for stage 1, 2 and 3, respectively). From stage 4 onward, queen larvae had significantly higher rates of JH biosynthesis than worker larvae ($P=0.03$ and $P=0.006$, respectively). JH biosynthesis rates in queen larvae reached their maximum during stage 3 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 for worker larvae remained relatively constant during the third and fourth instars (approximately 2-3 pmol JH/hr/pr) 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 were 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 characterize 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 chapter 5). 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.

VI. EFFECTS OF SOCIAL CONDITIONS ON JUVENILE HORMONE-MEDIATED REPRODUCTIVE DEVELOPMENT IN *BOMBUS terrestris* WORKERS

INTRODUCTION

Social insects are characterized by a division of reproductive efforts whereby oviposition is limited to a single or selected group of females while other females are engaged in brood care and other non-reproductive activities. In primitive social insects, reproductive dominance is achieved and maintained by aggressive interactions (e.g. Strambi, 1990; Michener, 1990; Fletcher and Ross, 1985; Röseler et al., 1980). However, it is thought that aggression cannot be effective in highly social insects because colonies are populated with thousands or even tens of thousands of individuals. It has been suggested that pheromones are used by highly social insects to regulate reproduction (Fletcher and Ross, 1985; Velthuis et al., 1990). Studies with *B. terrestris* suggest that this species represent an intermediate stage in which reproduction is regulated by a combination of pheromones and behavior (van Honk et al., 1980). This intermediary mode of queen control makes it a good model for studying both the underlying proximate mechanisms of this control and the evolution of queen dominance.

The main source of the putative queen pheromone is apparently the queen mandibular glands (Röseler et al., 1981). van Honk et al., (1980) reported that the competition phase started earlier in colonies headed by a queen whose mandibular glands were removed as compared to colonies with a sham operated queen. It was suggested that the onset of the competition phase in queenright colonies results from a decline in queen pheromonal output (Duchateau, 1989; van Doorn and Heringa, 1986; van Doorn, 1987; Pomoroy and Plowright, 1982). However, some authors (e.g. Owen and Plowright, 1982; van der Blom, 1986; van Honk and Hogeweg, 1981) have argued that a decrease in queen inhibition cannot fully explain the onset of the competition phase. Other hypotheses proposed to explain the onset of worker reproduction include changes in colony size or

worker density (van Honk et al., 1981; van Doorn and Heringa, 1986) and the onset of laying haploid eggs by the queen (van der Blom, 1986).

In this section we examine the hypothesis that the onset of the competition phase is associated with a decrease in queen inhibition. We studied this by performing analyses of juvenile hormone (JH), because results from earlier experiments with *B. terrestris* have suggested that JH is involved in oogenesis (Röseler, 1977). As mentioned above the earlier findings that JH is a gonadotropin in *B. terrestris* were achieved by an assay for estimating CA synthetic activity that was done under suboptimal conditions (Röseler and Röseler, 1978). Having validated the JH assay specifically for *B. terrestris*, we aimed at determining whether JH correlates with reproductive development.

We first determined whether JH release rates are correlated with ovarian development in workers. We then indirectly assessed the inhibitory effects of queenright colonies on worker reproduction by measuring JH release rate and ovarian development for workers in queenright colonies as compared to queenless groups. We further assessed the effect of intact colonies at different phases on JH release rates in callow workers. Finally, we compared the inhibitory effect of queens, separated from the effect of the colony. The queens were removed from colonies at different phases of development and their effects on the behavior and JH release rates of callow workers were compared.

MATERIALS AND METHODS

Bees: see above

Ovarian development

Bees were dissected in the adjusted "bee saline". Ovaries were removed and fixed in 4% formalin for at least 2 h, washed twice in 20% ethanol, and then incubated for a few h in 15% glycerin-ethanol. This procedure causes no detectable tissue shrinkage, making it possible to measure the size of even small oocytes. Oocytes were measured using an ocular micrometer. The mean length of the 8 terminal oocytes was used as an index of ovarian development.

Effects of social conditions on JH release, ovarian development and aggressive behavior by callow workers

Ontogeny of reproductive development under queenless and queenright conditions

Callow workers were collected, marked and placed into normal queenright colonies or reared in queenless groups. A queenless group was formed by confining three callow worker bees in a small nesting box. Bees were examined for rates of JH release and for ovarian development after 0, 1, 3, or 6 days.

Effect of queen type on JH release by callow worker

For testing the effects of queen type on JH release by callow workers they were placed in experimental groups containing four individuals in the laboratory. A "queenless group" consisted of 4 callow workers; a "virgin queen" group, 3 callow workers and a virgin queen; a "pre-competition phase" group, with a queen from a colony that had not yet entered the competition phase plus 3 callow workers; and a "competition phase" group, with a queen from a colony at the competition phase plus 3 callow workers. A separate set of experiments using pre-competition phase and competition phase queens were also conducted with groups of 10 callow workers. These conditions mimic better a natural colony that can undergo competition, but the number of workers was too large to enable particulate observations. Therefore we first verified that there were no differences in the queen effect whether housed with 3 or 10 workers. All subsequent experiments were done with groups of 3 workers. A colony was considered to be in the competition phase if on at least two successive days one or more of the following events were seen: two or more open egg cells, laying workers, aggression between workers and the queen, or oophagy. Before the competition phase there is no more than one open egg cell at any one time and there is no oophagy. Queen from colonies at the competition phase were taken no less than 5 days after the onset of the competition phase.

Queenless groups were composed of 4 workers to make them equal in size to the various groups with a queen. To help ensure that only three of the workers would be involved in dominance hierarchy interactions, as in the groups with a queen, one of the workers was replaced with a callow worker every day or two, and this individual was not analyzed. Workers were collected for JH release assays on day 3.

Effect of colony type on the rate of JH release by callow workers

Reproductive development in normally developing colonies was examined in a separate experiment. Individually marked callow workers were inserted into each of three types of colonies: queenright pre-competition phase, queenright at the competition phase, and queenless colonies. After 3 days, these workers were collected and examined for JH release.

Effect of queen type on callow worker behavior

Behavioral observations were conducted with individually marked workers using the same experimental groups described in section II above. Observations were performed daily for 30 min over a period of 14 days, under dim red light. Data were collected using a Psion organizer and analyzed using the "Observer" software (Noldus Information Technology). Agonistic behavior was classified into two major categories: threatening behavior and overt aggressive behavior.

"Threatening behavior" included buzzing and pumping. "Buzzing" was characterized by a bee opposing a nestmate while making fast, short, wing vibrations. (This is in contrast to ventilation fanning which is performed for a longer period). Buzzing was performed either toward a worker or the queen, and was frequently performed in combination with pumping (see below). Buzzing has previously been restricted to actions solely towards the queen (van Doorn and Heringa, 1986, Blom, 1986, Duchateau, 1989, Free, 1955, Röseler and Röseler, 1977). "Pumping" was characterized by a bee standing, facing another bee, and performing a distinct dorso-ventral pumping movement with her abdomen (Duchateau, 1989). Threatening behavior often preceded overt aggression.

"Overt aggression" included darting movements and attacks, biting or grappling with another bee, and pulling on a wing or hairs.

A Dominance Index was calculated from the analysis of all encounters. For each encounter, we recorded which bee advanced and which retreated. The Dominance Index was defined as: 1 - retreats/ total meetings (see also: van Doorn and Heringa, 1986; van Honk et al., 1981).

In previous studies, it has been reported that *B. terrestris* workers discriminate between larval sexes (Fisher and Pomoroy, 1989). Because this experiment was performed over a longer period it was necessary to avoid possible effects of larval sex on social interactions (van der Blom, 1986; Fisher and Pomoroy, 1989). Therefore, egg cells were removed 3 to 4 days after oviposition (preliminary observations indicated that larvae emerged 4 to 5 days after oviposition).

After all observations were finished, the workers were collected and examined for ovary development. Terminal oocytes were measured and examined for signs of resorption. Resorption of eggs was assumed in cases where eggs were shrunken and irregularly shaped remnants (Medler, 1962, Duchateau and Velthuis, 1989).

Fecundity of pre-competition phase and competition phase queens

Queen fertility was compared by counting the number of egg cells produced during the 3 day experiments and the 14 day behavioral observation periods. Queen fecundity was calculated as the total number of egg cells built by the queen during the experiment divided by the days of the experiment, minus one (the queens did not oviposit on the first day of an experiment).

RESULTS

Effects of social conditions on JH release, ovarian development and aggressive behavior by callow workers

Ontogeny of reproductive development under queenless and queenright conditions

Bombus terrestris workers emerged with undeveloped ovaries and low rates of JH release *in vitro* (Fig. 14). An increase in JH release rates was measured for CA from both queenless and queenright workers 24 h later, but the increase was larger for queenless workers. Rates of JH release were higher for queenless than queenright workers CA from day 1 to 6 (significant on day 3, Mann-Whitney test $P=0.007$, Fig. 14a). By day 6, ovarian development was significantly higher in queenless than in queenright workers (Mann-Whitney test $P=0.003$, Fig. 14b) (queenless workers reared under these conditions generally oviposited on the seventh day after eclosion). Rates of JH release and ovarian development were positively correlated in 6 day old queenless workers (Fig. 15; $r^2=0.35$, $P=0.002$, $n=24$). The association of JH release rate and ovarian development was further demonstrated in the linearity experiment; the CA of workers with developed ovaries produced significantly more JH already beginning from the first hour (Mann - Whitney of each hourly comparisons, $p<0.05$, Fig. 7).

Not all 6 day old queenless workers had elevated rates of JH release or an increase in ovarian development. Rather, usually only 1 or 2 workers in each group showed these increases. Larrère et al., (1993) reported that the dominant worker in each queenless group has the highest rates of JH release. The average rate of JH release for the one 6 day old worker with the highest rate of JH release in each group was $49.9 (\pm 8.5 \text{ SE})$ pmol/h/pair, and the terminal oocyte length was $2.44 (\pm 0.54 \text{ mm SE})$. The remaining workers had a rate of JH release of $26.2 (\pm 2.83 \text{ SE})$ and terminal oocyte lengths of $0.53 (\pm 0.1 \text{ mm SE})$. These values for the remaining workers were similar to those of queenright workers of the same age. The heterogeneity of CA activity among queenless workers was already clear on days 1 and 3 but at those ages there were no clear differences in ovarian development.

Effect of queen type on JH release by callow worker

There were significant differences in rates of JH release for 3 day old workers reared under different social conditions (ANOVA, $P < 0.0001$). Workers in queenless groups or in groups with virgin queens had significantly higher rates of JH release than workers in pre-competition phase or competition phase groups (Fisher PLSD post hoc test, $P < 0.01$, Fig. 16). There was no significant difference in JH release rates between workers from queenless and virgin queen groups, nor between workers in pre-competition phase and competition phase groups.

Additional experiments were conducted using groups of 10 callow workers. These condition mimic young colonies in which sometime competition can occur. No differences in JH release rates were detected for workers reared in groups of 3 or 10 individuals (t-test, $P = 0.6$ for competition phase groups, $P = 0.32$ for pre-competition phase groups), so data were pooled for the above analyses.

Effect of colony type on the rate of JH release by callow workers

Callow workers placed in either queenright colonies before the competition phase, queenright colonies during the competition phase, or queenless colonies, produced comparable amounts of JH after 3 days (Table 5, ANOVA: $P = 0.75$). Rates of JH release were relatively low and similar to those of workers from other queenright colonies and workers reared with mated queens in artificial groups in the laboratory. These low rates of JH release are not due to foreign callow workers being inhibited differently from native callow workers. No difference was observed in the rate of JH release between three day old workers reared either in native or foreign colonies (t-test, $df = 30$, $P = 0.59$).

Effect of queen type on callow worker behavior

There were significant differences in overt aggression and threatening behavior for workers reared in different experimental groups (Kruskal-Wallis test, $p < 0.01$). Workers from queenless and virgin queen groups showed the highest levels of threatening behavior and overt aggression throughout the first week of observation (Fig. 17). Egg cells were first observed usually after 7 or 8 days. Most (88-100%) of the overt aggression was performed by a single dominant worker. All of the overt aggression was performed by the dominant worker in 4 out of the 6 queenless groups and 3 of 4 virgin queen groups. Pumping was more typical of the dominant worker, with 98-100% of the pumping performed by her (with an exception of one of the queenless groups in which only 80% of the pumping was performed by a single worker). Buzzing toward another bee was also typical of the dominant worker, with 50-100% of total buzzing performed by the dominant

worker (generally more than 75%). On the other hand, levels of threatening behavior and overt aggression were low in the pre-competition phase and competition phase groups (Fig. 17).

There also were group differences in behavior over time. Levels of agonistic behavior in pre- competition phase and competition phase groups were significantly lower than in queenless and virgin queen groups from days 2-12 ($p < 0.05$, Mann-Whitney test). Overt aggression was significantly lower in pre- competition phase and competition phase groups than in virgin queen groups on days 2-3 and lower than in queenless groups on days 2-5.

The dominance index was positively correlated with ovarian development for workers from queenless and virgin queen groups (Spearman-rank correlation, $P < 0.05$) but not for workers from pre-competition phase or competition phase groups. Dominance rank was correlated with ovarian development rank in all experimental groups (Spearman-rank correlation, $P < 0.05$). Pre-competition phase and competition phase queens were always in the dominant position (dominance rank = 1) and had high and similar dominance index (0.92 ± 0.015 SE, $n=5$ for pre-competition phases and 0.88 ± 0.025 SE, $n=5$ for the competition phase queens, Mann-Whitney test, ns). Virgin queens had lower dominance ranks (mean = 1.5 ± 0.29 , $n=4$) and significantly lower dominance index (mean = 0.69 ± 0.05 SE, $n=4$) compared to pre- competition phase and competition phase queens (Mann-Whitney test, $p < 0.05$).

Workers reared with pre-competition phase queens had slightly less developed ovaries, with fewer signs of resorption compared to workers in the other groups (Table 3), but these differences were not statistically significant (ANOVA: $P=0.1$ for terminal oocyte length, $P=0.24$ for absorption signs, after square root transformation).

Fecundity of pre-competition phase and competition phase queens

Pre-competition phases queens had slightly greater fecundity than competition phase queens, but this difference was not statistically significant. Pre-competition phase queens constructed $1.04 (\pm 0.18$ SE, $n=15$) egg cells compared to $0.63 (\pm 0.15$ SE, $n = 14$) for the competition phase queens ($t=-1.75$, $df=27$, $P=0.09$).

DISCUSSION

Measurement of JH release in 6 day old queenless workers indicates a correlation between high CA activity and ovarian development. These results are consistent with earlier studies demonstrating a correlation between ovarian development and CA volume and between CA volume and CA activity (Röseler, 1977; Röseler et al., 1981). In callow

workers, JH release peaked three days before full ovarian development, suggesting a causal relationship between JH and ovarian development.

Our studies show that both CA activity and ovarian development are influenced by the social environment. Behavioral observations demonstrated that threatening behavior and aggressive interactions accompanied reproductive development. In queenless groups or groups with a virgin queen, at least one of the workers exhibited high CA activity, had developed ovaries, and oviposited as early as day 6.

Mated queen, separated from their colony and brood, inhibited the reproductive development of 3 day old workers to the same extent as in workers that developed in intact queenright colonies. There was no significant difference between mated queens from a colony before or during the competition phase. It is interesting to note that queens from colonies before the competition phase were a little more effective in inhibiting worker ovarian development but this difference was not statistically significant, and its biological importance, if any, is unknown. Queen at the competition phase were just as effective as pre-competition phases in inhibiting worker CA activity and aggressive behavior. Since virgin queens did not inhibit the CA activity and aggressive behaviors of callow workers, this effect can not be explained by the larger body size of the queen. Rather, it seems likely that virgin queens do not possess the same pheromonal blend as mated queens, or they may behave differently.

Our results support previous reports that dominance is correlated with ovarian development and can predict reproductive success in intact *B. terrestris* colonies (van Honk and Hogeweg, 1981; van Doorn and Heringa, 1986; van Doorn, 1987) as well as in queenless groups (van Doorn, 1989). All oviposition events observed in this study were performed by the dominant bee: the queen in the pre-competition phase and competition phase groups and the dominant worker in the virgin queen and queenless groups. Furthermore, workers in groups with mated queens were never observed to construct and open egg cells, behaviors that are typical of laying workers in colonies during the competition phase. Perhaps more extensive behavioral observations would have revealed oviposition by workers in pre-competition phase and/or competition phase groups because they do occur in nature, at least in colonies during the competition phase.

High dominance status in queenless *B. terrestris* colonies has previously been found to be positively correlated with rates of JH release (Larrère and Couillaud, 1993) and CA volume (van Doorn, 1989). Nevertheless, it is not clear whether those correlations indicate for causal relations, and whether JH controls dominance behavior or dominance behavior controls JH levels. In an earlier study, it was shown that an injection of JH I did

not effect the dominance position of queenright workers (van Doorn, 1987). However, queenless workers injected with JH I were more likely (though not significantly) to become dominant workers (van Doorn, 1989). In the primitively social wasp *Polistes gallicus*, dominance hierarchies among foundresses are determined behaviorally and are affected by hormones titer. Dominance status was positively correlated with JH and ecdysone titters; furthermore, injection of JH I and 20-hydroxyecdysone, separately and simultaneously, significantly increased the probability that the treated female would become dominant (Röseler et al., 1984, Röseler et al., 1985).

Workers inserted into queenright colonies before or during the competition phase or into queenless colonies exhibited relatively low and similar rates of JH release. The low CA activity in queenright workers is not surprising and was also seen when workers were confined with queens in the laboratory. Low JH release rates in queenless workers is a surprising finding, and may be the result of inhibition exerted by older dominant workers present in such colonies. Similar inhibition, presumably by the dominant workers, was also found in our studies with queenless groups. In these groups, one of the workers usually became dominant and had high JH levels and ovary development. The dominant worker was apparently able to inhibit the same physiological changes in the other two workers (see also Larrère and Couillaud, 1993). These results are consistent with earlier studies showing that ovaries of 5 day old workers born in colonies 10-14 days after queen elimination were as undeveloped as those of queenright workers of the same age (Röseler et al., 1981).

It is not clear from our studies whether the inhibition of ovarian development is the sole factor regulating worker reproduction. In our experiments some of the workers that were reared with queens for 14 days had mature oocytes but they were never seen to oviposit; if their oviposition behavior really was inhibited (rather than just not observed) then the regulation of worker reproduction can not be explained solely by the inhibition of ovarian development (Röseler, 1977). Workers with ripe eggs were also found in normally developing colonies before the competition phase, yet they apparently do not lay eggs either (Duchateau and Velthuis, 1989). While this study and others clearly implicate JH in the regulation of ovary development in bumble bees, it is not clear whether JH also regulates oviposition. van Doorn (1987) reported that the proportion of laying workers was not affected by JH I injection. Likewise, Larrère and Couillaud (1993) reported that laying queens have a low rate of JH release. However, Röseler (1977) reported high JH titters in the haemolymph of reproductive queens. We found comparably high rates of JH release for workers collected just after oviposition (unpublished data). However, there are no explicit

experiments that address the question of whether high JH titters are necessary for oviposition in bumble bees, or whether the high JH release rate found in laying workers is just an indication of the further development of penultimate oocytes.

A prevailing hypothesis concerning the initiation of queen-worker conflict and worker reproduction in *B. terrestris* colonies is a reduction in the queen's pheromonal output (Duchateau, 1989; van Doorn and Heringa, 1986; Pomoroy and Plowright, 1982; Röseler and Röseler, 1977). Our results do not support this hypothesis. Queens taken from colonies during the competition phase (e.g., when the worker-queen conflict is at its peak) were as effective in inhibiting callow worker reproduction as were queens from colonies before the competition phase. This inhibition occurred at the same magnitude in groups with either 3 or 10 workers, reducing the possibility that queens from colonies at the competition phase are less inhibitory than pre-competition phases queens but still able to inhibit small groups of workers. Although we can not preclude the possibility that in larger colonies weakness of the queen is better expressed. Two anecdotal observations indicate that even "weak" queens can partly inhibit worker reproduction. In one case, an old queen died on the tenth day of an experiment and in the second case, a queen was injured and was attacked by the workers starting from the third day. In both cases, laying workers were not observed until the 10th and 11th days, respectively. The experiments described above examine the effect of queens on reproductive development in young workers. However, it is possible that the response of worker to the queen inhibitory effect is age dependence and older workers react differently to queens of colonies before or at the competition phase. Although our experiments do not refute the hypothesis of a decline (pheromonally or behaviorally) in older queens, they clearly demonstrate that competition phase queens still have inhibitory ability, suggesting that there may be another mechanism regulating worker reproduction during colony development.

