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RESEARCH ARTICLE

Precocene-I inhibits juvenile hormone biosynthesis, ovarian activation, aggression and alters sterility signal production in bumble bee (*Bombus terrestris*) workers

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ABSTRACT

Juvenile hormone (JH) is an important regulator of development and physiology in insects. While in many insect species, including bumble bees, JH functions as gonadotropin in adults, in some highly eusocial insects its role has shifted to regulate social behavior including division of labor, dominance and aggression. Studying JH functions across social insect species is important for understanding the evolution of sociality; however, these studies have been limited because of the inability to reduce JH levels without surgically removing its glandular source, the corpora allata. Precocene is known to inhibit JH biosynthesis in several non-social insects, but has been poorly studied in social insects. Here, we tested whether precocene-I can effectively reduce JH levels in *Bombus terrestris* workers, and examined its effects on their physiology and behavior. Precocene-I treatment of three-worker groups decreased JH titer and ovarian activation, irrespective of the bees' dominance rank within the group, and was remedied by JH replacement therapy. Precocene-I also decreased aggressiveness and increased ester-sterility signal production; these changes were rank-dependent, and affected mainly the most reproductive and the least aggressive workers, respectively, and could not be remedied by JH replacement therapy. These results clearly confirm the role of JH as a gonadotropin and mediator of aggression in *B. terrestris*, and indicate that JH effects are associated with worker dominance rank. The ability to chemically reduce JH titer provides us with a non-intrusive method to probe the evolutionary changes associated with JH and the hormonal mechanisms that are associated with reproduction and behavior in social insects.

KEY WORDS: Social insects, Hormones, Reproduction, Pheromones, Aggressive behavior, Dominance

INTRODUCTION

Reproductive division of labor is a hallmark of social insects, where only one or a few females reproduce while the others remain sterile, raising both proximate and ultimate questions about the mechanisms regulating reproduction and the origin of sociality. Hormones are thought to play a central role in regulating reproduction in insects, but were hypothesized to undergo several changes during the evolution of sociality (Nijhout, 1994). Thus, investigating the roles of hormones in social insects, and especially in primitively eusocial species, can not only contribute to our understanding of questions pertaining to the proximate mechanisms underlying reproduction, but also shed light on the ultimate mechanisms leading to the transition from solitary to social life.

Juvenile hormone (JH) is a principal regulator of physiological processes in insects, exerting a wide variety of functions during the individual's life cycle. In pre-adult stages it regulates developmental processes and metamorphosis, while in adult females it induces vitellogenesis (the production of the major yolk protein) and reproduction (Nijhout and Wheeler, 1982; Robinson and Vargo, 1997; Hartfelder, 2000). In social insects, the hormone has acquired new roles such as regulating aggression and dominance in females of primitively eusocial insects such as bumble bees and wasps, regulating polymorphism and caste determination in isopteran and hymenopteran species, and regulating division of labor and behavioral maturation in the honey bee (Barth et al., 1975; Hartfelder and Engels, 1998; Bloch et al., 2000b; Hartfelder, 2000; Giray et al., 2005; Amsalem et al., 2014). Thus, the functions of JH were hypothesized to have changed during the transition from solitary to social lifestyles in insects.

Manipulation of JH titer is one of the most common methods employed to understand its mode of action and functions in insects (Sláma, 1971; Howard and Haverty, 1979; Ramaseshadri et al., 2012). The existence of several analogs to JH that both mimic the hormone action and are more stable than the hormone has facilitated experimentation aimed at understanding its function (Robinson, 1985; Robinson and Ratnieks, 1987; Schulz et al., 2002). However, in some cases where the levels of JH are already very high, e.g. in queenless groups of social insect workers, reducing JH levels is a much more efficient method to study its effects. While allatectomy [i.e. removal of the corpora allata (CA), a pair of glands attached to the brain where JH is being produced] may provide an insight into JH functions (Shpigler et al., 2014), the surgical trauma involved may in some cases (e.g. behavioral studies) render the interpretation of the results more difficult (Sullivan et al., 2003).