The initiation of worker reproduction could be affected by changes in the queen, in the workers or in both. A decline in queen inhibitory ability may be apparent even if the queen quality does not change dramatically. It is possible for example that in dense colonies the spreading efficacy of the queen pheromone declines or it is diluted due to the high numbers of workers. Increased colony density may also result in fewer encounters between the queen and the workers. The demonstrated ability of older workers to inhibit JH release in younger workers implies changes in worker-worker interactions as a factor in the onset of worker reproduction. Perhaps older workers become less sensitive to the inhibitory effects of a queen pheromone than do young workers (those used in our experiments), or they are more sensitive to changes in queen pheromone output that signal

queen quality (Keller and Nonacs, 1993). These issues can perhaps be explored by focusing on those "elite" workers, that are most likely to initiate reproduction (van Doorn and Heringa, 1986; van Honk and Hogeweg, 1981). Our results indicate that other social factors must be examined to gain a more complete understanding of the regulation of worker reproduction during colony development.

VII. JH titers, *in vitro* JH biosynthesis rates, ovarian development and social environment in *Bombus terrestris*

INTRODUCTION

As seen in the last chapter JH is implicated in the regulation of oogenesis and workers that initiate ovarian development also have high rate of JH production as measured in the radiochemical assay. We have also seen that there is some correlation between the radiochemical assay data and JH titer, but it is not always evident. It was therefore important to repeat some of the experiments while measuring both the blood titer of JH and its production by the CA *in vitro*.

MATERIAL AND METHODS

Bees, Radiochemical assay, RIA, and ovarian development: see above.

Effect of the queen on JH titers in young workers

Callow workers (< 12 h after eclosion and before full coloration), were collected, marked with colored tags, and introduced into other queenright colonies (QR), or placed in groups of 3 in small plastic cages (95 x 107 x 107 mm, with a cardboard bottom) to form queenless groups (QL). To reduce possible intragroup genetic variation, callow bees from different source colonies were collected together and then assigned randomly to queenless groups or queenright colonies. Measurements were made for callows and 1, 3 and 6 day-old bees.

Effect of dominance on JH biosynthesis, JH titers, and ovarian development

Dominant egg-laying workers were identified under dim red light in queenright and queenless colonies at the competition phase. A worker was defined as an egg-layer if seen to perform oviposition behavior. Typically, oviposition behavior followed the construction of a new egg cup, or the repairing of an already existing one. Oviposition behavior is

characterized by a bee inserting her abdomen tip into a egg cup for ≥ 2 min while moving her hind legs. In most cases this behavior results in egg laying (see next section for detailed description of egg laying behavior performed by workers). A short time after collecting each egg-laying worker, a non-egg-laying worker of comparable size was collected from the same colony. A worker was defined as a non-egg-layer if she was not seen performing behaviors characterizing dominant egg-laying workers during a 5 to 10 min observation. These behaviors include: oviposition behavior, egg cup construction, agonistic behaviors toward other bee, egg cup destruction or oophagy. Measurements of ovarian development, rates of JH biosynthesis and JH titers were made for each bee collected.

JH titers and biosynthesis rates in queens

JH titers and biosynthesis rates were measured for functional queens collected from colonies before or during the competition phase, and JH biosynthesis rates were made for 3- and 6 day old virgin queens (after confining pupae or collecting callow queens in small cages without males). Because functional queens have fully developed ovaries and virgin queens do not, we only made qualitative evaluations of ovarian development in queens.

RESULTS

Effect of the queen on JH titers in young workers

Bombus terrestris workers emerged with low JH titers, which increased with age. This age-related increase occurred more rapidly in queenless workers (Fig. 18). There were no significant difference in JH titers between queenless and queenright 1 day old workers, but queenless workers had significantly higher JH titers by day 3 (Mann-Whitney test, $P = 0.013$, $n = 14$). A similar trend was evident for 6 day old bees, but the difference was not significant (Mann-Whitney test, $P = 0.08$, $n = 26$).

The high mean JH titer in queenless groups does not reflect homogenous increase in all three workers. Rather, one worker generally had high JH titer while the other two workers had titers similar to those in queenright workers. For example, 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 to 174.6 ± 58.1 ng/ml ($n = 9$) of the remaining bees. As a result of the JH differentiation in the queenless workers, variance in JH titers in both 3- and 6 day old queenless bees was significantly higher than that of the queenright bees (variance ratio = 155 and 20.6, respectively; equality of variance F test, $P < 0.0001$). This difference was

almost statistically significant in 1 day old bees as well (variance ratio = 3.9; equality of variance F test, $P = 0.051$).

Effect of dominance on JH titers, biosynthesis rates and ovarian development

No significant differences in JH titers, biosynthesis rates, or ovarian development were found between egg laying workers from queenless and queenright colonies (Two-way ANOVA, effect of colony type $P = 0.33$, $P = 0.71$, $P = 0.26$, respectively). Therefore data collected from workers from the two colony types were pooled for the analysis.

Egg laying workers had significantly higher JH titers, biosynthesis rates and greater ovarian development than did non-egg laying workers from the same colonies (Table 6). All egg laying workers had developed ovaries (largest oocyte length > 2.56 mm) and 82% of them had JH titers higher than 100 ng/ ml, while in non-egg laying workers only 40% had developed ovaries. These differences were not due to differences in worker size in the two groups. Head widths were similar for egg laying and non-egg laying workers ($4.39 \text{ mm} \pm 0.1$; 4.53 ± 0.08 , respectively, $n=16$ for both groups; unpaired t-test, $t=1.07$, $P=0.29$).

To evaluate whether rates of JH biosynthesis were higher in egg laying workers independent of ovarian development status, we compared groups of egg laying and non-egg laying workers with mean terminal oocyte length > 2.5 mm (egg laying = $2.93 \text{ mm} \pm 0.05$, $n=31$; non-egg laying 2.87 ± 0.06 , $n=15$; Mann-Whitney test, $P=0.6$). JH biosynthesis rates did not differ for the two groups (egg laying = $31.5 \text{ pmol/h/pair} \pm 3.1$, $n=31$; non-egg laying, 24.7 ± 4.4 , $n=15$, $df=44$, $P=0.22$).

JH titers and biosynthesis rates in queens

All functional queens had developed ovaries. They also had high JH titers but low rates of JH biosynthesis (Table 7). Most (72%) of the functional queens had JH titers higher than 100 ng/ ml. Virgin queens had undeveloped ovaries; they also showed low rates of JH biosynthesis that was similar statistically to that of functional queens. There was no significant difference in JH titers or rates of biosynthesis between functional queens from colonies before or after the CP.

The relationships between JH biosynthesis rate, JH titer and ovarian development

To assess the relationships between JH biosynthesis rate, JH titer and ovarian development we performed linear regression analysis for pairs of values measured from the

same individual bee. Analyses were performed within defined group of females (within group regression) and among the means of defined group of bees (between group regression).

The relationship between rates of in vitro JH biosynthesis and JH titer in the haemolymph

JH biosynthesis rates *in vitro* explained only 4% of the variance in JH titers in worker's hemolymph (Table 8). To test whether the low regression coefficient is because the heterogeneity of sample that include worker at different ages and that developed under different social conditions we examined the regression separately for queenless and queenright worker and for worker at different ages. The regression was significant only when we restricted the analysis to 0 to 3 day old workers.

To reduce the effects of individual variation and sampling error we further examined the relationship between means of defined groups of bees with similar age that have developed under similar social conditions. Both JH biosynthesis rates and JH titers increase for workers 0 - 3 days of age (queenless and queenright workers) producing a significant regression (dash line in Fig. 5, $r^2=0.94$, $P<0.001$, $n=6$). However, means of 6 day old, egg-laying and non-egg-laying workers deviated from this regression line by having higher JH titers than expected. This trend was more profound in functional queens that had low JH biosynthesis rates and high JH titers (Fig. 19).

The relationship between JH titer in the haemolymph and ovarian development

Significant linear regression between JH titers and worker ovarian development was obtained irrespective whether we examined all the worker together or limited our analysis to queenright or queenless workers. Lower regression coefficients were obtained when we analyzed together queenless and queenright workers at the ages of 0 - 3 days or 6 days (Table 8).

Callow *Bombus terrestris* workers are born with low JH III titer and undeveloped ovaries. During the following days both showed correlated increase (Fig. 5). Increase rates depend on the social environment, and was significantly faster in queenless workers. The highest JH titers were obtained for bees with highly developed ovaries: functional queens and egg-laying workers. The comparison of the means of the different groups produced a highly significant regression of ovarian development on JH titers (Fig. 20,

$P < 0.0001$). Mean JH titers explained about 78% of the variance in mean ovarian development ($Y = 1.43X - 3.24$, $r^2 = 0.78$, $n = 9$).

DISCUSSION

In these experiments we measured JH titers, JH biosynthesis rates and ovarian development in the same individuals under various social conditions. With this approach we found that titers of JH III were correlated with the length of the terminal oocytes in the ovary. However, JH biosynthesis rates *in vitro* were correlated with JH titers only in bees under specific social conditions. These results suggest that in adult bees rates of JH biosynthesis *in vitro* can not be generally used as index for hemolymph. These results support the finding presented in other parts of this report pertaining to the effects of the social environment on reproductions are mediated by JH.

Different JH titers were obtained for young workers that developed under queenless or queenright conditions. These differences are compatible with the differences in ovarian development in workers under the same condition and agree with the measurements of JH production *in vitro* (Bloch et al., 1996; section III of this report). However the relationship between the titer of JH in the blood and the different social and physiological states of the bees are more complex. Bees introduced into colonies at various social stages showed ovarian development only at a particular stage of colony development, a few days before the competition phase started (see next section of this report for a full description of the results). Differences in JH titers between these workers were not as evidence. There was a slight increase in the titer in workers introduced just before the competition phase, and a marked reduction in bees introduced at the competition phase, but these differences were not significant. Nonetheless, highest JH titers were obtained for dominant bees with developed ovaries: egg-laying queens (irrespective whether obtained from colonies before or during the competition phase) and workers, whereas low ranked workers from the same colonies and of similar size (non-egg-laying, Table 1) had low JH titers and ovarian development.

JH was also suggested to be involved in the modulation of dominance behavior in bumble bees (Larrere and Couillaud, 1993; Doorn, 1989). Although we also found an association between JH titers and dominance in queenright colonies we attribute this association to ovarian development rather than dominance hierarchy. Dominant workers had higher JH titers, JH biosynthesis rates *in vitro* and ovarian development than their lower ranked nestmates, but when the association of JH with dominance behavior is separated from its association with ovarian development, there were no differences between

dominant and low ranked workers. These results suggest association between JH titers and worker ovarian development irrespective her dominance status. This finding also explain the failure to increase worker dominance under queenless or queenright conditions by JH I injections (Doorn, 1989; Doorn, 1987). On the contrary, in *Polistes* wasps JH separately, or simultaneously with 20-hydroxy ecdysone increase the probability that a treated individual will be dominant (Roseler et al., 1984).

The lack of correlation between JH biosynthesis rates *in vitro* and its titer in the blood in bees of specific social status but not under other conditions warrant further studies. The titer of JH in the blood reflects both its biosynthesis and its degradation. Regarding the biosynthesis, it is possible that the CA *in vivo* is modulated by brain hormones and when removed from the body behaves differently. Since there are significant differences in JH release rate between queenless and queenright workers, we assume that the CA *in vitro* behaves in a similar manner as *in vivo*. The peak of JH production however precedes that of oocyte maturation, suggesting that it had a triggering effect after which the CA is turned off. JH however persists in the blood longer time which can explain the correlation with ovarian development. However, we know little on the half life of JH in the blood or the regulation of JH specific esterase in *B. terrestris*. We also have no clue on the mode of CA regulation. All these awaits further research.

VIII. REGULATION OF REPRODUCTION BY DOMINANT WORKERS IN QUEENRIGHT COLONIES

INTRODUCTION

In the majority of social insects reproduction is highly biased in favor of one or more individuals, the queens, whereas most other individuals, the workers, serve as non-reproductive helpers. In most species workers are capable of reproduction and will do so under certain social conditions, e.g. after the queen dies or at a certain stage of colony development. This suggests that under normal social conditions the reproduction of workers is suppressed. The proximate as well as the ultimate mechanisms for the maintenance of this reproductive bias constitute basic problems in insect sociobiology and the evolution of sociality in general.

Kin selection theory predicts that the queen-worker conflict will be expressed at two levels; who reproduces, and the sex ratio of the colony (Hamilton, 1964; Hamilton, 1972; Trivers and Hare, 1976; Bourke and Franks, 1995; Crozier and Pamilo, 1996). Since workers are generally more related to their sisters than to their daughters, conflict over the

parentage of females should be selected against according to kin selection. This has presumably resulted in the worker's loss of ability to produce female progeny in many social species, due to the loss of spermatheca and therefore the ability to mate. Furthermore, in species that workers do possess a spermatheca they rarely mate. Thus, queen-worker conflict over reproduction centers primarily over the parentage of the male progeny. Overt conflict is not always evident and depends largely on the social structure of the species. The conflict is expected to be the strongest in monogynous monandrous species, where workers are more related to their sons or random nephews than to their brothers. In this case, workers should compete with the queen over male production. Workers should also compete among themselves, albeit to a lesser degree, creating the possibility for a few dominant workers to take over reproduction. In contrast, when the queen is multiply mated, workers become more related to their brother than to a random nephew resulting in worker policing whereby the workers support the queen's but not their sisters' male progeny. Furthermore, if the chances of workers to reproduce is very low due to effective policing, reproductive self-restraint should be selected for (Ratnieks, 1988).

The inhibition of worker ovarian development is believed to be the proximal mechanism for a queen to maintain control of worker reproduction (Alexander, 1974; West-Eberhard, 1981). In species with small and simple colonies, it is believed that the queen dominance is achieved by direct behavioral interactions. In contrast, in species with larger colonies queen control is achieved indirectly by the dissemination of special "queen substances" (reviewed in: Wilson, 1971; Hölldobler and Bartz, 1985; Fletcher and Ross, 1985; Michener, 1990; Spradbery, 1991; Velthuis et al., 1990; Heinze et al., 1994). An alternative hypothesis recently proposed that the queen does not control worker reproduction, directly or indirectly, but it is the workers that behave so as to maximize their inclusive fitness. In this case the putative queen pheromone is merely a signal indicating the presence of the queen (Keller and Nonacs, 1993).

We studied worker-queen conflict over male production in the bumble bee *Bombus terrestris*. This species is most suitable for this study since it is accepted that the single queen in the colony mates only once (Röseler, 1973; Estoup et al., 1995). Moreover, because of its annual life history, any possible conflict over reproduction should be manifested rather early in colony development. From its social structure it is predicted that overt aggression between the queen and workers should erupt, and that workers will also compete with each other over the right to reproduce. It is further predicted that early in the colony life cycle the queen should dominate reproduction (irrespective whether the workers concur with this domination (Keller and Nonacs, 1993) or are actively inhibited by the

queen), whereas later when competition between the queen and the workers erupts reproduction may be shared by additional dominant workers (Bourke, 1994).

The reproductive behavior and physiology of *B. terrestris* are well studied (Röseler, 1974; Röseler, 1977; Röseler and Röseler, 1978; Röseler et al., 1981; Röseler and Honk, 1990). Colony development is characterized by two distinct phases of reproduction. During the initial phase, the queen is the only reproductive female. Ovarian development in young workers and oviposition in older ones with mature eggs are inhibited at that stage. The "competition phase" that follows is characterized by reproduction of workers and by antagonistic interactions and oophagy among workers and between workers and the queen (Cumber, 1949; Free and Butler, 1959; Honk and Hogeweg, 1981; Honk et al., 1981; Duchateau and Velthuis, 1988; Röseler and Honk, 1990). The initiation of the competition phase was previously defined as the "competition point" (CP, Duchateau and Velthuis, 1988). Queen control was assumed to be attained by combined behavioral (aggressive dominance) and pheromonal means that inhibit the biosynthesis of JH, the presumed gonadotropic hormone, in the workers resulting in low JH titer in the blood and consequently slower ovarian development (reviewed in: Röseler and Honk, 1990). Worker reproduction at the competition phase was interpreted as the result of decrease in queen dominance status. However, detailed behavioral observations demonstrated that the queen is still active, fertile and has the highest dominance position in colonies during the competition phase (Honk and Hogeweg, 1981; Doorn and Heringa, 1986; Duchateau, 1989). The queen's decreased inhibition was therefore attributed mainly to changes in her pheromonal output (Honk et al., 1981; Honk and Hogeweg, 1981; Owen and Plowright, 1982; Doorn and Heringa, 1986; Doorn, 1987; Duchateau, 1989; Doorn and Chrambach, 1989; Röseler and Honk, 1990). Recent experiments using small queenright groups of callow workers demonstrated that queens taken from colonies before or during the competition phase were similarly effective in inhibiting worker reproduction and aggressive behavior (Bloch et al., 1996). Moreover, body extracts of the two queen types have similar inhibitory effect on rates of JH biosynthesis in callow workers. These findings are not consistent with the hypothesis of queen inhibition decline. Although we can not exclude the possibility that in large colonies queen pheromone spreading is less effective (Honk and Hogeweg 1981; Doorn and Heringa 1986; Röseler and Honk 1990), these results lend credence to the alternative hypothesis that centers around changes in workers response to the presence of the queen.

In the following report we first tested the hypothesis that the competition phase in *B. terrestris* initiates in response to a decrease in queen inhibition. This hypothesis predicts

that the lowest inhibition on worker JH mediated ovarian development occurs during the competition phase. Worker reproductive potential was assessed by introducing callow workers into intact colonies at different stages of social development. Having found that worker reproduction was inhibited in queenright colonies during the competition phase we tested an alternative hypothesis that emphasizes the importance of the dominant workers. Mutual inhibition might be one of the means in the competition predicted to occur among workers in single mated monogynous colonies. The ability of dominant workers to inhibit reproduction of their nestmates was assessed in colonies dequeened at different stages of development and in small queenless groups. Worker reproduction was evaluated by direct observation of worker oviposition behavior, by following worker ovarian development, and by measuring the rates of juvenile hormone biosynthesis *in vitro*.

MATERIAL AND METHODS

Bees, determination of social events, and rates of JH biosynthesis: see above

Ovarian development

Bees were dissected in "bee saline" (Huang et al., 1991) with molar ion ratio of 1.5Na:1K, as found in *B. terrestris* worker hemolymph (Bloch et al., 1996). The ovaries were removed and the length of each terminal oocyte was measured with scaled ocular lens under a dissecting microscope (X25 - 50). The mean length of all terminal oocytes or only that of the largest terminal oocyte served for indexing ovarian development. In addition, we recorded the number of mature oocytes with a clearly visible chorion.

For size comparisons we also measured the length of mature, worker borne, eggs collected after their deposition in queenless colonies, and that of chorionated oocytes dissected out of workers' ovaries. In addition, we tested whether egg size is correlated with body size (using head width as a measurement of body size), by measuring the length of all chorionated oocytes from individuals of various sizes. Only workers with two or more chorionated oocytes in their ovaries were included in the analysis.

Worker oviposition behavior in intact colonies

Detailed observations of worker oviposition behavior during different stages of colony development were performed to determine the pattern of worker reproduction in normally developing *B. terrestris* colonies. In four young developing colonies all newly eclosed workers were marked with plastic colored tags. The colonies were observed daily for a five to ten minutes to follow their normal development. Starting from day 20 after the first worker eclosed, each colony was observed daily for 45 minutes to 3 hours.

Observations were performed until day 94. In one colony (#193) the queen was found dead on day 36, while in another two colonies queens were removed by the experimenter on days 62 and 72 respectively (#194 and #196).

Oviposition behavior was defined as a worker seen inserting her abdomen into an open egg cup for a few minutes while moving it up and down, along with typical kicking movement of the hind legs. In 65 out of 74 incidences of this described behavior at least one egg was laid. Oviposition behavior was thus classified as equivalent to oviposition. Oviposition behavior was often preceded by a period of new egg cup construction or by opening and preparing an already existing one. The duration of oviposition behaviors of individually marked bees, and whether the bee closed the cell after oviposition, were also recorded.

Effect of queen removal on rate of worker oviposition

The competition phase is characterized by worker reproduction, indicating that at least some of the workers are not inhibited anymore. However, this does not preclude the possibility that the queen continues to affect worker oviposition behavior. To evaluate the queen effect, worker oviposition was monitored two days before the queen was removed and two days thereafter, in 11 mature colonies, mostly a short time after gyne or male eclosion. Each daily observation lasted for two hours. Monitoring worker oviposition in the same colony as a function of the presence of the queen (rather than comparing queenless and queenright colonies of the same age) was done in order to overcome the variability in the rate of worker oviposition among colonies.

Worker reproductive potential at different stages of colony development

Worker reproductive potential was estimated by comparing their levels of ovarian development and JH production, the presumed gonadotropic hormone. Callow workers (less than 12 h old, before full coloration), were marked and introduced into queenright colonies at different stages of development. In order to maintain the same worker density no more than two workers were introduced simultaneously into young colonies, replacing a comparable number of resident workers. When the introduced workers were collected for analysis, they were replaced by an equal number of callow workers. In large colonies after the competition point, up to five workers were introduced without any compensation. We also examined whether the introduction of callow workers into foreign colonies affected their survival and reproductive development. Nestmates and alien workers introduced into the same colony had similar survival rates (chi square test, $P=0.18$, $n = 30$) and ovarian

development (t-test, $df=32$, $P=0.75$). Consequently, data of all introduced workers were pooled irrespective of their origin.

We defined three stages of colony development: stage I - more than two weeks before the competition point; stage II - two weeks or less before the competition point; and stage III - after the competition point. For the analysis of ovarian development workers were dissected at the age of 7 days. At this age worker ovaries exhibit the entire range of oocyte development without any signs of egg resorption (Duchateau and Velthuis, 1989; our observations). Analyses of JH were performed in 3 days old workers, when there are clear differences in JH biosynthesis rates between queenright and queenless workers (Röseler and Röseler, 1978; Bloch et al., 1996).

In a separate set of experiments we evaluated the impact of the queen on the inhibition of worker reproduction. Callow workers were introduced into colonies at various stages of development immediately after the queen was removed. Seven days later they were collected and monitored for ovarian development.

Effect of dominant workers on ovarian development in callow workers

To test the ability of dominant workers to inhibit reproduction of callow workers, we constructed small queenless groups, each composed of 4 workers of which 0, 1 or 3 were dominant. Workers were defined as dominant if they were seen to oviposit or to construct egg cups in their original colony. After 7 days, all the workers of the queenless groups were dissected and analyzed for ovarian development.

RESULTS

Workers oviposition behavior in intact colonies

To study regulation of worker reproduction we first characterized the pattern of oviposition behavior in normally developing colonies. We paid special attention to worker age at oviposition, as this can be used as an indication for the strength of inhibition. For example, when inhibition is low, workers should have faster ovarian development and therefore will be able to oviposit at a younger age.

The number of workers performing oviposition behavior increased after the competition point, until it peaked approximately when sexuals (young gynes and males) started to emerge (Fig. 21). It was positively correlated with worker density ($r^2 = 0.64$, $P < 0.0001$, worker oviposition after square root transformation), probably due to an increase in the number of egg-laying workers (Fig. 21, bottom).

Worker oviposition behavior was characterized in four colonies, observing a total of 489 events. It generally began with egg cup construction or by opening and reusing an already existing egg cup. An ovipositing worker typically inserted her abdominal tip into an open egg cup for a few minutes while moving her abdomen and hind legs. Examination of the egg cups ($n = 74$ events) immediately following such behavior revealed that in 90.5% of the cases at least one egg was laid. Observation on oviposition events in four colonies revealed that not all workers oviposited. Only 28.3% to 45.3% (13 to 87 bees) of the entire worker population were observed laying at least once. However, 57% to 100% of workers of the first brood (the oldest workers in the colony) were seen ovipositing.

The number of eggs laid in each egg cup by workers was lower and more erratic than by queens. Queens laid 9.5 ± 0.33 eggs per cup, as revealed from opening egg cups after the queen had oviposited (mean $\pm$ SE, $n = 39$ egg cups made by 11 queens). On the other hand, in over half (52.7%) of the cases worker laid one or two eggs, in 23% of the cases three or four eggs, and only 14.8% of the egg cups contained more than four eggs. A small percentage (9.5 %) of the workers that performed oviposition behavior did not lay eggs. These workers were of similar age as workers that did oviposit (mean $\pm$ SE = 29.4 ± 4.4 , $n = 7$ and 29.6 ± 1.3 days respectively, $n = 67$, Mann-Whitney test, $P = 0.94$). In most cases oviposition behavior performed by workers was followed by egg cup sealing. Some workers did not seal the egg cup after oviposition due to interference by other workers or for other unknown reasons. Sealing behavior might be age related because workers that did not seal the egg cups were significantly younger at oviposition than those that did seal (means $\pm$ SE = 35.3 ± 1.3 , 40.9 ± 1.8 days respectively; Mann - Whitney test, $P = 0.02$).

Oviposition was performed by workers of a wide range of ages (6 to 81 days-old), but most were two weeks to two months old (Fig. 22). The sharp decline in egg laying among workers that were older than 60 days was not due to mortality, since 71.6% of the egg laying workers lived longer than 60 days and 12.9% lived longer than 80 days. Examination of individual workers from their first to last observed oviposition revealed that for 18% of the workers the oviposition period was longer than 21 days, with the longest period being 66 days. Median age of workers at their first observed oviposition was 20 days ($n = 212$ workers). Egg laying workers younger than ten days old were seen only at the first month of the competition phase.

Effect of queen removal on rate of worker oviposition

The impact of the queen's presence on worker oviposition during the competition phase was tested in 11 colonies that were rendered queenless around the period of sexual eclosion (to assure that the colony is well into the competition phase). Changes in worker oviposition in the presence of the queen were assessed by comparing oviposition on day 2 with that of day 1 before her removal. Worker oviposition between these two days was not significantly different although a insignificant decrease occurred on the second day (Table 9). On the other hand, oviposition on each of the two days after queen removal was significantly higher than on the day before queen removal. However, worker oviposition two days before queen removal and during the two days following queen removal were not significantly different.

Worker reproductive potential at different stages of colony development

The reproductive potential of workers was assessed by introducing callow workers into intact colonies at different social stages. Comparison between introduction of nestmates and alien workers revealed that they had similar survival rates (chi square test, $P=0.18$, $n=30$) and ovarian development (t-test, $df=32$, $P=0.75$). Callow workers were selected for these experiments since they were all in the same physiological state and any difference in their reproductive output, therefore, reflects the effect of the social environment under which they were reared. Two different experiments were done using 1). Queenright colonies; 2). Colonies from which the queen was removed at the time of worker introduction. Reproductive development was evaluated by measuring the length of the terminal oocyte and the *in vitro* JH biosynthesis rates in the introduced workers. For comparison, the mean size of deposited eggs collected from queenless colonies was $3.28 \text{ mm} \pm 0.05 \text{ SE}$ ($n=25$ eggs), and that of chorionated oocytes dissected out of workers was $3.33 \text{ mm} \pm 0.03$ (mean $\pm$ SE, $n=61$ bees). These were not significantly different from each other (t-test, $t=0.98$, $df=86$, $P>0.2$).

Although egg size (after Ln transformation for normality) was significantly correlated with worker head width (Linear regression analysis, $P=0.0075$, $r^2=0.12$, measurements were taken only from workers with two or more chorionated oocytes), body size explained only 12% of the variance in oocyte size. We therefore did not correct oocyte length according to worker head width.

There was no association between the "switch point" (SP, estimated retrospectively after the first male eclosed as described in the Materials and Methods section) and worker ovarian development. Workers introduced into colonies two to one week before the SP

(since they were dissected at the age of 7 days, they were removed from the colony before the switch point), and workers introduced 0 to 7 days after the SP had similar mean terminal oocyte length (0.56 ± 0.05 , $n = 86$ after SP vs 0.66 ± 0.09 , $n = 45$, before SP; Mann-Whitney, $P = 0.43$).

Workers introduced into colonies during stage II (less than two weeks before CP) had more developed ovaries than those introduced during the other two stages (Table 10, Kruskal-Wallis test, $P < 0.0001$, post hoc test according to Siegel 1988, $P < 0.001$). JH biosynthesis rates were significantly lower for workers introduced during stage III (after the CP) (ANOVA, $P < 0.05$; Fisher PLSD, $P < 0.05$), but did not differ between workers introduced during the other two stages. Figure 23 A - C describe the frequency distribution of ovarian development in these callow workers. During stage I (Fig. 23A) the distribution was more or less symmetric with only about 10% of the workers possessing oocytes larger than 2 mm, and none had chorionated eggs. At stage II (Fig. 23B) a larger percentage of workers had developed ovaries and a few of them had chorionated eggs, with less than 10% of the workers exhibiting totally undeveloped ovaries (largest terminal oocyte < 0.25). In contrast, during the competition phase (stage III; Fig. 23C) the distribution was skewed to the left with the majority of the workers having undeveloped ovaries (a significantly higher percentage of workers at this stage had undeveloped oocytes: Chi square test, $P < 0.001$). Nevertheless, about 4% of the workers possessed chorionated eggs.

When callow workers were introduced into colonies in which the queens were removed at the time of introduction (dequeened colonies), results were quite different (Figs. 3D and 3E). Most of the workers that were introduced into colonies that were dequeened before the competition phase had developed oocytes (largest terminal oocyte length > 2 mm) and over 40% of these workers had chorionated eggs (Fig. 23D). In contrast, the majority of workers that were introduced into colonies that were dequeened after the competition phase had undeveloped ovaries. Although about 8% of these workers did have chorionated eggs (Fig. 23E).

Figure 24 depicts the temporal changes in ovarian development of callow workers in the above mentioned queenright and dequeened colonies. Workers introduced into dequeened colonies either more than 6 days before, or around the competition point had significantly larger mean terminal oocytes than workers introduced to the corresponding queenright colonies (Mann-Whitney, $P < 0.0001$). In contrast, after the competition point ovarian development was similarly low in workers introduced into either queenright or queenless colonies (Mann-Whitney, ns). Moreover, workers introduced into dequeened colonies before the competition point had significantly more developed ovaries than

workers introduced into dequeened colonies during the competition phase (Kruskal-Wallis test, $P < 0.0001$, post hoc test, $P < 0.05$). The differences in ovarian development of workers introduced into dequeened colonies were very profound. At the date of the competition point queen removal resulted in fast ovarian development in the introduced workers, while as early as one to two days into the competition phase the introduced workers failed to develop their ovaries.

Effect of dominant workers on ovarian development in callow workers

Possible inhibition of ovarian development exerted by dominant workers was tested by constructing queenless groups consisting of varying numbers of callow and dominant workers. Since the dominant workers were selected only on the basis of behavior, their reproductive activity was ascertained by dissecting them after the tests. All, but one, had developed ovaries with mature eggs. Ovarian development in callow workers was significantly lower when housed either with 1 or 3 dominant nestmates (Table 11, Kruskal-Wallis, $P < 0.0001$, multiple comparisons between treatments, $P < 0.0001$). In the groups comprising of purely callow workers, an average of 1.36 ± 1.69 chorionated oocytes developed as compared to none in the groups containing 3 dominant workers. The groups that contained only one dominant worker were intermediate in this respect, although the results were not statistically different from the groups with 3 dominant workers. A comparable number of egg cups was constructed during the experiment in the different groups.

DISCUSSION

Our results provide the first empirical evidence that in the presence of a functional, active, and fecund queen, the workers are the major factor that inhibit the reproductive development of their nestmates. Moreover, worker inhibitory effect is apparently limited to a certain temporal stage during colony development (the competition phase). Worker inhibition of reproduction by their dominant nestmates under queenless conditions was suggested previously for *B. terrestris* (Röseler and Röseler, 1977; Röseler et al., 1981; Bloch et al., 1996) and reported for other Hymenopteran species (reviewed in Michener, 1990; Spradbery, 1991; Velthuis et al., 1990). A similar inhibition in queenright colonies probably occurs in several ant species characterized by a high level of agonistic encounters among nestmate workers (Cole, 1981; Heinze et al., 1994).

First, we characterized worker oviposition during different stages of colony development. Determination of oviposition by workers often requires a close look at the egg cups involved. This is a time consuming procedure that can disturb the colony, and

therefore can not be done routinely. To bypass this problem we have characterized the oviposition behavior performed by workers and established that it is a good representation of actual oviposition. Using this estimate, we determined that *B. terrestris* workers oviposit at a wide range of ages, in contrast to the narrow age range reported for honey bee and stingless bee laying workers (Engels and Imperatriz-Fonseca, 1990; Velthuis et al., 1990, Hartfelder and Engels, 1998). The youngest (a conservative estimates, since the first egg laying of many workers might not occurred during observations time) and the oldest ages at oviposition were 6 days and 81 days respectively. Oviposition at such a young age is in accordance with the age of first oviposition observed in workers under queenless conditions (Duchateau and Velthuis, 1988; Röseler and Honk, 1990; Bloch et al., 1996). In this study the majority of the workers seen ovipositing for the first time were two to four weeks of age.

A prevailing hypothesis for explaining worker reproduction was that it initiates as a response to “queen control deterioration”. A prediction from this hypothesis is that removal of the queen during the competition phase will not affect the magnitude of worker reproduction. The results of our experiments do not give support to the “queen control deterioration” hypothesis. Even in the midst of the competition phase, indicated not only by worker behavior but also by the development of sexuals, the queen apparently do not lose her inhibitory capacity. Queen removal at this stage resulted in a significant increase in worker oviposition during the following two days when compared to the day before removal (but not to two days before). These findings are consistent with a previous report that in small worker groups, queens before or after the competition phase are as effective in inhibiting JH biosynthesis in callow workers (Bloch et al., 1996), and a recent finding that body extracts of queens, irrespective of their social status, had similar inhibitory effect.

Oviposition patterns of workers in unmanipulated colonies can not fully reveal the actual inhibition of worker reproduction because different factors can inhibit ovarian development and oviposition behavior (Duchateau and Velthuis, 1989). To overcome this limitation we have followed the reproductive development of callow workers introduced to either unmanipulated or manipulated colonies. The advantage of this approach is that all introduced workers are in the same initial physiological state. Moreover, we have established that survival rates and ovarian development (and in a previous study that JH biosynthesis, Bloch et al., 1996) in the introduced workers were similar whether they were nestmates or alien bees. It is therefore reasonable to assume that the observed measurements represent the actual changes in workers during the development of intact colonies.

The introduction experiments lead to several conclusions. Callow workers are able to develop ovaries at any stage of colony development. Our results demonstrated that young workers can have developed ovaries even earlier than the onset of the competition phase, yet they refrain from oviposition (see also Duchateau and Velthuis, 1988). In these cases workers may be inhibited from ovipositing by the queen, or may delay their oviposition because it maximizes their inclusive fitness to do so at this stage of development. Egg laying by workers at early stages of colony development may disrupt the ergonomic growth of the colony and consequently reduce the number of female sexuals (Keller and Nonacs, 1993, Bourke, 1994). These workers however, are at an advantage when the competition phase starts. They may lay eggs earlier and inhibit other workers from doing so (discussed below). It stems from these findings that in normal colonies the reproducing workers are not necessarily the oldest ones. Before the competition phase it is most likely that the queen inhibits worker reproduction. The stronger inhibition of workers introduced into colonies at the competition phase suggests that additional individuals, besides the queen, are actively inhibiting new workers from becoming reproductive. This hypothesis is strengthened by the results obtained from dequeened colonies. Almost half of the callow workers introduced into dequeened colonies before the competition phase succeeded in developing chorionated eggs, but during the competition phase worker ovarian development was similarly inhibited in queenright and dequeened colonies.

The experiments in which callows were housed with various numbers of dominant workers support the "worker inhibition" hypothesis. Dominant workers inhibit reproduction in callow workers to levels comparable to those found in queenright colonies at the competition phase. The apparent reduction in the queen's impact at the competition phase (worker ovarian development was similar in queenright and dequeened colonies at that stage) could result from increase relative inhibition by the dominant workers without any changes in her inhibitory capacity.

The worker introduction experiments gave no explicit support or rejection to the queen inhibition decline hypothesis. The reduced worker ovarian development during the competition phase apparently is not consistent with the predication of this hypothesis. However, we assume that the strong inhibition during the competition phase was due to the effect of the dominant workers. Therefore, it is not clear whether or not there were changes in the queen effect during this period. Before this stage, during the two weeks before the CP there was a mild, but significant increase in worker ovarian development (but not in JH biosynthesis rates). However, even during this stage ovarian development was clearly

reduced compared to queenless workers of similar age. Thus, there is a doubt whether this mild changes can explain the initiation of the competition phase.

In honey bees, behavioral mutual worker inhibition of reproduction is explained by the policing model (Ratnieks, 1988; Ratnieks and Visscher, 1989; Ratnieks, 1993). Although physiological inhibition is one of the mechanisms suggested by the policing model (Ratnieks, 1988), this does not adequately explain our findings in bumble bees. Worker policing is predicted to develop in polyandrous species where relatedness of the worker to the queen sons is higher than to their nestmate workers' sons (Ratnieks, 1988). In *B. terrestris* it is believed that the single queen mates only once (Röseler 1973; Estoup et al., 1995). Thus, all workers are full sisters and their relatedness to the queen's sons is lower than to their sisters' sons. This overrule the motivation for worker to police one another to the favor of the queen. A better kin selection based explanation seems to be that in species for which the single queen mates only once the worker are expected to gain better fitness by raising their own sons than those of their mother queen or their full sisters sons. Inhibition of endocrine mediated ovarian development is thus a part of this competition between the workers in *Bombus terrestris* colonies.

The observed changes in the inhibitory effect of dominant workers at the beginning of the competition phase were very fast and apparently associated with the initiation of egg laying. The mechanisms by which worker inhibits its nestmates are still unclear. These may involved special behavior, accumulation of an inhibitory pheromone, or a combination of both. The characteristic agonistic behaviors toward nestmates in queenright colonies (Free and Butler, 1959; Honk and Hogeweg, 1981; Doorn and Heringa, 1986, Duchateau, 1989), and even at increase strength in queenless groups (Free, 1955; Röseler and Röseler, 1977; Doorn, 1989; Bloch et al., 1996) might result in their reproductive inhibition, a mechanism which was previously suggested for the regulation of reproduction in primitively social insects (reviewed in: Wilson 1971; Fletcher and Ross 1985; Bourke 1988; Spradbery 1991;). Dominant workers might also be characterized by special odors. For example, queens and dominant workers seems to recognize, and are more aggressive toward, workers with developed ovaries (Michener 1974; Doorn and Heringa 1986; Duchateau 1989; Röseler and Honk 1990). In *B. hypnorum*, based on chemical analysis, Ayasse et al., (1995) reported that dominant workers were outstanding in possessing higher amounts of volatile secretions and special odor bouquets. Furthermore, the amount of detected odors was highly correlated with the size of the ovaries but not with body size. In the related honey bees, egg-laying workers under queenless conditions possess queen-

like substances in their Dufour's gland (Katzav-Gozansky et al., 1997), but not in their mandibular glands (Plettner et al., 1993).

By careful determination of male emergence in these colonies we have further confirmed earlier reports (Duchateau and Velthuis, 1989) that changes in worker ovarian development are not associated with the switch point, giving no support to the hypothesis that the switch to male production prompts worker reproduction (Blom, 1986). We have also confirmed that the workers do not reproduce during the earlier stages of colony development (Honk et al., 1981; Doorn and Heringa, 1986; Duchateau and Velthuis, 1988, reviewed in: Röseler and Honk, 1990).

In view of these results we can draw a more comprehensive picture of the social conflicts and their expression in *B. terrestris*. The conflict over reproduction in this species culminates in male production. Although the females possess a spermatheca, and despite the possible existence of males in the nest or the environment, workers do not mate. There are two stages with respect to the control of reproduction within the nest. In the early stages of colony development the queen is the dominant female; workers that escape this inhibition and develop eggs refrain from ovipositing. Whether this is due to a distinct queen pheromonal control or is a means for maximizing worker inclusive fitness (Keller and Nonacs, 1993) is not clear. The competition point marks the transition into the second stage in which inhibition of young workers' ovarian development is exerted mainly by dominant workers. Our data suggest that during the transition from the first to the second stage there is a temporal window of weakened worker inhibition. This prospective window was expressed in three different analyses: a) significantly larger mean terminal oocytes (but not JH biosynthesis) were found in workers introduced during the two weeks before the competition point; b) only workers introduced during the period 10 days before to 10 days after the competition point achieved oocyte maturation by the age of seven days; c) oviposition by young workers (less than 10 days) was mainly limited to the initial stage of the competition phase (data not shown). Röseler and Röseler (1977) and Doorn and Heringa (1986) further report that the initiation of the competition phase is characterized by an increased rate of agonistic behaviors. At the competition phase the queen-worker conflict becomes overt and is expressed in mutual aggression and oophagy. Who wins the conflict in *B. terrestris* is still not clear. The observation of oviposition patterns and individual behavior in intact colonies indicate that the queen may be winning by taking advantage of her physical superiority. She spends much of her time inspecting the egg cups, and is usually the most active in their destruction (unpublished observations). Furthermore, the larger number of eggs deposited by the queen in each egg cups gives her an additional

advantage. Workers that open a queen made egg cup destroy only a few of the eggs, in many cases adding their own to the clutch (unpublished observations). It seems therefore that in most colonies the queen may win the conflict and produce most of the male progeny. Crossing experiments with two morphologically distinct subspecies (Röseler and Honk, 1990; Duchateau, 1996) strongly suggest that indeed, while the queen remains alive she usually wins the conflict and produces most of the males (but see Sakagami 1976; Owen and Plowright, 1982; Röseler and Honk, 1990, and references therein for species in which reproductive success of workers may be higher). Matricide, as observed in colony #193, seems to be one of the options for the workers to increase their success (reviewed in Bourke, 1994).

Regulation of reproduction in bumblebees seems to be more complicated than appreciated before. We found that inhibition of worker ovarian development and JH biosynthesis were the strongest during the competition phase, giving no support to the hypothesis that worker reproduction starts in response to a decrease in queen dominance. We did not find any changes in worker reproductive physiology associated with the queen switch from females to male production (the switch point). Thus, the initiation of worker reproduction (the CP) can not be explained as a simple response of the workers to changes in queen's pheromone or brood sex. Our data strongly suggest that during the competition phase dominant workers play a central role in the regulation of their callow nestmates reproduction. This implies that their importance in controlling colony development is more significant than considered before.

IX. THE EXOCRINOLOGY OF THE QUEEN BUMBLE BEE

INTRODUCTION

In his pioneering work on the exocrinology of bumble bees, Bergström has reported extensively on the chemistry and function of the labial gland secretion of male bumble bees (Bergström et al., 1973, 1981, 1981a). Later studies since have concentrated on the chemical analysis of exocrine secretions of queens and workers. Genin et al., compared the composition of extracts obtained from the cuticle and from the labial glands of males, queens and workers of *B. hypnorum* (Genin et al., 1984). Investigations on the volatile constituents of the tarsal glands of *B. terrestris* revealed a complex mixture of hydrocarbons with which the bees seem to mark food sites that have been visited (Schmitt et al., 1991). In a comparative study Tengö et al., demonstrated that Dufour's gland secretion contains species specific multicomponent mixtures covering a wide range of

volatility (Tengö et al., 1991). The function of this glandular secretion in *B. hypnorum* is to mark a trail leading from the nest entrance to the inner parts of the nest (Hefetz et al., 1993). Chemical analysis of the secretion in this species also showed interesting intra-nidal differences between workers which are correlated with size polymorphism (Tengö et al., 1991). A recent comparative study of several species of bumble bees showed a congruency in the hydrocarbon constituents between cuticular washes and Dufour's gland secretions (Oldham et al., 1994).

The studies on bumble bee pheromones to date demonstrated that they constitute complex blends of chemicals. It is therefore imperative to gain a thorough knowledge of the chemical composition of the various exocrine secretions in order to understand their possible biological function. In this study we present a comprehensive inventory of the volatile constituents of several of the prominent exocrine glands of queen *B. terrestris*.

MATERIALS AND METHODS

Sample preparation

For glandular extraction queens were dissected under chilled water, and the glands were transferred into 2 ml pentane (Merck, Uvasol). The following queen exocrine glands were analyzed: mandibular, labial, hypopharyngeal, tarsal, and Dufour's glands. Cephalic glands including mandibular glands, hypopharyngeal glands and labial glands were removed in this sequence in order to avoid as far as possible cross contamination during the dissections.

Chemical analyses

As described earlier (Klimetzek et al., 1989), crude extracts were concentrated in microvials at 40°C to 30 µl. Analyses of volatiles were carried out by combined capillary gas chromatography / mass spectrometry (Fisons GC8000 equipped with a 30m 0.25mm DB-5 capillary column temperature programmed from 60°C for 3 min. to 300°C at a rate of 5°C/min.. For separation of enantiomers, a 2,6-heptakis-(2,6-di-O-methyl-3-O-pentyl)- β -cyclodextrin capillary column was used, temperature programmed from 50°C for 5 min. to 170°C at 5°C/min. and linked to a Fisons MD800 mass spectrometer, 70eV). The compounds were identified by their fragmentation patterns and by comparison with authentic samples (The Wiley, 1989). Hydroxylated compounds present in the crude extracts were either methylated with diazomethane or silylated with bis-(trimethylsilyl)-acetamide (Mielniczuk, 1992), followed by GC/MS. For methylation, 10 µl of a solution

of diazomethane in pentane were added to 10 μ l of the crude extract, concentrated (Klimetzek et al., 1989), and submitted to GC/MS analysis. Silylation was performed on a part of the methylated extract (5 μ l) that was treated with 3 μ l bis-(trimethylsilyl)-acetamide at room temperature for 30 min. Subsequently, the mixture was concentrated and analyzed by GC/MS.

Position of double bonds was elucidated by alkylthiolation using dimethyl disulfide (DMDS) (Vincenti, 1987). Double bond positions in unsaturated fatty acids were determined by methylthiolation of the methylated extracts. For alkylthiolation, 10 μ l of the extract were mixed with 50 μ l of carbon disulfide, 50 μ l DMDS, and 5 μ l of iodine solution (60mg in 1ml ether) and heated to 60°C for 12h. The excess of iodine was destroyed with 50-100 μ l aqueous sodium thiosulfate solution (0.5g in 10 ml H₂O). After addition of 300 μ l pentane and 50 mg sodium chloride the organic layer was separated, concentrated, according to the known procedure (Klimetzek et al., 1989), and submitted to GC/MS analysis.

Synthetic compounds

Reference compounds were either commercially available or prepared by standard procedures. Double bonds were produced from the corresponding triple bonds. For the synthesis of the optically active 3-hydroxy carboxylic acids, readily available 3-keto acids were stereoselectively hydrogenated with [S-(-)-2,2'-bis-(diphenylphosphino)-1,1'-binaphthalene]chloro (p-cymene) ruthenium chloride as a catalyst (Kitamura et al., 1992; Gnet et al., 1994, 1995). Esterification of the alcohol moiety in the hydroxy acids was carried out with butanoyl chloride according to the standard procedure.

RESULTS

Five prominent exocrine glands of *B. terrestris* were chemically analyzed revealing a wealth of compounds including hydrocarbons, alcohols, carbonyl compounds, carboxylic acids, various types of esters and oxygenated acids.

Hydrocarbons

A total number of 188 hydrocarbons could be identified (Table 12). Straight chain hydrocarbons ranging from dodecane to heptatriacontane formed a backbone in almost all secretions. As usual for hydrocarbon patterns from biological material, uneven numbered homologues always showed higher concentrations than the adjacent even numbered ones. Maximum concentrations were found in C₂₃, C₂₅, C₂₇, C₂₉ and C₃₁. A large number of unbranched alkenes forming clusters of up to 10 positional isomers (hentriacontene)

showed maximum concentrations at the same chain lengths as the corresponding alkanes. In a series of alkadienes, including positional isomers, the double bonds were interrupted by even numbers of methylene groups. The closest distance between double bonds was found in 9,15-heptacosadiene and 9,15-nonacosadiene (4 methylene groups), while double bonds were most remotely positioned in 5,21-nonacosadiene (19 methylene groups). Alkadienes, with nonacosadiene being most abundant, were predominantly found in labial, tarsal and Dufour's glands. The extreme complexities of the mixtures prevented the assignment of the double bond geometry in any of the unsaturated hydrocarbons.

Apart from straight chain hydrocarbons a multitude of methyl branched alkanes could be identified according to their mass spectra (Pomonis et al., 1978, 1980). While considerable amounts of 9-methyl heneicosane and 11-methyl heneicosane were found almost exclusively in the mandibular glands, 11-methyl and 13-methyl heptacosane as well as 13-methyl nonacosane could be identified in the secretions of mandibular and Dufour's glands. Gas chromatographic separation of enantiomers of the chiral methyl alkanes was impossible due to the similarities of chain lengths on both sides of the branching points.

Alcohols

Of the 18 alcohols identified, 4 represented chiral methyl carbinols, present in the mandibular glands (Table 13). Unsaturated primary alcohols, 9-decenol and 9-dodecenol were only found in the labial glands. A series of 10 bishomologue fatty alcohols occurred in the tarsal glands.

Carbonyl compounds

Among the carbonyl compounds, 6 methylketones of medium chain length occurred predominantly in the mandibular glands, accompanied by the corresponding secondary alcohols (Table 13). Long chain methylketones were found in the labial and tarsal gland only. Trace amounts of five aldehydes were more or less randomly distributed in several glands. In contrast, the spiroacetal spiroacetal 2-ethyl-7-methyl-1,6-dioxaspiro-[4.5]-decane, which is a cryptic ketodiol, is present in the mandibular glands only (Table 14).

Carboxylic acids

A total number of 67 carboxylic acids could be identified (Table 15). The "classical" fatty acids proved to represent the most prominent components among this group. Methyl branched carboxylic acids were exclusively found in the mandibular glands. The absolute configuration of these chiral compounds remains to be determined. Similar to the straight chain alkenes, unsaturated carboxylic acids formed clusters of up to 5 positional

isomers, the stereochemistry of which could not be assigned. Unsaturated acids with shorter chains were found in the labial glands, and those showing 16 and 18 carbon atoms were found in all glands investigated. The occurrence of acids with more than 20 carbon atoms was restricted to the mandibular and tarsal glands. Concentrations in Dufour's glands secretions reached trace amounts per component only.

Carboxylic acid esters

Esters form the second largest group (157 aliphatic esters listed in Table 15) of volatiles present in the various glands. Three types of aliphatic esters could be distinguished: Esters with a short alcohol component, esters with a short acid component, and wax type esters with similar chain lengths on either side of the functional. In addition, a small amount of fatty acid esters of the diterpene geranylcitronellol were found predominantly in Dufour's and labial glands (Table 14).

Esters with a short alcohol component prevailed in labial and Dufour's glands. Dufour's glands were also characterized by many acetates, albeit in small amounts. Among the acetates, tetracosyl acetate and hexacosyl acetate were found in fairly large amounts in tarsal glands. Among the medium volatiles, dodecyl hexanoate formed a major component in labial glands. With respect to the composition of wax type esters, labial and Dufour's glands secretions were most complex. These included a series of even numbered alcohols (ranging from C₈ to C₂₀) and a series of even numbered acids (between C₈-C₁₈). Decyl and dodecyl esters having a total of 24,26,28 and 30 carbon atoms were most abundant. While at the alcohol moiety desaturations were exclusively found in position 9, the acid moieties showed double bond positions at 7,9,11 and 13, again forming clusters of positional isomers like the dodecyl hexadecenoates in the labial glands. Double bond position in unsaturated esters including positional isomers could be distinguished by the mass spectra of their DMDS-derivatives. Isomeric esters such as formed by shifting of the ester group, as in the pair dodecyl dodecanoate and decyl tetradecanoate, can be easily identified even when they are not resolved by gas chromatography since they produce pronounced signals of the protonated acids in the EI-mass spectra (Tengö et al., 1985).

A special class of higher oxygenated compounds is represented by the hydroxylated acids, some of which are esterified with butanoic acid. 3-Hydroxydecanoic acid and its butyrate form major components of the mandibular glands secretion (Table 16). The EI spectra of the corresponding methyl esters are shown in Figure 25. In the mass spectrum of methyl 3-hydroxydecanoic acid (Fig. 25A) the base peak at m/z 103 is formed by α cleavage of the 3-hydroxy group giving rise to m/z 71 upon loss of methanol. The doublet

at m/z 152/153 is the result of elimination of water and methanol or a methoxy group, respectively. The signal at m/z 74 represents the characteristic Mc Lafferty fragment of methyl esters, while m/z 127 may be regarded as its complement (minus a proton). The mass spectrum of methyl 3-butyroxy decanoate (Fig. 25B) is highly dominated by m/z 71 which is represented by the acylium ion of the side chain. Other characteristic ions in this spectrum are the same as in the parent ester.

DISCUSSION

As in many social Hymenoptera queens of *B. terrestris* proved to be a rich source of a multitude of volatile compounds that may serve in chemical communication or have other biological functions. The extensive study of these products showed that glands cannot be characterized by possessing certain groups of chemicals but, on the contrary typically have a plethora of volatile substances.

The compounds listed in Tables 12-16 show the almost complete pattern of volatiles present in queens of *B. terrestris*. Apart from a group of higher boiling compounds only some trace components remained unknown. The mass spectra of these polar substances suggested the structures of steroids; their masses and base peaks of 70 eV spectra are shown in Tabel 14.

Among the compounds found in the glands of *B. terrestris*, aliphatic hydrocarbons were most abundant. Hydrocarbons are omnipresent in insects' exocrine secretion. They form components of the sex pheromone blends in many dipteran species (Howard and Blomquist, 1982; Blomquist et al., 1987), in the rove beetle *Aleochara curtula* (Peschke and Metzler, 1987), in the leafminers (Francke et al., 1988), and in geometrid moths (Li et al., 1993). Although they are very abundant in the social Hymenoptera, there is little experimental evidence as to their function. In ants, female's Dufour's gland produces tridecanone that functions as a sex pheromone (Walter et al., 1993). The solitary bee *Xylocopa virginica* produces alkanes in its Dufour's gland and uses them for marking flowers that have been depleted of their resources (Vinson et al., 1978). In *B. terrestris*, flower marking is also accomplished by using hydrocarbons, but in this species they are the products of the tarsal glands and deposited on flowers that are rewarding. In ants they comprise the bulk of the postpharyngeal gland secretion and are apparently applied to the cuticular surface, explaining the congruency between these two secretions (Bagneres and Morgan, 1991; Hefetz, 1992; Soroker et al., 1995). The report suggesting the postpharyngeal secretion as a source of recognition cues (Soroker et al., 1994), renders hydrocarbons as the natural candidates for forming such a signal. The same congruency in

hydrocarbons was also found in several species of *Bombus* (Oldham et al., 1994), lending credence to the recognition hypothesis. However, in bumble bees, in contrast to ants, there is no evidence that the glandular secretion is actively applied onto the cuticular surface. The quantities of hydrocarbons present in the glandular exudates of *B. terrestris* renders unlikely the possibility that they are simply lining the cuticular intima of the glands. It is also unlikely that these complex mixtures merely serve as a solvent for the other glandular components: for that function a simple mixture or even a single compound would suffice, as was shown for the defensive secretion of *Bledius* species (Dettner, 1987). Moreover, the glands possess not only straight chain alkanes, but also many alkenes, alkadienes, and branched alkanes, suggesting a specific function for these compounds. Whether hydrocarbons serve as integral components of the secretion in *B. terrestris* queens, or form a neutral group of compounds, awaits experimental evidence.

Apart from the hydrocarbons, fatty acids and their esters with straight chain alcohols form two other large groups of compounds identified from *B. terrestris*. Although both groups of chemicals are widespread among the various exocrine glands, some glandular specificity can be found. The mandibular gland secretion shows the most complex acid pattern, whereas the labial and Dufour's glands secretions show the broadest spectra of esters, although each gland possesses a very different composition. Among the acids "classical" saturated representatives like myristic, palmitic and stearic acids as well as oleic acid are always dominant, while vaccenic acid is an additional major product in the hypopharyngeal and in the labial glands. Similar to the alkanes, unsaturated acid form clusters of up to five geometric isomers. Only the mandibular glands produce chiral methyl branched acids, among which at least 12-methyl tetradecanoic acid is known to exhibit antibiotic properties (Hattori et al., 1987).

Esters are one of the most widespread classes of aliphatic compounds and they are particularly abundant in the exocrine secretion of bees (Wheeler and Duffield, 1988; Francke et al., 1983). They are the major components in Dufour's gland secretion of *Andrena* species (Tengö and Bergström, 1975; Cane, 1981) where they serve as a brood cell lining. In the anthophorid bee *Eucera palestinae* esters are also abundant and, in addition to the brood cell lining also serve to mark the nest entrance (Shimron et al., 1985). Ethyl and methyl esters are male specific components of the cephalic secretion of *Scaptotrigona postica* (Engels et al., 1990). They are also found on the cuticular surface of honey bee larvae and are reported to act as an attractant for the bee mite *Varoa jacobsoni* (Le Conte et al., 1989). The small group of 2-propylesters, in particular 2-propyl tetradecanoate are typical surfactants and may be used to spread the glandular content after

its secretion. Esters of unsaturated fatty acids and 2-propanol are pheromones of *Dermestes* species (Francke et al., 1979).

While wax type esters appear to be almost ubiquitous to bees, no specific function could be attributed to esters with less than 40 carbon atoms. They may be used, due to their high hydrophobicity, as lubricants, but the remarkably complex mixture suggests a communicative function. Octadecyl (Z)-9-tetracosanoate, for example, was identified as a pheromone in the cockroach *Nauphoeta cinerea* (Takahashi and Fukui, 1983).

As a constituent of the marking pheromone of male bumble bees, the diterpene geranylcitronellol is present in the labial gland of *Bombus* species (Bergström et al., 1973, 1981, 1981a), and of *Psithyrus rupestris* (Svensson and Bergström, 1977). Heads of queens and workers of *B. hypnorum* contain rather high amounts of esters of geranylcitronellol with unsaturated fatty acids, but the biological function of these esters is still unknown (Ayasse et al., 1995).

The carbonyl compounds identified, just like most of the compounds described from the queens, clearly originate from the acetate pool. The methyl ketones, methyl carbinols and the corresponding esters show distinct distribution among the glands. The ketones represent decarboxylated β -keto carboxylic acids which are formed either during anabolism or metabolism of common fatty acids. The majority of these methyl ketones are therefore composed of an uneven number of carbon atoms. It is interesting to note that two groups of ketones showing a gap of 10 carbon atoms were found. Three high boiling compounds which are seldom found in Hymenoptera were found in the labial and the tarsal glands of *B. terrestris*. The low boiling ketones, on the other hand, occur predominantly in the mandibular glands. These ketones are especially widespread among the Hymenoptera as constituents of volatile secretion in ants, and solitary as well as social bees. The corresponding methyl carbinols are also frequently found in bees. In the stingless bee *Scaptotrigona postica* they are produced by the queen and serve as attractants for workers. Interestingly, a group of acetates of long chain methyl carbinols occurs almost exclusively in Dufour's gland. The spiroacetal 2-Ethyl-7-methyl-1,6-dioxaspiro-[4.5]-decane, which also occurs in social wasps (Francke et al., 1979a), solitary bees (Francke et al., 1981), and in fruit flies (Fletcher and Kitching, 1995) represents a higher oxygenated degradation product of fatty acids, namely 2,9-dihydroxy-undecane-5-one. Spiroacetals of this type are known to be pheromones of bark beetles and fruit flies (Francke et al., 1977; Birgersson et al., 1995).

The most interesting compounds identified are oxygenated fatty acids and their derivatives (Table 16). A group of 10 hydroxy acids showing the hydroxyl group towards

the end of the chain occurs exclusively in the tarsal glands. These compounds form a link to the chemistry of lactone producing halictine and colletid bees (Hefetz et al., 1978, 1979), since these lactones are formed from ω -hydroxy and ω -1-hydroxy acids. The mandibular glands are especially rich in 3-hydroxy acids. The structure of these acids may be linked to the above mentioned methyl ketones and methyl carbinols. They clearly represent products of β -oxidation and are reported as a substructure of bacterial lipids (Imoto et al., 1983). β -Hydroxy decanoic acid and two lower homologues were also reported as antifungal agents from the metathoracic glands of the leaf cutting ant *Atta sexdens* (Schildknecht and Knoob, 1971).

Careful examination of the enantiomeric composition of the 3-hydroxy acids and their butyrates revealed that the hydroxy acids in *B. terrestris* do not show constantly the same purity: whereas 3-hydroxyhexanoic acid is represented by the pure (S)- enantiomer, 3-hydroxytetradecanoic acid is almost racemic. The main component in this group, 3-hydroxydecanoic acid shows an enantiomeric excess (EE) of more than 99% of the (S)-enantiomere. Corresponding, the butyric acid esters show at least this enantiomeric purity.

Especially intriguing in the finding of 3-hydroxy acids in the mandibular glands and 10-hydroxy acids in the tarsal gland is the analogy to the honey bee acids. In honey bees, 9-2-(E)-hydroxydecanoic acid and the corresponding 9-oxo compound are components of the queen pheromone (Slessor et al., 1988). In view of the indication that in *B. terrestris* a putative queen pheromone is present in the mandibular glands (van Honk et al., 1980), one may speculate that this function resides with the hydroxylated acid derivatives present in the gland. Whether these compounds with or without the association of the hydroxy acids from the tarsal glands are involved in queen control over the workers' behavior, is currently being investigated.

X. RE-EVALUATION OF THE QUEEN EXOCRINE GLANDS AS THE SOURCE OF A PHEROMONE REGULATING WORKER REPRODUCTION.

INTRODUCTION

Social insects are characterized by highly developed communication systems that enable them to regulate and co-ordinate complicated social processes. Among these, pheromones serve as the main route for social communication, and can be categorized into two types: "releaser" pheromones, eliciting a fast behavioral response; and "primer" pheromones, mediating prolonged physiological changes before evoking an eventual

behavioral response. Releaser pheromones include chemicals used for sexual attraction, aggregation, alarm, trail marking and recognition. The distinctive and usually “all or none” behavioral response to these pheromones enables a relatively simple and short identification procedure. Indeed, the chemical composition of many releaser pheromones has been successfully identified in many social Hymenoptera, revealing a plethora of chemical structures (Blum and Brand 1972; Free, 1987; Wheeler and Duffield 1988; Hölldobler and Wilson, 1990). On the other hand, only a few primer pheromones have been chemically identified with certainty (Dulka et al., 1987; Slessor et al., 1988; Arnold et al., 1994). These seemingly poor achievements can be attributed to the prolonged and complicated processes mediated by primer pheromones, such as the regulation of reproduction and caste determination. Accordingly, the need to construct a long term and complex bioassay has considerably hampered their identification.

In highly social insects where colonies are too large for an effective behavioral control, it is commonly believed that queen primer pheromones mediate regulation of reproduction (reviewed in: Wilson, 1971; Michener, 1974; Hölldobler and Bartz, 1985; Fletcher and Ross, 1985; Spradbery, 1991). This hypothesis relies exclusively on indirect evidence, including observations on species in which worker reproduction is inhibited without any apparent behavioral control; single and double mesh experiments; and experiments where worker reproduction was inhibited in the presence of dead queens or queen extracts (reviewed in: Hölldobler and Bartz, 1985; Fletcher and Ross, 1985; Spradbery, 1991; Free, 1987; Hölldobler and Wilson, 1990). However, all efforts to isolate and chemically identify such queen pheromones have failed. Even in the well known case of the honey bee queen substance, the reproductive inhibiting activity of the mandibular gland secretion or its major constituent 9-oxo 2-decenoic acid (Butler 1959, Barbier and Lederer, 1960; Callow and Johnson, 1960; Butler et al., 1961) could not be confirmed. The complete identification of the chemical composition of the queen honey bee mandibular pheromone confirmed that it has several releaser effects on workers: suppression of new queen rearing (Pettis et al., 1995); inhibition of swarming (Winston et al., 1991); and enhancing worker foraging (Currie et al., 1994). However its function as a primer pheromone which inhibits worker reproduction was refuted (Willis et al., 1990), leaving only the possibility of primer effect on queen rearing (Slessor et al., 1998).

The multiple failures to show a causative effect of a queen pheromone in the social insects, led to the development of an alternative hypothesis: namely, “queen signal and not queen control”. According to this hypothesis, queen presence is conveyed by a queen signal and the workers behave so as to maximize their inclusive fitness (Keller and Nonacs,

1993). This hypothesis emphasizes the ultimate mechanism for the evolution of worker sterility, but leaves an open question regarding the nature of the queen signals. In such cases queen presence may be conveyed by a complex set of signals that encompasses several communicative channels. For example, recent findings demonstrated that the queen honey bee may produce a queen specific signal in head glands other than the mandibular glands (Slessor et al., 1998). In this study we re-evaluated the reported role of the queen *B. terrestris* mandibular gland secretion in inducing worker sterility.

Colonies of the bumble bee *Bombus terrestris* are characterized by two distinct phases of regulation of reproduction. During the first phase, the queen is the only reproductively active female, while the following competition phase (CPh) is characterized by reproductive competition between the queen and dominant egg laying workers (Doom and Heringa, 1986; Duchateau and Velthuis, 1988; Röseler and Honk, 1990). Several lines of evidence have suggested that the mandibular gland secretion of the queen possesses a primer pheromone that delays ovarian development in workers. Workers from colonies headed by queens whose mandibular gland had been surgically removed, started to reproduce earlier than workers from colonies with intact, or sham operated, queens (Honk et al., 1980). Corpora allata (CA) volumes and *in vitro* biosyntheses of juvenile hormone (JH) were lower in callow workers exposed to dead queen bodies, whole queens or mandibular glands extracts (Röseler et al., 1981). Since in these studies queen mandibular gland extracts or cuticular washes had similar inhibitory effects to those of living queens, it was suggested that the putative queen pheromone is produced in these glands and then spread over her body by self grooming (Röseler et al., 1981; Röseler and Honk, 1990). These studies, however, used non-specific assays for JH, and in some cases the statistical analysis may have been inadequate. Moreover, queens of *B. impatiens* from which the mandibular glands were surgically removed retained their full reproductive dominance (Pomeroy, 1981, as cited in Plowright and Lavery, 1984).

In this paper we re-examine the importance of exocrine secretions produced by various glandular sources in the regulation of reproduction in *B. terrestris*. The parameter used in this study was the rate of JH biosynthesis *in vitro* in workers exposed to different queen derived secretions, using a radiochemical assay (RCA), validated specifically for adult *B. terrestris* (Bloch et al., 1996).

MATERIAL AND METHODS

Bees : See above

JH biosynthesis rates: See above

Extract preparation

Extracts were made from queens collected from normally developing colonies that were either before or after the competition point. The queens were killed by freezing (30-60 min) at -20°C , and were either extracted immediately or stored at -80°C until extraction. Total body extracts were prepared by cutting the bees into small pieces and immersing them in a glass scintillation vial (20 ml) filled with the extraction solvent. Two different extraction procedures were used: 1) 24 hours with pentane (Merck) followed by an additional 10 - 12 hours in absolute ethanol (Riedel de Hen) (pentane - ethanol extract). The pentane and ethanol extracts were applied separately, one following the other on the dummies before the bioassay; and 2) Three successive extraction periods, each for two hours, in chloroform - methanol 2: 1 (Blight and Dyre, 1959); (Chloroform - methanol extract). Cuticular washes were obtained by extracting frozen queens, to reduce extraction of fat body and internal organs (Gilby, 1980), for five minutes in chloroform - methanol 2: 1. Extracts were first concentrated in a vacuum evaporator and then under nitrogen gas flow.

For glandular extraction, queens were dissected under chilled water, and the glands were transferred into 1.5 ml chloroform - methanol 2: 1. The following exocrine glands were extracted: mandibular (MG), hypopharyngeal (HPG), head salivary (SG), Dufour's (DG) and tarsal (TG). The cephalic glands (MG, HPG and SG) were removed in sequence to prevent cross contamination during the dissections.

Dummy preparation

In most of the experiments, dead virgin queens were used as dummies for extract application. They were prepared by two different methods: 1). After emptying the gaster the queens were dried for one to two weeks in a vacuum oven at 45°C ("oven dried dummies"); or 2). Washing for 7 to 12 hours in a soxhlet distillator with 2: 1 chloroform-methanol as solvent ("soxhlet washed dummies"). The effects of queen cuticular washes and mandibular gland extracts were also examined by treating living callow virgin queens collected at the day of eclosion, still before yellow pigmentation. In these experiments, the chloroform - methanol extracts were evaporated to dryness and then reconstituted in pentane. Following application the queens were introduced into the queenless group. The introduced queens were replaced once a day to prevent their possible integration into the group social hierarchy (Bloch et al., 1996). In two experiments we tested the effect of queen mandibular gland extracts after application on a glass slide or a Petri dish covered with melted honeybee wax.

Bioassay

Dummies were treated with one queen equivalent (in 50 - 150 μ l solvent on dead dummies, or 20-50 μ l on living queens), or solvent in the control groups. After letting the solvent evaporate they were housed with three callow workers, that were less than 12 h after eclosion and before full coloration, in an experimental cage (107x107x95 mm). To reduce inter-group genetic variation, the workers were selected randomly from a pool of callow workers originating from different colonies. Dummies were replaced every day by freshly treated ones. After three days, or unless otherwise specified, the callow bees were sacrificed and analyzed for *in vitro* JH biosynthesis rates.

The biological activity of the hydroxy acid constituents of the mandibular glands (Hefetz et al., 1996) was tested using a blend containing 9 μ g 3-hydroxyoctanoic acid (3HOA), 15 μ g hydroxydecanoic (3HDA) and 1.5 μ g 3- hydroxydodecanoic acid (3HDDA) in methylenc chloride. Thirty μ l of the solution, approximately equivalent to the quantity found in the mandibular glands of six queens (6 Qeq.), were applied to an oven dried dummy or a pea size wax ball. Newly treated dummies were introduced twice a day into the experimental groups, exposing the workers to a daily amount of approximately 12 Qeq. hydroxy acids. After three days the bees were sacrificed and the *in vitro* rates of JH biosyntheses were measured as described above.

JH biosynthesis was expressed either as the mean of all the bees together (Index-1), or as the mean of the bees that had the highest rate in each group (Index-2). These latter bees probably represent the dominant bees established in each group (Larrere and Couillaud, 1993). Groups for which the data set was not complete were excluded from the analysis.

We also tested the effect of total body extracts on worker behavior. These experiments were performed blind in marked cages without any other sign indicating the type of treatment involved. On the second day of extract exposure, each group was observed for 20 minutes under dim red light. Agonistic interactions registered among workers included the number of threatening behavior and overt aggression events. Data were collected using a Psion organizer and analyzed using the "Observer" software (Noldus Information Technology). Agonistic behavior was classified into two major categories: threatening behavior and overt aggressive behavior (Bloch et al., 1996). Briefly, "threatening behavior" included buzzing (fast short wing vibrations while opposite a nestmate), and pumping (distinct dorso-ventral pumping movements with her abdomen) (Duchateau, 1989). "Overt aggression" included darting movements and attacks, biting or

grappling with another bee, and pulling on a wing or hairs. (Duchateau, 1989; Doorn and Heringa, 1986; Bloch et al., 1996).

Chemical analyses and quantification

Mandibular gland extracts, or their methylated products, were separated on a Tracor 540 GC using a 30-m SE 54 capillary column that was kept at an initial temperature of 60 °C for two minutes and then heated to 250 °C at 5 °C/ min, kept at 250 °C for 5 min, and then raised to 295 °C at 20 °C/ min. The presence of the hydroxy fatty acids was ascertained by co-injection with standard hydroxy acids or their corresponding methyl esters. Quantitative estimation was based on peak integration using 2.5 µg eicosane as an internal standard.

Methylation of MG extracts was achieved with diazomethane prepared from 1 mmol (147 mg) MNNG (1-methyl-3-nitro-1-nitrosoguanidine) using Aldrich Diazomethane Kit. The reagent was placed in the inner tube and about 2 ml extracts in the outer tube. The reaction was initiated by slowly adding (1 drop per 5 seconds to prevent overheating) 0.6 - 1.0 ml of 5N sodium hydroxide to the inner tube while immersing the apparatus in liquid nitrogen. After 5 min the apparatus was removed from the liquid nitrogen container and kept at room temperature for at least one hour to ensure acceptable yield of diazomethane and effective methylation.

RESULTS

Exposure of callow workers to queen total body extract

In a preliminary experiment we examine the effect of exposing callow workers for one day to queen total body extracts using "oven dried dummies". The rates of JH biosynthesis in this experiment were similarly low for treatment (22.19 ± 1.8 SE, $n = 29$) and control groups (24.1 ± 2.7 , $n = 25$; unpaired t-test, $P = 0.55$). We therefore tested the effect of queen total body extracts over a longer period of three days. This is the period for which the differences in rates of JH biosynthesis between queenless and queenright workers are the highest (Röseler and Röseler, 1978; Bloch et al., 1996). In these experiments we used either "oven dried dummies" or "soxhlet washed dummies". Since there were no differences between these two modes of extract presentation (t-test, index-1: $t = 1.62$, $df = 76$, $P = 0.11$; index-2: $t = 1.48$, $df = 24$, $P = 0.15$), the data were pooled. For the same reasons, the results of the experiments using chloroform-methanol and pentane - ethanol extracts were also pooled (t-test, index-1: $t = 0.34$, $df = 43$, $P = 0.73$; index-2: $t = 0.13$, $df = 13$, $P = 0.15$).

Table 17 shows the effects of worker exposure to total queen extracts on the rates of JH biosynthesis *in vitro* and worker behavior. Biosynthesis rates are expressed as the mean of all the workers tested (Index-1), or the mean of the individuals with the highest rates in each group (Index-2). Irrespective of the index used, these rates in workers exposed to the extract were lower than in workers exposed to solvent alone. In this respect, extracts made from queens collected from colonies before or after the onset of the competition phase were similarly effective (index-1: 36.9 ± 5.4 SE, $n=15$; 39.6 ± 2.4 SE, $n=34$ for queens before and after CP respectively, t-test, $df = 47$, $P = 0.6$; index-2: 53.8 ± 7.2 SE, $n=5$; 50.4 ± 3.2 , $n=11$ for queens before and after CP respectively, $df = 14$, $P = 0.63$).

The behavioral observations following exposure to total queen extract revealed a more equivocal effect. Threatening behavior and overt aggression were slightly more frequent in the control groups than in the test group, but these differences were not statistically significant (Table 17). The data presented in Table 17 comprise pooled data from different treatments and modes of exposure to extracts. Separate analyses demonstrated that there were no significant differences between workers from groups exposed to the different dummies (Mann-Whitney test, threatening behavior: $n = 36$, $P = 0.22$; overt aggression: $n = 36$, $P = 0.4$), or between experiments using the two extraction procedures (Mann-Whitney test, threatening behavior: $n = 36$, $P = 0.76$; overt aggression: $n = 36$, $P = 0.89$).

The effect of queen glandular extracts

The effects of various queen glandular extracts were tested using the above-described 3 day bioassay with “oven dried dummies” or “soxhlet washed dummies” (Table 18). In the experiments using “oven dried dummies” the rates of JH biosynthesis between workers exposed to queen glandular extracts were similar to those observed in bees that were exposed to solvent alone (unpaired t-test, after Bonferroni correction, $P > 0.01$). When index-1 was used the control group had, for unknown reasons, lower rates of JH biosyntheses (34.6 pmol/h/pair) than in control groups in the previous experiment (Table 17), and those from previously reported for queenless workers (Bloch et al., 1996). In contrast, when index-2 was used, the levels in the control group were as high as those measured in the previous experiment. Using index-2, JH production rates in groups exposed to mandibular glands extracts were reduced when compared to the control group (t-test, $df = 11$, $P = 0.04$), but this difference became non-significant after Bonferroni correction (P value has to be lower than 0.01).

In the experiments using “soxhlet washed dummies”, rates of JH biosynthesis in the control group were higher than in the “oven dried dummies” (t-test, Index-1: $t = 2.1$, $df = 37$, $P = 0.047$; Index-2: $t = 0.45$, $df = 11$, $P = 0.66$), and comparable to those of the control group in the total body extract experiment (Table 17). However, we failed to detect any differences as a consequence of exposure to any of the queen glandular extracts (Table 18). There was a slight reduction in the rates of JH biosynthesis in workers exposed to SG or DG extracts (t-test, $P = 0.049$, $P = 0.046$, respectively), but these proved to be insignificant after Bonferroni correction.

The possible effect of the mandibular glands was specifically assayed by applying the extracts on glass slides, or onto small Petri dishes covered with honey bee wax (Table 19). In these experiments too the rates of JH biosynthesis were similar for workers exposed to mandibular glands extracts or to solvent.

In a separate set of experiments live callow virgin queens were applied with either cuticular washes or mandibular gland extracts and housed with three callow workers. Cuticular washes from functional queens resulted in significant inhibition of JH biosynthesis in the callow worker (Fig. 26, t-test, index-1: $t = 2.74$, $df = 28$, $P = 0.01$; Index-2: $t = 2.57$, $df = 8$, $P = 0.03$). Mandibular glands extract, on the other hand, had little effect. Similar rates of JH biosynthesis were observed in callows exposed to functional queen MG extracts and to clean solvent (26.7 ± 3.5 SE, $n = 12$ and 28.9 ± 3.6 SE, $n = 18$, respectively). In this particular experiment the rates of JH biosynthesis in the solvent control were unusually low. To test whether these low rates were due to inhibition of the CA or due to low performance of the specific incubation (e.g., bad methionine lot), we repeated the assay under the same incubation conditions and using the same methionine lot, but using callow workers (day-0) and 3 day old queenless workers from the same colonies. These two groups represent minimal and maximal CA activities (Bloch et al., 1996). The results of these assays were 33.5 ± 7.5 SE for 3 day old workers and 10.8 ± 1.6 SE for the callow workers. These are lower than those previously reported for similarly aged workers in our laboratory (49.8 ± 6.7 SE and 52.1 ± 5.4 SE for 3 day old and 15.4 ± 3.6 SE for callows), but they still reflect the expected physiological differences between 3 day old queenless and callow workers.

The effect of mandibular gland hydroxy-acids

The mandibular glands of queen *B. terrestris* are rich in 3-hydroxy fatty acids. We therefore tested in our bioassay whether they possess an inhibitory activity. We first estimated their amount in functional queens by GC analysis of the methylated extracts.

Queen glands possessed an average of $1.53 \mu\text{g} \pm 0.39$ 3HOA, $2.38 \mu\text{g} \pm 0.68$ 3HDA and $0.23 \mu\text{g} \pm 0.08$ 3HDDA (mean $\pm$ SE for $n = 6$ queens). Accordingly, callow workers were daily exposed to about 12 queen equivalents of synthetic hydroxy acids. There was a slight, but not significant decrease in the rates of JH biosynthesis in workers exposed to the hydroxy acid blend (Two-way ANOVA, treatment: $df = 1$, $P = 0.09$; replicates: $df = 4$, $P = 0.06$; Fig. 27).

DISCUSSION

Primer pheromones regulating reproduction are predicted to affect the physiological processes involved in ovarian development. In *B. terrestris* workers, reproduction is effectively inhibited until a particular period in colony development. It was postulated that the queen actively inhibits the workers from reproducing by a combined behavioral dominance and pheromone production (Honk et al., 1980; Röseler et al., 1981). The onset of worker reproduction was accordingly assumed to be the result of pheromonal output decline by the queen (Duchateau, 1989; Doorn and Chrambach, 1989; Doorn, 1987; Pomoroy and Plowright, 1982). Based on surgical removal and *in vitro* assays the mandibular glands have been pinpointed as the source of the putative pheromone, but the chemistry of its secretion has not been elucidated. However, a recent finding that queens before the competition phase (a period when worker reproduction is effectively inhibited) and queen after the competition phase (when worker reproduction takes place) can equally inhibit worker reproduction in small groups (Bloch et al., 1996) casts doubts on these conclusions. In an effort to identify the putative mandibular gland pheromones, a comprehensive study of the chemistry of the secretion in mated queens was performed (Hefetz et al., 1996). In this article we re-evaluate the occurrence of a primer pheromone that is responsible for the inhibition of worker reproduction. Because JH is apparently a gonadotropin in *Bombus terrestris* (Bloch et al., In prep.; Röseler and Honk, 1990), we used rates of JH biosynthesis to assess the inhibitory activity of queen secretions.

Rates of JH biosynthesis were significantly inhibited in queenless workers exposed daily to total body extracts or cuticular washes of a functional queen. This suggests that the extract contains substances that may act as primer pheromones and that these are apparently spread on the surface of the queen's body. The same extracts, however, did not significantly alter the aggressive behavior that usually erupts among workers under queenless conditions, at least until dominance hierarchy is achieved. Threatening behavior and overt aggression in groups of workers exposed to queen total body extracts were low, but did not differ from the control groups. It is thus possible that the putative pheromone

affects the physiology, but not the behavior of the workers. Aggressive acts by the queen, on the other hand, can effectively depress any aggression that may develop in workers. This lend credence to earlier suggestions that in *B. terrestris* the queen uses mixed behavioral and pheromonal means to inhibit workers reproduction (Honk et al., 1980; Röseler et al., 1981).

To identify the glandular source of the prospective pheromone, we repeated the assay by exposing callow bees to dummies treated with extracts of five major queen exocrine glands. When we used "oven dried dummies" we experienced low rates of JH biosynthesis in the control, significantly lower than those obtained previously. The rates of JH biosynthesis in workers exposed to the glandular extracts were similarly low, but because of the low control rates we could not conclusively state that the glands are devoid of activity. The effect of the non treated "oven dried dummies" could have been due to odors emitted by the dummy that affected the workers irrespective of the treatment. We therefore tried to eliminate all possible odor from our dummy queens by extensively washing them in a soxhlet apparatus. This procedure resulted in higher levels of JH biosynthesis in the control. Nevertheless, none of the examined gland extracts exerted significant inhibition on worker CA activity. Thus, irrespective of the dummies used no extracts of any gland did not possessed the primer pheromone activity.

These results are in contrast to the previous reports that the mandibular glands are the source of the primer pheromone that inhibits worker reproduction (Honk et al., 1980; Röseler et al., 1981). We therefore further examined the effect of queen MG extracts in supplementary experiments, using different application methods. The results confirmed that the mandibular gland secretion has no effect on *in vitro* biosynthesis of JH. There were no significant differences between callows exposed to queen MG extracts or to pure solvent applied on glass slides, on a Petri dish covered with wax, or on a live callow gyne body. We also tested the activity of three major hydroxyacids present in the mandibular glands of queen *B. terrestris* (Hefetz et al., 1996). Application of 12 Qeq/day did not change the rates of JH biosynthesis when compared to the control. In the experiments using virgin queens as carriers for the tested material the control was rather low. Thus although the levels of JH were not significantly different from the control, the results are equivocal. In order to validate these results we measured the rates of JH biosynthesis for 3 day old queenless workers (expected to have high JH synthesis) and for callow workers (expected to have low JH synthesis) under the same incubation conditions and the same lot of radioactive methionine. The synthesis rates for JH in the 3 day old queenless workers and in callows were indeed lower than observed in earlier studies. The synthesis in the callow workers

was, as expected, significantly lower than for the 3 day old queenless workers. This indicates that the results obtained by using virgin queens as carriers are valid. Moreover, when we used this system in the application of cuticular washes we obtained significance inhibition of JH biosynthesis. Therefore our findings are not consistent with the hypothesis that the mandibular glands secretion does not contain a primer pheromone activity that interferes with ovarian development.

Earlier reports regarding the activity of the mandibular glands as a queen pheromone were mainly based on two types of experiments. In the first, worker oviposition started earlier in colonies headed by queens deprived of their MG, compared to colonies headed by sham operated or unmutated queens (Honk et al., 1980). The second is a report that 1 day old queenless workers exposed to queen MG extracts had lower CA volume than workers exposed to pure solvent (Röseler et al., 1981). The different results obtained in the present study could be due to differences in performing the bioassays: 1). Röseler et al., (1981) measured CA volume as opposed to our direct measurement in the *in vitro* assay. We consider direct measurement of JH biosynthesis to be more reliable. Studies with a wide variety of species, including the related honey bee, demonstrated a poor correlation between these indices (e.g. Feyereisen, 1985; Lanzerein et al., 1978; Dale and Tobe, 1986; Huang et al., 1991; Kaatz et al., 1992); 2). The workers assayed in Röseler et al., (1981) were 1 day old, vs 3 days old in the present study. We selected this age because the CA of 3 day old, but not that of 1 day old, workers show a maximum difference in activity between queenless and queenright callow workers (Bloch et al., 1996; Röseler and Röseler, 1978); 3). In the present study the queen dummies and workers were maintained without any brood, whereas Röseler et al., (1981) added to each experimental group ten to twenty pupas. Although we do not know the precise role of brood in social interactions in bumble bees, a brood pheromone that inhibits ovarian development in workers was described for honey bees (Arnold et al., 1994); 4) The dummies in our study constituted virgin queens, while Röseler et al., (1981) used bodies of functional queens that had not caused inhibition in previous assays; 5) Röseler et al., (1981) mixed wax obtained from normally developing colonies with their extract preparation, while in this study only the solvent was used. It is possible that the supplementary components that originate from normally developing colonies in the assays performed by Röseler et al., (1981) affected the results.

The question of whether queen *B. terrestris* possesses a primer pheromone that inhibits worker reproduction is still unresolved. Supporting the presence of such a pheromone are the inhibitory activities of the total body extract or of cuticular washes. We

have not been able to discriminate whether the pheromone is produced by an internal exocrine gland and is applied onto the epicuticle of the bees by self grooming, or whether it is an integral part of the cuticular lipids. According to our results, none of the commonly known exocrine glands, mandibular gland included, are active. It is possible that either another gland, not studied by us, produces the substances, or that the activity of the pheromone comprises combined secretion from several glands.

XI. BRAIN BIOGENIC-AMINES, DOMINANCE AND REPRODUCTION IN *BOMBUS TERRESTRIS*

INTRODUCTION

Biogenic amines are known as neurotransmitters in synaptic clefts, and as neurohormones that circulate in the blood and act as neuromodulators in the central nervous system (reviewed in: Orchard, 1982; Erber et al., 1993; Kravitz, 1988; Hasselmo, 1995; Izquierdo and Medina, 1997). In arthropods and specifically in insects they are involved in the regulation of several behavioral and physiological processes (Dedecker et al., 1994; Orchard, 1982; Roeder, 1994). In crustaceans, serotonin is associated with increased agonistic interactions and with high dominance status, while octopamine with reduced aggression and low social rank (Huber et al., 1997; Kravitz, 1988). Likewise, serotonin levels were associated with increase in inter and intra-species aggression of the ant *Formica rufa* (Kostowski et al., 1975). However, artificial application of the chemical cause increase only in the conspecific aggression (Kostowski and Tarchalska, 1972).

Little is known about the role of biogenic amine in the regulation of insect reproduction. Several studies showed that dopamine or octopamine regulate biosynthesis rates of JH, the gonadotrophic hormone in most insect species (Hentschel, 1981; Woodring and Hoffmann, 1994; Pastor et al., 1991b). Other reports suggest that biogenic amines are involved in other processes that affect insect ovarian development (Grillot and Raabe, 1989; Pastor et al., 1991a; Harris and Woodring, 1995). In *Drosophila*, for example, inhibition of tyrosine hydroxylase resulted in reduced dopamine levels and abnormally developed ovaries (Neckameyer, 1996; Pendleton et al., 1996). Dopamine levels were also correlated with ovarian development for queenless workers of the honey bee (Harris and Woodring, 1995).

This part of the study aimed at exploring the relationship between biogenic amines, behavior and reproduction in *B. terrestris*. This was done by comparing brain levels of three principle insect biogenic amines, dopamine, octopamine and serotonin, for bees at

different reproductive and dominance status. In the first experiment we compared young queenless and queenright workers. Then we compared dominant and lower ranked workers from queenright colonies during the competition phase. In the last experiment, we compared functional queens from normally developing colonies with young virgin queens.

MATERIALS AND METHODS

Bees, ovarian development, determination of social events: see above

Dissections and sample preparation

All the bees used in these experiments were completely frozen within 1-2 seconds of collection by immersing them into liquid nitrogen. This minimized the possibility of stress-induced changes in biogenic amines. Collected bees were then stored frozen at -80°C to prevent amine degradation. Brains were dissected in a dissecting dish cooled by dry ice and remained frozen during the entire dissection procedure to minimize amine degradation. The hypopharyngeal glands, optic lobes and subesophageal ganglion were removed during dissection. Each brain was then placed individually in a 1.5 ml Eppendorf tube and stored in a -80°C until analysis. The size of each bee was determined by measuring the length of the medial vein of the front wings that was mounted on a glass slide.

For sample preparation, 50 µl of chilled perchloric acid (0.2 M) were added to each tube while on ice (4°C). The perchloric acid contained dihydroxybenzylamine (DHBA, 50 pg/µl) and synephrine (25 pg/µl) as internal standards. DHBA was used to quantify dopamine and serotonin, and synephrine was used to quantify OA. Each sample was sonicated, rechilled for 20 min, and centrifuged at 13,000 rpm for 10 min at 4°C (Hermle 2360 centrifuge). The sample was then transferred to a 0.22 µm nylon membrane filter and centrifuge again for 6 min. The filtered sample was then transferred to microvial, precooled to 2°C, for analysis by high-performance liquid chromatography (HPLC).

Measurements of biogenic amine levels was achieved by subjecting the brain extracts to HPLC separation. The HPLC apparatus consisted of refrigerated ESA automatic injector, ESA model 580 pump, a 80 mm x 4.6 mm high efficiency reverse phase ESA Catecholamine HR-80 column (#316 stainless steel and packed with 3µm spherical octadecylsilane) and an Coulochem II electrochemical detector coupled to a model 5014B microdialysis analytical cell. Channel 1 of the detector was set at 425 mV for detecting dopamine and serotonin and channel 2 was set at 650 mV for detecting OA. The mobile phase (pH = 5.6) was composed 15 % methanol, 15% acetonitril, 75 mM sodium dodecyl sulfate, 1.5 nM sodium phosphate monobasic and trace amounts of citric acid (Sigma Chemical Co. St. Louis, MO), with water purified by a Nanopure II system (Millipore).

The HPLC system was connected to an EZChrom Chromatograph Data System version 6.0 (Scientific Software) for analysis of peak areas using both internal and external standards. External standards were run before and after each set of biological samples.

Brain biogenic amine levels in callow QR and QL worker

Callow workers (< 12 h after eclosion and before full coloration), were collected from different colonies. They were then marked with colored tags and introduced into queenright colonies (QR), or housed to form groups of three queenless bees (QL). Queenless groups were placed in small plastic cages (95x107x107 mm) with a cardboard bottom. Measurements of brain biogenic amine levels were made for callow (day 0) 1 day old and 3 day old bees.

Brain biogenic amine levels in dominant and low ranked QR workers

In the first experiment we used workers of unknown age. Dominant workers where defines as workers that performed one of the following behaviors: oviposition behavior, construction of egg cups, destruction of egg cups or oophagy. Workers were defined as low ranked if over five to ten minutes of observation they did not performed any agonistic behaviors or any the other dominance behavior described above. Low rank workers were generally engaged in larvae nursing, brood incubation or were calmly exploring the colony (in contrast to the typical excitement of dominant workers). Two bees were collected for each analyses, dominant individual and one low ranked, from the same colony and of approximately of the same size. In the second experiment workers of known age were used, so the bees compared were also of the same approximate age.

Brain biogenic amine levels in functional and virgin queens

Functional and virgin queens were collected from normally developing colonies. To ensure that the eclosing queens do not mate within the nest they were removed from the nest as pupae or callows and confined to small cages. They were sacrificed for analysis at 3 to 4 days of age.

RESULTS

Brain biogenic amine levels in callow QR and QL worker

Levels of dopamine, octopamine and serotonin in the brains of 1 or 3 day old queenless and queenright workers were not significantly different (Fig. 28, unpaired t-test, $P>0.05$). Octopamine levels in 3 day old queenright workers were significantly higher than

for callows and for 1 day old queenright workers (ANOVA, $P=0.002$; Scheffe post hoc test, $P=0.004$, $P=0.033$, respectively; Fig. 28b). Dopamine, serotonin, and octopamine in queenless groups did not show similar increase with age (Fig. 28., ANOVA, ns).

Brain biogenic amine levels in dominant and low ranked QR workers

Dominant workers had both significantly more developed ovaries and higher octopamine brain levels (Table 20A), but there were no differences in the levels of dopamine and serotonin. To test whether the differences in octopamine levels were age related rather than social status, we further compared dominant and low ranked workers of similar age. As in the first replicate octopamine levels were higher in dominant workers irrespective of age (Table 20B), but in this case the bees had similar high developed ovaries. This suggests that the differences in octopamine levels can be attributed to the dominance hierarchy between the workers and not to their age or ovarian development.

Of the three biogenic amines tested, only dopamine brain levels were significantly correlated with ovarian development (Spearman rank correlation, first replicate: $Rho=0.41$, $n = 24$, $P < 0.05$; second replicate, $n = 27$, $Rho=0.42$, $P < 0.05$). In a two-way ANOVA, ovarian development ($P=0.019$) but not dominance ($P=0.17$) or the interaction between the two factors were significant. To better evaluate the relationship between dopamine and ovarian development, we pooled the data for queenright worker in experiments I and II while classifying ovarian development into four categories: a) undeveloped ovaries (largest terminal oocyte $\leq 0.5\text{mm}$), moderately developed ovaries ($0.5 < \text{largest terminal oocyte} \leq 1.0\text{mm}$), developed ovaries ($1.0 < \text{largest terminal oocyte} \leq 2.5 \text{ mm}$), and highly developed ovaries (largest terminal oocyte $> 2.5 \text{ mm}$). Dopamine levels were significantly greater for workers with highly developed ovaries than for workers with reduced or moderately developed ovaries (Fig. 2., ANOVA, $P=0.001$, Fisher's PLSD post hoc test $P<0.05$). we further explored possible age-dependent changes in biogenic amine levels for worker of known age. Age was not correlated with any of the biogenic amine tested (correlation Z-test, $P>0.05$).

Brain biogenic amine levels in functional and virgin queens

Functional queen had highly developed ovaries (mean largest oocyte length: $3.55 \pm 0.11\text{SE}$, $n=10$), whereas the ovaries of virgin queens were undeveloped (0.09 ± 0.09 , $n = 11$; unpaired t-test, $P<0.0001$). Brain biogenic amine levels, however, were not significantly different between the two queen types (unpaired t-test, $P>0.05$; Fig. 30).

Levels of brain biogenic amine were higher for queens than for workers (compare Fig. 2. with Fig.1 and Table 1). This was probably related to their bigger body and brain

size. While worker mean radial cell length (as index for body size) was 20.7 (SE=0.2, n=79), queen mean radial cell length was 29.6 (SE=0.1, n=23), significantly higher (unpaired t-test, df=100, $P<0.0001$).

DISCUSSION

The current report represent the first study of biogenic amines in bumble bees, and among the few performed with social insects other than the honey bee. Our results suggest that biogenic amines might be involved in a complex regulatory system of dominance and reproduction in *B. terrestris*. In queenright colonies, octopamine levels in the brain were associated with dominance status and dopamine levels were correlated with oocyte development. The brain levels of biogenic amines measured for worker *B. terrestris* were comparable to those reported for honey bee workers (Taylor et al., 1992; Harris and Woodring, 1995). In contrast to worker honey bee brain biogenic amine levels in *B. terrestris* do not seem to increased with age. Although octopamine levels in queenright workers increased during the first three days after eclosion, they did not increase any further in older queenright workers (8 to 39 days old).

All our analyses of biogenic amines were done with whole brains, and probably represent averages of the levels that might be differentially produced in the various brain parts. We should therefore consider the results as general trends rather than specific mechanisms or regulatory processes. Dopamine levels seem to be correlated with ovarian development, as judged fro the higher levels in queenright workers that had developed ovaries (largest terminal oocyte length > 2.5 mm). Similar association between elevated brain dopamine levels and ovarian development was reported previously for honey bee workers under queenless conditions (Harris and Woodring, 1995). In *B. terrestris*, the levels of dopamine in young bees (6 and 12 days old) were not different between queenless and queenright. Since at this age the ovaries of the queenless workers are at the initial stage of development, it is unlikely that dopamine is related to the initiation of oogenesis. It is possible therefore that the involvement of dopamine in ovarian development is expressed only at the satges of oocyte maturarion.

The levels octopamine in dominant queenright workers were significantly higher than in their low ranked sisters. This was demonstrated in two independent experiments, using bees from different colonies. In the first experiments we used bees of similar size but with unknown age, and therefore could not separate between the effects of age and social status. For the same reason, we could not determine whether levels of octopamine are associated with ovarian development or a reflection of dominance hierarchy. The study

with bees of known age revealed that ovarian development of dominant and low ranked bees of the same age were similar, yet dominant workers had elevated octopamine levels in the brain. This latter results therefore suggest that the observed differences in levels of octopamine in the brain are related to behavioral differences. We can not exclude the possibility that some dominant workers were defined as "low-ranked" because they did not performed characteristic "dominance" behaviors during observation time. Therefore, the observed differences between dominant and lower ranked workers should be considered as a conservative estimation. The exact role, if any, of octopamine in the establishment of dominance hierarchy is still unclear. This awaits further studies encompassing the measurements of biogenic amine in bee of well defined social status on the one hand, and the effect of externally applied biogenic amine on the development of dominance hierarchy and reproduction on the other hand.

CONCLUDING REMARKS

The aims of this research were to investigate the hormonal and pheromonal processes associated with queen-worker conflict in *B. terrestris*. This species can serve as an excellent model for testing both ultimate theories pertaining to social evolution and proximal mechanisms that were selected by these evolutionary processes. The conflict between the queen and her workers over the production of male progeny plays a central role in the social organization of many social species. However in the highly advanced eusocial species like the honey bee or many of the ants, some of the traits have become fixed and irreversible and it is therefore difficult to create experimental paradigms that will manifest this conflict. In bumble bees, on the other hand, queen-worker conflict erupts overtly also under natural conditions, and can be readily mimicked in the laboratory. This provides a good tool for assessing the proximal mechanisms responsible for maintaining the cohesiveness of the colony before the conflict occurs, and the changes in social organization thereafter.

Our first objective was to elucidate some of the endocrine events that mediate ovarian development, thereby assisting us to probe into the process of worker reproduction. We have reconstructed the radiochemical assay for determining the production of JH by the corpora allata *in vitro* and validated it specifically for bumble bees. We complemented these studies with determination of JH levels in the blood by adapting the chiral specific radioimmunoassay for JH to bumble bees.

The measurements of JH revealed a clear correlation with ovarian development, and therefore could be used as a measure of the initiation of reproduction. Measuring JH rather

than assessing ovarian development has the advantage of being a more direct and explicit measure of the initiation of reproduction in bees. It also shortens the bioassay to three days, rather than the six days needed when using oocyte development as a measure of reproduction. Throughout this project we used both parameters for the assessment of worker reproduction.

The central dogma in the published literature regarding reproductive conflict in *B. terrestris* was that the queen dominates her workers by mixed behavioral and pheromonal means. It was further presumed that the decline in the queen pheromonal output is the direct cause for worker reproduction at the competition phase. We based our working hypotheses according to this dogma. In our laboratory reared colonies the initiation of the competition phase occurred at a predicted stage in colony development, and we were able to manipulate the colonies by relying on this prediction. Our first finding did not support the above dogma. Queens before the competition point or after it were equally as effective in inhibiting JH release or ovarian development in small worker groups. We corroborated these findings by demonstrating that queens effectively inhibited ovarian development in callow workers inserted into intact colonies. These studies also revealed that dominant workers after the competition point can also inhibit their nestmates from reproducing. This inhibition was also demonstrated in small queenless group. Thus we conclude that in a developing *B. terrestris* colony there is a small window in which workers have the opportunity to reproduce. This finding fits nicely with the genetic theories of social evolution. Kin selection theory predicts that if the colony is headed by a single queen and all workers are full sisters (from a singly inseminated queen) they will compete with the queen and among themselves for the parentage of male progeny.

We thus refuted the prevailing hypothesis of “queen deterioration” for explaining the onset of worker reproduction. Instead, we hypothesize that it is the workers that change and become insensitive to the queen signal. In fact, the workers behave in a way that maximize their inclusive fitness. They help the queen to rear workers until the colony is built up, but enter into conflict with her when the reproductive castes are ready to be manufactured. The significant correlation between the onset of queen production and the competition phase support this hypothesis. At the competition phase two events occur: the queen starts to lay haploid eggs, and mutual oophagy occurs. Consequently diploid eggs become scarce, and since larval commitment to worker or queen occurs at the age of 5 days, a queen must be produced at this stage. We do not know yet who controls the fate of the diploid larvae: the queen, the workers, or independent physiological events in the

larvae? However it is in the interest of both workers and the queen for many queens to be produced at this particular time of the colony life cycle.

Another hypothesis that prevails in *B. terrestris* literature is that a queen pheromone exists and that it is produced by the mandibular glands. During this study we investigated the chemistry of all the prominent exocrine glands, including the mandibular glands. We also developed a bioassay to test the activity of the various glands as a queen pheromone. The chemical analyses revealed a plethora of compounds, but we can not assign a function to these at the moment. We were also unable to repeat the finding that the mandibular gland secretion possesses a pheromone that inhibits worker reproduction. While total body extracts or cuticular washes have an inhibitory effect on worker production of JH, none of the glands had such an effect. It is possible that an assembly of glands is involved, or that the queen simply produces a signal stating her presence; the workers then sense the queen presence and the demography of the nest, and behave so as to maximize their own fitness. This hypothesis fits the above described behavioral phenomena and supports a recent hypothesis that the queen pheromone constitutes a queen signal rather than manifest queen control.

Finally, we initiated a study of brain biogenic amine. This important class of biologically active compounds is seemingly involved both in ovarian development and establishment of dominance hierarchy. We do not know the exact roles or mode of action of these substances as yet, but the preliminary results presented in this report set solid ground for further research.

Bumble bees have become an important vector for pollination of greenhouse as well as outdoor crops. As the number of crops that are pollinated by bumble bees increases, the demand for specific colonies is growing. For some of the crops it is desirable to have heavily populated colonies, whereas in others such colonies may pose a disadvantage. Understanding the biology and social behavior of this species will undoubtedly pave the way to improving their management and will meet future demands for custom made colonies at reasonable prices to the grower.

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studies in subjects that are directly linked to this project. We wish to thank them for their important contribution to the success of this project.

DESCRIPTION OF COOPERATION

All the experiments concerning live bees were done in Israel. The RCA was also done in Israel. It was validated last year in the US, and all subsequent assays are accomplished in Israel. The RIA for assessing JH titers in the blood and the determination of brain biogenic amines were done in the laboratory of Gene Robinson in Illinois. The chemical analyses of the exocrine glands were done in collaboration with Prof. Wittko Francke of the University of Hamburg, Germany. Although not part of the project, Prof. Francke was mentioned as a collaborating scientist in the original proposal. The cooperative investigator spent six months on sabbatical in Israel and visited the principle investigator laboratory regularly. He participated in our group discussion, assisted in some of our experiments and participated in writing the papers resulting from the proposed research.

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TABLES AND FIGURES

Table 1. Egg laying rates of queens *Bombus terrestris* during nest development.

Days of observations	Interval (hr.) between two successive egg cells			
	Mean	Std. Deviation	Std. Error	n
Nest 1 6	18.1	7.3	2.5	8
Nest 2 7	20.4	14.8	5.2	8
Nest 3 7	12.2	3.3	0.86	15
Nest 4 4	12.5	2.8	0.98	8
nest 5 6	12.9	5.6	1.7	10
All nests	14.7	7.9	1.1	47

Table 2. Social changes in normally developing *B. terrestris* colonies in the laboratory.
* - Estimated, based on subtracting the appropriate egg to adult developmental time from male or queen observed eclosion date (see text for details).

Event	Number of colonies	Days after first worker eclosion	
		Mean $\pm$ se	Range (min. - max.)
The competition point (CP)	92	24.0 $\pm$ 0.7	7 - 42
First male eclosion	85	40.8 $\pm$ 0.9	20 - 57
First queen eclosion	71	43.1 $\pm$ 0.7	28 - 58
Egg developed into first male *	85	14.1 $\pm$ 0.7	-3 - 27
Egg developed into first queen *	71	13.2 $\pm$ 0.6	2 - 24

Table 3. PH, osmolarity, and ion concentrations in haemolymph *B. terrestris* females

	pH		osmolarity		K		Na	
	mean	n	mean $\pm$ sd	n	mean $\pm$ sd	n	mean $\pm$ sd	n
workers	6.79	17	484.7 $\pm$ 32.6	17	21.96 $\pm$ 1.96	6	32.61 $\pm$ 8.2	6
Queens	6.69	17	481.9 $\pm$ 19.7	18	18.72 $\pm$ 0.83	4	22.08 $\pm$ 2.5	4

Table 4. 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 $\pm$ SD	
	Queen	Worker	Queen	Worker
1	7.8	6.8	0.710 $\pm$ 0.030 (23)	0.703 $\pm$ 0.024 (51)
2	64.0	39.5	0.963 $\pm$ 0.038 (121)	0.959 $\pm$ 0.026 (67)
3	256.2	95.8	1.336 $\pm$ 0.050 (91)	1.276 $\pm$ 0.044 (40)
4	1291.0	375.3	1.933 $\pm$ 0.068 (31)	1.669 $\pm$ 0.061 (41)

Table 5. Ovary development (mean $\pm$ SE) of 14 -day old workers that were reared with different queens or queenless. Pre-CPh and CPh queens are queens before the competition phase and queens at the competition phase respectively.

Group	n	Terminal oocyte length (mm)	Number oocytes with absorption signs
Queenless	24	1.63 $\pm$ 0.21	2.5 $\pm$ 0.49
Virgin queens	10	1.22 $\pm$ 0.38	2.1 $\pm$ 0.84
Pre-CPh queens	15	0.81 $\pm$ 0.21	1.07 $\pm$ 0.55
Cph queens	15	1.37 $\pm$ 0.26	2.33 $\pm$ 0.66

Table 6. Rates of JH biosynthesis in vitro, JH titers and index of ovarian development (mean terminal oocyte length) in egg-laying and non-egg-laying workers obtained from the same colonies (mean $\pm$ SE, sample size in parentheses). The last two rows summarized statistical analyses (Mann-Whitney test for JH titers and ovarian development analyses, Unpaired t-test for JH biosynthesis rates).

Behavioral definition	JH biosynthesis rates (pmol/ h/ pair)	Haemolymph JH titer (ng/ ml)	Mean terminal oocyte length (mm)
Egg-laying workers	34.13 $\pm$ 3.22 (32)	394.6 $\pm$ 81.4 (17)	2.7 $\pm$ 0.06 (39)
Non-egg-laying workers	22.57 $\pm$ 2.45 (30)	149.7 $\pm$ 40.6 (15)	1.33 $\pm$ 0.17 (38)
Significance	P= 0.006	P= 0.003	P < 0.0001

Table 7. JH titers and biosynthesis rates in queens (mean $\pm$ SE, sample size in parentheses). Pre-CPh - colony before the phase of worker reproduction (the competition phase), CPh- colonies during the competition phase. The last two rows summarized statistical analyses (ANOVA for JH biosynthesis rates, Mann-Whitney test for JH titers).

Queen type	JH biosynthesis rates (pmol/h/pair)	Haemolymph JH titer (ng/ ml)
Pre-CPh functional queens	16.8 $\pm$ 2.3 (16)	181.9 $\pm$ 46.7 (14)
CPh functional queens	18.8 $\pm$ 3.2 (21)	279.6 $\pm$ 46.5 (22)
Virgin queens	10.8 $\pm$ 1.65 (16)	Not determined
Significance	P= 0.09	P= 0.18

Table 8. The relationships between rates of JH biosynthesis in vitro, JH titers and ovarian development in *B. terrestris* workers. The table summarized linear regression analysis based on the a-priori assumptions that JH titer and ovarian development depend on JH biosynthesis rates and that ovarian development depends on JH titers. Data for JH titers and ovarian development were analyzed after Log transformation. Significance of the variance explain by linear regression: *, P<0.05; **, P<0.01; ***, P<0.001; ns, not significant.

Correlation	All workers	queenright	queenless	0 to 3 day old	6 day old
JH biosynthesis - JH titers	r ² =0.04 n=151, *	r=0.007 n=97, ns	r=0.075 n=40, ns	r=0.07 n=97, ***	r=0.0001 n=23, ns
JH biosynthesis - ovarian development	r=0.013 n=156, ns	r=0.0001 n=50, ns	r=0.12 n=49, *	r=0.07 n=55, ns	r=0.4 n=25, ***
JH titers - ovarian development	r=0.25 n=95, ***	r=0.51 n=42, ***	r=0.22 n=38, ***	r=0.1 n=39, *	r=0.14 n=26, ns

Table 9. Worker performing oviposition behavior (mean $\pm$ SE, sample size in parentheses) in colonies before and after queen removal. Different letter indicate statistically significant different (Wilcoxon sign test, P < 0.05).

Days to queen removal	-2	-1	1	2
Oviposition/ 2h	8.1 $\pm$ 1.55 (10) ab	5.64 $\pm$ 1.38 (11) b	8.82 $\pm$ 1.81 (11) a	8.25 $\pm$ 1.92 (10) a

Table 10. Ovarian development of 7-day-old and JH biosynthesis rates of 3-day-old workers introduced into colonies at different stages of development. Different letters indicate significant differences in the post hoc test (ovarian development: Kruskal-Wallis test, $P < 0.0001$, Siegel post hoc test, $P < 0.001$; JH biosynthesis rates: ANOVA, $P = 0.027$, Fisher PLSD, $P < 0.05$).

Stage of introduction	Time and social state of the recipient colony	Terminal oocyte length (mm)		JH biosynthesis rates (pmol/h/pair)	
		n	mean $\pm$ SE	n	mean $\pm$ SE
Stage I	More than 2 weeks BCP	82	0.50 $\pm$ 0.05 a	15	29.02 $\pm$ 2.56 a
Stage II	Less than 2 weeks BCP	176	0.76 $\pm$ 0.05 b	41	26.90 $\pm$ 2.02 a
Stage III	After CP	165	0.55 $\pm$ 0.05 a	25	19.62 $\pm$ 2.39 b

Table 11. Ovarian development of 7-day-old workers and egg cups constructed in mixed queenless groups of callow and dominant workers (mean $\pm$ SE). Different letters indicate significant difference in the post hoc test.

Number of dominant workers in group		Ovarian development in the callow workers		Total number of egg cups constructed
	n	Terminal oocyte length (mm)	Chorionated oocytes number	
3	9	0.49 $\pm$ 0.11 a	0 ab	2.11 $\pm$ 0.68
1	21	0.86 $\pm$ 0.18 a	0.87 $\pm$ 0.19 a	2.43 $\pm$ 0.81
0	28	2.32 $\pm$ 0.17 b	1.36 $\pm$ 1.69 b	2.29 $\pm$ 0.52
Kruskal-Wallis test		$P < 0.0001$	$P = 0.0005$	$P = 0.93$
Post hoc test		$P < 0.0001$	$P < 0.05$	NS

Table 12. List of hydrocarbons identified in exocrine glands of *Bombus terrestris*.
M-: Mandibular gland; H-: Hypopharyngeal gland; L-: Labial gland; T-: Tarsal gland; D-:
Dufour's gland; ****: major; ***: medium; **: minor; *: trace

A. Alkanes	M	H	L	T	D
Dodecane					*
Tridecane					*
Tetradecane		*			*
Pentadecane		*	*		*
Hexadecane	*	*			*
Heptadecane		*	*		*
Octadecane	*	*	*	*	*
Nonadecane	*	*		*	*
Eicosane	*	*	**	*	*
Heneicosane	***	**	**	***	***
Docosane	**	***	**	**	**
Tricosane	***	***	***	****	***
Tetracosane	**	***	**	**	**
Pentacosane	***	***	**	****	***
Hexacosane	**	**	**	***	**
Heptacosane	***	***	***	****	****
Octacosane	**	**	**	***	**
Nonacosane	***	***	***	****	****
triacontane	**	**	**	**	**
Hentriacontane	*	***	***	***	***
Dotriacontane	*	*	**	**	
Tritriacontane	*	*	*	*	
Tetratriacontane	*	*	*	*	
Pentatriacontane		*	*	*	
Hexatriacontane		*	*	*	
Heptatriacontane		*			
B. Alkenes					
1-Decene	*	*	*		
2-Decene	*	*	*		
1-Tridecene		*			
4-Tridecene		*			
Tetradecene		*			
Pentadecene					*
5-Pentadecene			*		
4-Hexadecene					*
5-Hexadecene			*		
6-Hexadecene					*
7-Heptadecene					*
8-Heptadecene					*
8-Nonadecene			*		*
9-Nonadecene		*	*		*
8-Eicosene					*
9-Eicosene					*
10-Eicosene					*
1-Heneicosene					*

	M	H	L	T	D
7-Heneicosene	*	*	*	*	*
8-Heneicosene				*	*
9-Heneicosene	*		*	*	*
10-Heneicosene			*	*	*
8-Docosene					*
9-Docosene				*	*
10-Docosene					*
11-Docosene					*
5-Tricosene		*		*	*
7-Tricosene	*	*	**	*	***
8-Tricosene		*			*
9-Tricosene	**	**	**	**	***
10-Tricosene		*	*	*	**
11-Tricosene		*	*	*	**
7-Tetracosene					**
9-Tetracosene				*	**
10-Tetracosene				*	*
11-Tetracosene				*	*
12-Tetracosene					*
5-Pentacosene	*	*		*	*
7-Pentacosene	**	**	**	**	***
8-Pentacosene	*	*	*	*	*
9-Pentacosene	**	**	**	**	***
10-Pentacosene	**	**	**	*	***
11-Pentacosene	**	**	**	*	***
12-Pentacosene	**	**	**	*	***
5-Hexacosene		*	*	*	*
7-Hexacosene	*	*	*	**	***
9-Hexacosene	*	*	*	**	***
10-Hexacosene		*	*	**	**
11-Hexacosene	*	*	*	**	***
12-Hexacosene	*	*	*	*	**
13-Hexacosene		*	*	**	***
5-Heptacosene	**	*	**	**	**
7-Heptacosene	***	***	***	****	****
8-Heptacosene	**	**	**		***
9-Heptacosene	***	***	***	****	****
10-Heptacosene	**	***	***	****	****
11-Heptacosene	**	**	**	****	***
12-Heptacosene	***	***	***	****	****
13-Heptacosene	***	***	***	****	****
5-Octacosene					**
7-Octacosene		*	**	**	****
8-Octacosene			*	*	
9-Octacosene	*	*	**	**	****

	M	H	L	T	D
10-Octacosene	*	*	*	*	**
11-Octacosene	*	**	*	*	***
12-Octacosene	*	*	**	*	**
13-Octacosene	*	**	**	*	***
14-Octacosene	*	*	**	*	**
5-Nonacosene	*	**	**	***	***
7-Nonacosene	***	***	***	****	****
8-Nonacosene			**	***	***
9-Nonacosene	***	***	***	****	****
10-Nonacosene	**	**	**	***	****
11-Nonacosene	***	**	**	***	****
12-Nonacosene	**	**	**	***	***
13-Nonacosene	***	**	**	***	****
14-Nonacosene	**	**	**	***	****
B. Alkenes					
7-Triacontene			*	**	
9-Triacontene			*	**	**
10-Triacontene					*
11-Triacontene					*
12-Triacontene					*
13-Triacontene					*
14-Triacontene					*
15-Triacontene					*
5-Hentriacontene			**	**	
7-Hentriacontene		*	**	***	***
8-Hentriacontene			***		
9-Hentriacontene	*	**	**	***	****
10-Hentriacontene	*	*	**	**	**
11-Hentriacontene	*	**	***	***	***
12-Hentriacontene	*	*	**	**	**
13-Hentriacontene	*	*	**	**	**
14-Hentriacontene	*	*	**	**	**
15-Hentriacontene	*	*	**	**	**
Dotriacontene	*				
Trtriacontene					*
9-Trtriacontene			*	*	
10-Trtriacontene			*	*	
Pentatriacontene					*
C. Alkadienes					
Heneicosadiene				*	
Tricosadiene				*	*
Pentacosadiene				*	
7,17-Pentacosadiene					*
8,16-Pentacosadiene					**
7,15-Hexacosadiene			*		*
7,17-Hexacosadiene				**	*
Heptacosadiene		**			
5,13-Heptacosadiene					*
5,19-Heptacosadiene					*
7,15-Heptacosadiene	*			**	**
7,17-Heptacosadiene			**		***
7,19-Heptacosadiene				**	***

	M	H	L	T	D
8,16-Heptacosadiene	*		**	**	***
8,18-Heptacosadiene	*			**	**
9,15-Heptacosadiene	*				
9,17-Heptacosadiene	*		**	**	***
Octacosadiene			*		
7,19-Octacosadiene				*	**
9,15-Octacosadiene					*
9,17-Octacosadiene				*	***
9,19-Octacosadiene				*	**
Nonacosadiene		**			
5,19-Nonacosadiene			*		*
5,21-Nonacosadiene					*
7,15-Nonacosadiene				*	*
7,17-Nonacosadiene					**
7,19-Nonacosadiene	***		***	***	****
7,21-Nonacosadiene			**	**	
8,16-Nonacosadiene					**
8,18-Nonacosadiene	***		***	**	***
8,20-Nonacosadiene				***	***
9,15-Nonacosadiene			***		
9,17-Nonacosadiene			***	***	****
9,19-Nonacosadiene	***		***	***	****
10,16-Nonacosadiene			***		
10,18-Nonacosadiene				**	***
9,17-Triacontadiene					*
9,19-Triacontadiene					*
11,17-Triacontadiene					*
11,19-Triacontadiene					*
Hentriacontadiene		*			
7,19-Hentriacontadiene					**
8,20-Hentriacontadiene					**
8,22-Hentriacontadiene				**	**
9,17-Hentriacontadiene				*	**
9,19-Hentriacontadiene					**
9,21-Hentriacontadiene				**	**
10,18-Hentriacontadiene				*	**
10,20-Hentriacontadiene			***		***
11,19-Hentriacontadiene			***	**	***
Trtriacontadiene			*		*
D. Methylalkanes					
3-Methyl pentadecane		*			
4-Methyl pentadecane		*			
4-Methyl heptadecane					*
5-Methyl heptadecane					*

	M	H	L	T	D
3-Methyl nonadecane					*
9-Methyl heneicosane	***	*			
11-Methyl heneicosane	***	*			
9-Methyl tricosane					*
11-Methyl tricosane					*
11-Methyl pentacosane					*
13-Methyl pentacosane			*		*

	M	H	L	T	D
3-Methyl heptacosane				*	
11-Methyl nonacosane					**
13-Methyl nonacosane	**				**
15-Methyl nonacosane	**				

Table 13: List of alcohols and carbonyl compounds found in exocrine glands of *Bombus terrestris*. M-: Mandibular gland; H-: Hypopharyngeal gland; L-: Labial gland; T-: Tarsal gland; D-: Dufour's gland; ****: major; ***: medium; **: minor; *: trace

Alcohols	M	H	L	T	D	Undecan-2-ol	*				
9-Decenol			*			Ketones	M	H	L	T	D
Decanol			*		*	2-Heptanone	*				
9-Dodecenol			**			2-Nonanone	**	*			
Dodecanol		*	**			2-Decanone	*				
Tetradecanol		*	*	*		2-Undecanone	*	*			
Hexadecanol		*		*		2-Tridecanone	*				*
Octadecanol		*		*	*	2-Tricosanone			*	*	
Eicosanol			*	*		2-Pentacosanone			*	*	
Docosanol				*		2-Heptacosanone			*	*	
Tetracosanol				*		Aldehydes					
Hexacosanol				*		Nonanal		*			
Octacosanol				*		Decanal		*			
Triacontanol				*		Dodecanal			*		
Dotriacontanol				*		Tetradecanal	*		*		
Nonan-2-ol	*	*				Octadecanal					*
Decan-2-ol	*	*									

Table 14. List of terpene and esters and miscellaneous compounds found in exocrine glands of *Bombus terrestris*. M-: Mandibular gland; H-: Hypopharyngeal gland; L-: Labial gland; T-: Tarsal gland; D-: Dufour's gland; ****: major; ***: medium; **: minor; *: trace

Terpene esters	M	H	L	T	D
Geranylcitronellyl decanoate					*
Geranylcitronellyl dodecanoate	*		*		*
Geranylcitronellyl tetradecanoate			*		*
Geranylcitronellyl tetradecanoate			*		
Geranylcitronellyl hexadecanoate			*		*
Geranylcitronellyl hexadecanoate			*		*
Geranylcitronellyl octadecatrienoate					*
Geranylcitronellyl octadecanoate					*

Misc. Compounds					
Limonene		*	*		
Squalene	***	***			**
Geranylcitronellol					*
2-Ethyl-7-methyl-1,6-dioxaspiro-[4.5]-decane	*				
mass / base peak					
398/314 (Steroid?)	***				*
400/43 (Steroid?)	*				
400/95 (Steroid?)	*				
412/55 (Steroid?)	*				
414/43 (Steroid?)	*				
414/382 (Steroid?)	*				
428/396 (Steroid?)	*				

Table 15: List of acids and esters found in exocrine glands of *Bombus terrestris*. M-: Mandibular gland; H-: Hypopharyngeal gland; L-: Labial gland; T-: Tarsal gland; D-: Dufour's gland; ****: major; ***: medium; **: minor; *: trace

Acids	M	H	L	T	D
Hexanoic acid	*	*	*	*	
Heptanoic acid				*	
7-Octenoic acid			*		
Octanoic acid	*	*	*	*	*
Nonanoic acid		*		*	*
Decenoic acid					*
7-Decenoic acid			*		
9-Decenoic acid			*		
Decanoic acid	*	*	*	*	*
Undecenoic acid					*
Undecanoic acid				*	*
7-Dodecenoic acid					*
9-Dodecenoic acid			**		
11-Dodecenoic acid			*		
Dodecanoic acid	**	*	**	*	*
Tridecanoic acid	*			*	*
5-Tetradecenoic acid					*
6-Tetradecenoic acid	*				*
7-Tetradecenoic acid					*
9-Tetradecenoic acid		*		*	*
11-Tetradecenoic acid					*
Tetradecanoic acid	***	*	*	**	*
Pentadecenoic acid	*				

	M	H	L	T	D
Pentadecanoic acid	*	*	*	*	*
6-Hexadecenoic acid	**				*
7-Hexadecenoic acid	**	*	**		*
9-Hexadecenoic acid	**	**	**	**	*
11-Hexadecenoic acid	**	*	**	**	*
13-Hexadecenoic acid	*				
Hexadecanoic acid	***	**	*	***	*
6-Heptadecenoic acid	*				
8-Heptadecenoic acid	*				
Heptadecanoic acid	*		*	*	*
Octadecatrienoic acid	*	*			*
9,12-Octadecadienoic acid	***	*		***	*
8-Octadecenoic acid	*				
9-Octadecenoic acid	****	****	****	****	*
11-Octadecenoic acid	***	****	****		*
13-Octadecenoic acid	*				*
Octadecanoic acid	****	**	****	***	*
Nonadecanoic acid	*			*	
9-Eicosenoic acid					*
11-Eicosenoic acid	*			**	*
13-Eicosenoic acid				*	*
Eicosanoic acid	*		*	**	*
Heneicosanoic acid	*			*	

	M	H	L	T	D					
Docosanoic acid	*			*	*	Ethyl tetradec-11-enoate			**	
Tricosenoic acid	*					Ethyl tetradecanoate			**	*
Tricosanoic acid	*			*		Ethyl hexadec-7-enoate			**	*
Tetracosanoic acid	*			*		Ethyl hexadec-9-enoate			**	*
Pentacosanoic acid				*		Ethyl hexadec-11-enoate	*		***	*
Hexacosanoic acid	*			*		Ethyl hexadec-13-enoate			**	
Heptacosanoic acid	*			*		Ethyl hexadecanoate	*		***	*
Octacosanoic acid	*			*	*	Ethyl octadecatrienoate				*
Nonacosanoic acid	*			*		Ethyl octadeca-9-12-dienoate				*
Triacontanoic acid	*			*		Ethyl octadec-9-enoate		*	***	***
Dotriacontanoic acid				*		Ethyl octadec-11-enoate	*	*	***	*
4-Me-tetradecanoic acid	*					Ethyl octadec-13-enoate			*	*
8-Me-tetradecanoic acid	*					Ethyl octadec-15-enoate			*	
12-Me-tetradecanoic acid	*					Ethyl octadecanoate			**	**
10-Me-pentadecenoic acid	*					Ethyl eicosanoate				*
10-Me-pentadecanoic acid	*					2-Propyl esters				
12-Me-pentadecanoic acid	*					2-Propyl dodecanoate			*	*
4-Me-hexadecanoic acid	*					2-Propyl tetradecanoate	**	**	*	*
8-Me-hexadecanoic acid	*					2-Propyl hexadec-11-enoate			*	
8-Me-octadecanoic acid	*					2-Propyl hexadecanoate	*		*	*
Methyl esters						2-Propyl octadec-9-enoate			*	*
Methyl octanoate		*	*			2-Propyl octadecanoate				*
Methyl dec-9-enoate			*			Acetates				
Methyl decanoate			**		*	Dodecyl acetate			*	
Methyl dodec-9-enoate			*			Octadecyl acetate				*
Methyl dodecanoate		*	**		*	Docosyl acetate				*
Methyl tetradec-5-enoate					*	Tetracosyl acetate			***	
Methyl tetradec-6-enoate					*	Hexacosyl acetate			***	
Methyl tetradec-7-enoate					*	Octacosyl acetate			*	
Methyl tetradec-9-enoate		*			*	Triacetyl acetate			*	
Methyl tetradec-11-enoate			*			Nonadec-10-en-2-yl acetate				*
Methyl tetradecanoate		*	**	*	*	Nonadec-12-en-2-yl acetate				*
Methyl hexadec-6-enoate	*				*	Heneicosen-2-yl acetate				*
Methyl hexadec-7-enoate	*	*	**		*	Heneicos-2-yl acetate				*
Methyl hexadec-9-enoate	*	*	*		*	Tricosen-2-yl acetate				*
Methyl hexadec-11-enoate	*	*	***	*	*	Tricos-2-yl acetate				*
Methyl hexadec-13-enoate	*		**	*		Pentacosen-2-yl acetate				*
Methyl hexadecanoate	*	*	***	*	**	Pentacos-2-yl acetate				*
Methyl octadecatrienoate			*		*	Heptacos-9-en-2-yl acetate		*	*	*
Methyl linoleate		*	*		*	Heptacos-2-yl acetate				*
Methyl octadec-9-enoate	*	*	****	*	****	Nonacosadien-2-yl acetate				*
Methyl octadec-11-enoate	*	*	****	*	****	Nonacos-9-en-2-yl acetate		*	*	**
Methyl octadec-13-enoate	*		**	*		Nonacos-2-yl acetate				*
Methyl octadecanoate			***	*	***	Butyrates				
Methyl eicosanoate					*	Decyl butyrate			*	*
Ethyl esters						Dodec-9-enyl butyrate			*	
Ethyl octanoate	*		*			Dodecyl butyrate			***	*
Ethyl decenoate			*			Tetradecyl butyrate			*	
Ethyl decanoate	*		*		*	Hexanoates				
Ethyl dodecadienoate			*			Dec-9-enyl hexanoate			*	
Ethyl dodecenoate			*			Decyl hexenoate			*	
Ethyl dodecanoate			**		*	Decyl hexanoate	*		*	*

	M	H	L	T	D		M	H	L	T	D
Dodecenyl hexanoate					*	Dodecyl hexadec-11-enoate	***	**	****	**	
Dodec-9-enyl hexanoate			*			Dodecyl hexadec-13-enoate			*		
Dodec-11-enyl hexanoate			*			Dodecyl hexadecanoate			****	*	**
Dodecyl hexanoate	*	*	****	*	*	Dodecyl octadecatrienoate	*		***		
Tetradecyl hexanoate			*			Dodecyl octadecadienoate	*		***		*
Wax type esters						Dodecyl octadec-9-enoate	**	**	***	**	**
Octyl dodecanoate			*			Dodecyl octadec-11-enoate	***	**	****	**	**
Octyl octadec-9-enoate				*		Dodecyl octadec-13-enoate			**		
Octyl octadec-11-enoate			*	*		Dodecyl octadecanoate					*
Dec-9-enyl decanoate			*			Dodecyl eicosenoate	*				*
Decenyl hexadecanoate	*					Dodecyl eicos-13-enoate			**		
Decyl octanoate	*		**		*	Tetradecyl octadecenoate	*				
Decyl dec-9-enoate			*			Tetradecyl octadec-9-enoate			*		*
Decyl decanoate	*		**			Tetradecyl octadec-11-enoate			*		*
Decyl dodecanoate	*		**		**						
Decyl tetradecanoate	***		**		*	Tetradecyl octadec-13-enoate			*		
Decyl hexadec-11-enoate			*								
Decyl hexadecanoate	*					Tetradecyl octadecanoate					*
Decyl octadec-9-enoate	**	*	*	*	***	Hexadecenyl octadecenoate			*		*
Decyl octadec-11-enoate			*		**	Hexadecyl dodecanoate					*
Dodec-9-enyl octanoate			*			Hexadecyl hexadecenoate	*				
Dodec-9-enyl decanoate			*			Hexadecyl octadienoate					*
Dodec-9-enyl dodecanoate			**			Hexadecyl octadecenoate				*	*
Dodec-9-enyl tetradecanoate			*			Hexadecyl octadec-9-enoate			*		**
Dodec-9-enyl tetradecanoate			*			Hexadecyl octadec-11-enoate			*		**
Dodecenyl hexadecadienoate			*			Hexadecyl octadecanoate			*		*
Dodecenyl hexadecenoate	*					Octadecenyl octadecatrienoate					*
Dodecenyl octadecadienoate	*		***		*	Octadecenyl octadecenoate					*
Dodecenyl octadecenoate			*			Octadecyl dodecanoate					*
Dodecyl octanoate	*		***	*	*	Octadecyl tetradecanoate					*
Dodecyl decenoate				*	**	Octadecyl hexadecanoate				*	*
Dodecyl dec-7-enoate			*			Octadecyl octadec-9-enoate					*
Dodecyl dec-9-enoate			*			Octadecyl octadec-11-enoate					*
Dodecyl decanoate	*		***		**	Octadecyl octadecanoate					*
Dodecyl dodec-9-enoate			*			Eicosyl tetradecanoate				*	
Dodecyl dodec-11-enoate			*			Eicosyl octadecenoate				*	*
Dodecyl dodecanoate	***		****	*	*	Eicosyl octadec-9-enoate			*		
Dodecyl tetradec-9-enoate			*								
Dodecyl tetradec-11-enoate	*		***	*							
Dodecyl tetradecanoate	*		****	*	*						
Dodecyl hexadecadienoate	*		***		*						
Dodecyl hexadecenoate					**						
Dodecyl hexadec-7-enoate			*								
Dodecyl hexadec-9-enoate			*								

Table 16. List of oxygenated carboxylic acids found in the exocrine glands of *Bombus terrestris*. M-: Mandibular gland; H-: Hypopharyngeal gland; T-: Tarsal gland; ****: major; ***: medium; **: minor; *: trace

OXYGENATED ACIDS	M	H	T
2-Hydroxypropionic acid	*		
3-Hydroxyhexanoic acid	*		
3-Hydroxyoctanoic acid	***		
3-Hydroxydecanoic acid	****	*	
3-Hydroxydodecanoic acid	*		
3-Hydroxydodecanoic acid	***		
3-Hydroxytetradecanoic acid	*		
3-Hydroxytetradecanoic acid	*		
11-Hydroxydodecanoic acid			*
12-Hydroxytetradecanoic acid			*
13-Hydroxytetradec-5-enoic acid			*
13-Hydroxytetradec-7-enoic acid			*
14-Hydroxyhexadecanoic acid			*
15-Hydroxyhexadecanoic acid			*
15-Hydroxyhexadec-7-enoic acid			*
15-Hydroxyhexadec-9-enoic acid			*
17-Hydroxyoctadec-9-enoic acid			*
3-Butyroxooctanoic acid	**		
3-Butyroxodecanoic acid	***		
3-Butyroxododecanoic acid	*		

Table 17. Rates of *in vitro* JH biosynthesis and frequencies of threatening behaviour and overt aggression in 3-days-old workers exposed daily to 1 Qeq total body extract or to the solvent. JH biosynthesis rates are expressed by two different indices: index-1 - all workers, index-2 - workers with the highest rates from each experimental group. Statistical tests included unpaired t-test for JH biosynthesis rate data and mann-whitney test for behavioural data.

Extract	JH biosynthesis rate (pmol/h/pair) mean $\pm$ se (n)		Threatening behaviour (events/ h) mean $\pm$ se (n)	Overt aggression (events/h) mean $\pm$ se (n)
	Index-1	Index-2		
Solvent	47.3 $\pm$ 3.5 (27)	62.8 $\pm$ 4.5 (9)	11.4 $\pm$ 3.6 (15)	1.4 $\pm$ 0.7 (15)
Queen's total body	38.9 $\pm$ 2.4 (45)	51.3 $\pm$ 3.3 (15)	5.2 $\pm$ 1.6 (17)	1.2 $\pm$ 1.0 (17)
Statistic significance	P= 0.049	P= 0.046	P= 0.32	P= 0.5

Table 18. Rates of *in vitro* JH biosynthesis by CA of 3-day-old queenless worker exposed daily to queen glandular extracts on different dummies (mean $\pm$ SE). Index-1 and Index-2 as as described in Table 1. MG - mandibular glands, HPG - Hypopharyngeal glands, SG - Head salivary glands, DG - Dufour's gland, TG - Tarsal glands. For description of the different types of dummies see Material and Methods

Extract	Oven dried dummies		Soxhelt washed dummies	
	Index I	Index II	Index I	Index II
Solvent	34.6 $\pm$ 5.3 (15)	59.2 $\pm$ 5.85 (5)	48.1 $\pm$ 3.9 (24)	62.7 $\pm$ 5.0 (8)
MG	33.0 $\pm$ 3.1 (24)	41.4 $\pm$ 4.9 (8)	49.1 $\pm$ 3.4 (17)	59.4 $\pm$ 4.7 (6)
HPG	38.7 $\pm$ 2.6 (24)	49.3 $\pm$ 3.6 (8)	41.5 $\pm$ 4.6 (18)	57.2 $\pm$ 6.25 (6)
SG	34.7 $\pm$ 4.6 (24)	52.5 $\pm$ 7.7 (8)	35.3 $\pm$ 5.0 (21)	58.2 $\pm$ 8.1 (7)
DG	38.9 $\pm$ 5.4 (15)	56.8 $\pm$ 10.2 (5)	36.5 $\pm$ 3.9 (21)	50.5 $\pm$ 4.9 (7)
TG	49.6 $\pm$ 6.3 (12)	63.2 $\pm$ 9.7 (4)	42.3 $\pm$ 4.3 (27)	60.8 $\pm$ 6.8 (9)

Table 19. Rates of *in vitro* JH biosynthesis (mean $\pm$ SE) in CA of three-days-old workers exposed daily to 1 queen equivalent mandibular glands (MG) extract or to solvent. Extracts were applied on glass slides or on Petri dishes covered with wax. Index-1 and Index-2 are as described in Table 1.

Experiment	Solvent		Queen's MG extract	
	Index-1	Index-2	Index-1	Index-2
Glass slide	65.3 $\pm$ 9.9 (9)	89.3 $\pm$ 12.7 (3)	49.9 $\pm$ 11.5 (9)	89.7 $\pm$ 14.9 (3)
Petri dish	37.2 $\pm$ 3.3 (42)	56.7 $\pm$ 3.8 (12)	35.25 $\pm$ 3.22 (36)	57.2 $\pm$ 4.0 (11)

Table 20. Total brain biogenic amine levels, size, age and ovarian development in dominant and lower ranked queenright workers from colonies during the competition phase (mean $\pm$ SE, sample size in parentheses). A. First repetition, worker of unknown age. B. Second repetition, worker of known age. The last row outline the results of statistical analyses (A. unpaired t-tests, Mann-Whitney test for oocyte length; B. paired t-test, Wilcoxon sign test for oocyte length measurements).

A. Workers of unknown age						
Dominance position	Dopamine (pg/ brain)	Serotonin (pg/ brain)	Octopamine (pg/ brain)	Largest oocyte length (mm)	wing length (mm)	Age (days)
High	1685.3 $\pm$ 203.0 (15)	367.6 $\pm$ 50.9 (15)	834.9 $\pm$ 39.6 (15)	2.85 $\pm$ 0.22 (15)	21.5 $\pm$ 0.5 (14)	--
Low	1598.1 $\pm$ 175.8 (15)	367.9 $\pm$ 49.6 (15)	678.6 $\pm$ 54.3 (15)	1.53 $\pm$ 0.28 (9)	21.0 $\pm$ 0.2 (9)	--
P value	0.75	0.98	0.02 *	0.004 **	0.45	--
B. Workers of known age						
High	1391.3 $\pm$ 203.8 (15)	363.6 $\pm$ 37.3 (15)	719.8 $\pm$ 25.3 (15)	3.02 $\pm$ 0.11 (12)	19.4 $\pm$ 0.36 (14)	19.2 $\pm$ 2.4 (13)
Low	1231 $\pm$ 204.6 (15)	353.2 $\pm$ 44.8 (15)	604.8 $\pm$ 32.9 (15)	2.38 $\pm$ 0.32 (12)	19.8 $\pm$ 0.42 (14)	19.6 $\pm$ 2.7 (13)
P value	0.61	0.76	0.038 *	0.32	0.42	0.57

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

Figure 1b. Age distribution of larvae related to their head capsule size. Note that age heterogeneity increases with development.

Figure 2. Colony development of *Bombus terrestris* in the laboratory. Four colonies which were closely watched are presented.

Figure 3. Regression analysis depicting the relationship between: A. Estimated switch point (first haploid egg laid) and the competition point, B. First-queen pupation and the competition point, C. First queen pupation and the switch point.

Figure 4. JH secreted by CA of 6 day old *B. terrestris* queenless workers that were incubated in different incubation media. Different letters indicate statistically significant differences ($P < 0.05$).

Figure 5. JH secreted by CA of 6 day old *B. terrestris* queenless workers that were incubated in different methionine concentrations. Different letters indicate statistically significant differences ($P < 0.05$).

Figure 6. JH secreted by CA of 6 day old *B. terrestris* queenless workers that were incubated with different incubation temperatures. Different letters indicate statistically significant differences ($P < 0.05$).

Figure 7. Total amount of JH secreted by CA of 6 day old queenless workers of *B. terrestris*. Glands were transferred to a new medium after 1, 2, and 3 h, and the incubation was terminated after 6 h. Linearity of synthesis is present for workers with developed (mean oocyte length > 1.5 mm, $n=5$) and undeveloped (mean oocyte length < 1.5 mm, $n=4$) ovaries (statistical analyses in text).

Figure 8. HPLC fractionation of the incubation medium including homogenized CA. The arrows denote the elution times of the three species of JH.

Figure 9. Immunoactivity of HPLC separated adult *B. terrestris* worker hemolymph samples (n=6). Fractions were collected every minute. Immunoactivity was determined using RIA with antibodies against JH III. For comparison, the horizontal open bar represent immunoactivity of JH III sample separated using similar procedure.

Figure 10. Correlation between blood titer of JH and its synthesis by the CA *in vitro* in adult and larva *Bombus terrestris*. Measurements were performed on the same individual.

Figure 11. Regression analysis of log larval weight as a function of larval age. Regression equation for queens larvae: $Y=0.274X-0.95$; for workers larvae: $Y=0.275X-1.134$.

Figure 12. 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)

Figure 13. JH biosynthesis rates as a function of larval development. In each of the four instars larvae were divided into 5 stages (for details see "Results"). Each point represents the mean $\pm$ SE for each developmental stage. The number near each point is the number of CA pairs analyzed.

Figure 14. JH release rate (a) and ovarian development (b) in *B. terrestris* workers that were reared under queenright or queenless conditions. Each point represents the mean ($\pm$ SE) for 8-24 individuals. Asterisk indicate statistically significance ($P < 0.01$).

Figure 15. Regression analysis of ovary development and JH release rates in 6 day old *B. terrestris* queenless workers. $Y=0.03X + 0.42$, $r^2 = 0.35$, $P=0.023$.

Figure 16. JH release rate (mean $\pm$ SE) by CA of 3 day old *B. terrestris* workers reared in different social conditions. QL - a queenless group, VQ - reared with a virgin queen, pre-CPh - reared with a queen from a colony before competition phase. CPh - reared with a queen from a colony during the competition phase. Different letters indicate that means are significantly different ($P < 0.01$).

Figure 17. Threatening behaviour (a) and overt aggression (b) in *B. terrestris* workers reared for 14 days in different social conditions. See figure 4 for details of groups.

Figure 18. JH III titer in the hemolymph of callow *B. terrestris* workers reared under queenless and queenright conditions. Each point represents the mean ($\pm$ SE) of 7 to 14 individuals. * indicate statistically significant difference ($P < 0.05$), # - indicate almost significant different ($P = 0.08$).

Figure 19. The relationship between *in vitro* JH biosynthesis rates and hemolymph titer among means of defined groups of bees. Each point represents the mean $\pm$ SE of 6-55 individuals. C - callow workers less than 12 hours old, QR - queenright, QL - queenless, LW - laying workers, NLW - non laying workers, FQ - functional queens, the numbers indicate the bee age (days). The dash line represents the regression line for groups of bees 0 to 3 days old ($Y=1.02+0.02X$, $r^2=0.94$, $n=6$).

Figure 20. The relationship between hemolymph JH titer and ovarian development among means of defined groups of bees. Each point represents the mean $\pm$ SE of 7 - 15 individuals. C - callow workers less than 12 hours old, QR - queenright, QL - queenless, LW - laying workers, NLW - non laying workers, the numbers indicate the bee age (days). The solid line outlines the regression line for the two variables ($Y=3.2X - 7.27$, $r^2=0.79$, $P<0.001$).

Figure 21. Worker oviposition behavior relative to colony development in *B. terrestris* colonies (n=3). A: worker numbers: continues line = actual number (born minus dead); dash line = cumulative number of egg-laying workers. B: The number (mean $\pm$ se) of workers performing oviposition behavior observed during one hour. CP - mean time to the competition point. R - mean time to first reproductive (males and gynes) eclosion.

Figure 22. Frequency distribution of the ages of workers observed while performing oviposition behavior. Filled part represents the frequencies of workers that were observed ovipositing for the first time.

Figure 23. Frequency distribution of ovarian development of callow workers introduced into colonies at different stages of development. Workers were analysed for ovarian development at the age of seven days. Left column 3A-3C - queenright colonies. Right column 3D-3E- dequeened colonies. Filled bars depict coronated oocytes.

Figure 24. Ovarian development (mean $\pm$ SE) of callow workers introduced into colonies at different stages of development. Filled bars = queenright colonies, open bars = dequeened colonies. Workers were analyzed for ovarian development at the age of seven days. *** statistically significant different ovarian development among queenright and queenless colonies (Mann-Whitney test $P < 0.0001$).

Figure 25. Mass spectra of A) methyl-3-hydroxydecanoic acid and B) Methyl 3-butyroxy decanoate

Figure 26. Rates of *in vitro* JH biosynthesis by CA of 3 day old queenless worker exposed daily to queen cuticular washes or to clean solvent applied on live callow gyne body (mean $\pm$ SE). Dotted bars - Index-1 = all the bees in the experimental groups, filled bars - Index-2 = just the bees with the higher rate from each experimental group. Sample sizes are indicated above each bar. Using both incidences, differences between cuticular wash treatment and the control group were significant (Unpaired t-test, $P < 0.05$).

Figure 27. Rates of *in vitro* JH biosynthesis by CA of 3 day old queenless worker exposed daily to hydroxy acids blend or to clean solvent applied on oven dried dummy and a wax ball (mean $\pm$ SE). Dotted bars - Index-1 = all the bees in the experimental groups, filled bars - Index-2 = just the bees with the higher rate from each experimental group. Sample sizes are indicated above each bar. Using both incidences, differences between cuticular wash treatment and the control group were not significant (Unpaired t-test, $P > 0.05$).

Figure 28. Total biogenic amine brain levels (mean $\pm$ SE, sample size within bars) in zero to three days-old workers developed under queenless (open bars) or queenright (shaded bars) conditions. a. Dopamine (DA), b. Octopamine (OA), c. Serotonin (5-HT).

Figure 29. Dopamine levels (mean $\pm$ SE, sample size within bars) in the brains of *B. terrestris* queenright workers at different categories of ovarian development. Bars with different letters are significantly different (ANOVA, $P=0.001$, Fisher's PLSD post hoc test $P<0.05$).

Figure 30. Brain biogenic amine levels (mean $\pm$ SE) in callow virgin (n=12) and functional (n=11) *B. terrestris* queens. a) Dopamine (DA), b) Octopamine (OA) and c) Serotonin (5-HT).

LIST OF PUBLICATION RESULTING FROM THE GRANT

PUBLISHED

- Hefetz, A., Taghizadeh, T. and Francke W. 1996 The exocrinology of the queen bumble bee *Bombus terrestris* (Hymenoptera: Apidae, Bombini). Z. Naturforsch. 51:409-422.
- Bloch, G., Borst, D.W., Huang, Z.-Y., Robinson, G.E. and Hefetz, A. 1996 Effects of social conditions on JH-mediated reproductive development in *Bombus terrestris* workers. Physiol. Ent. 21: 257-267
- Cnaani, J., Borst, D., Huang, Z.-Y., Robinson, G., and Hefetz, A. 1997 Differences in development and rates of JH biosynthesis between queen and worker larvae in *Bombus terrestris*. J. Insect Physiol. 43: 373-381

SUBMITTED FOR PUBLICATION

- Bloch G, and Hefetz A. Regulation of Reproduction by Dominant Workers in Bumble Bee (*Bombus terrestris*) Queenright Colonies
- Bloch, G and Hefetz, A. A re-evaluation of the role of the mandibular glands in the regulation of reproduction in bumblebee colonies
- Bloch, G. Borst, W.D., Huang, Z.-Y. Robinson, G.E. and Hefetz, A. JH titers, *in vitro* JH biosynthesis rates, ovarian development and social environment in *Bombus terrestris*

IN PREPARATION

- Bloch, G. Simon, T. Robinson, G.E., Hefetz, A. Brain biogenic-amines, dominance and reproduction in the bumble bees *Bombus terrestris*