As a primitively eusocial species, the bumble bee *Bombus terrestris* (Linnaeus 1758) is an excellent model system to investigate the functional transition of JH from solitary to social species. During their annual colony cycle, *B. terrestris* colonies display both cooperation and competition over reproduction and exhibit characteristics of both primitively and highly eusocial insects. Initially, the queen goes through a solitary phase after termination of the winter diapause and before establishing a colony, and behaves like a solitary species (Michener, 1974). When the first workers emerge, in accordance with advanced eusociality, the queen is the sole reproducer (Michener, 1974; Duchateau and Velthuis, 1988), there is an extensive use of pheromones (Ayasse and Jarau, 2014) and workers present partial division of labor (Amsalem et al.,

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Received 27 April 2014; Accepted 17 June 2014

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The Journal of Experimental Biology (2014) doi:10.1242/jeb.107250

2013). As the population of the colony increases, the so-called 'competition phase' starts in which there is extensive worker reproduction and aggression (Duchateau and Velthuis, 1988; Duchateau, 1989; Amsalem et al., 2009). In addition, unlike in the honey bee, where JH regulates division of labor and behavioral maturation rather than its traditional role as a gonadotropin (Hartfelder and Engels, 1998; Robinson and Vargo, 1997; Hartfelder, 2000), in *B. terrestris* it retained its original role as a gonadotropin and is positively correlated with reproduction in both queens and workers (Roseler, 1977; Roseler and Roseler, 1988; Van Doorn, 1989; Bloch et al., 2000b). Interestingly, however, vitellogenin (vg), the major yolk protein in the ovaries, which is positively regulated by JH in most insects [but not in the honey bee, some termites and ants (Robinson and Vargo, 1997; Brent et al., 2005; Guidugli et al., 2005; Elliott and Stay, 2008; Amdam and Page, 2010)], appears to have been co-opted to regulate aggressive behavior in queenless *B. terrestris* workers (Amsalem et al., 2014). The interrelationship between JH and aggressive behavior in *B. terrestris* remains equivocal: while some positive correlations were found between JH titer and dominance behavior in workers (Roseler, 1977; Bloch et al., 2000b), a direct treatment with JH-III did not increase aggressive behavior or dominance rank in queenless workers (Van Doorn, 1989; Amsalem et al., 2014).

Thus, although it is clear that there is a link between ovarian activation and aggression in *B. terrestris* (Amsalem and Hefetz, 2010), the role that JH plays in regulating these two parameters remains to be fully determined. Furthermore, the associations between JH, ovarian activation and aggression may be influenced by worker dominance rank under a queenless regime, where reproduction, aggression and pheromone production are not equally distributed among nestmates (Amsalem and Hefetz, 2011).

The discovery of the precocenes by Bowers (Bowers, 1976) provided an interesting alternative to surgical removal of the CA that is seemingly less traumatic. Precocenes-I and -II (7 methoxy-2,2-dimethylchromene and 6,7-dimethoxy-2,2-dimethylchromene, respectively) are chromene derivatives of plant origin (Bowers, 1976; Soderlund et al., 1980; Haunerland and Bowers, 1985) that have been shown to have multiple effects on metamorphosis during the pre-adult stages of different non-social insect species (Nemec et al., 1978; Unnithan and Nair, 1979; Kozhanova and Nemec, 1991; Khan and Kumar, 2000; Khan and Kumar, 2005; Gaur and Kumar, 2009) and on reproduction in adults of several insect orders where they prevent normal vitellogenic development of the oocytes, leading to sterility (Pratt and Bowers, 1977; Deb and Chakravorty, 1982; Bradley and Haynes, 1991; Kozhanova and Nemec, 1991; Kumar and Khan, 2004; Ringo et al., 2005; Amiri et al., 2010). In these non-social insect species, precocenes also affect several aspects of behavior such as aggression (Chen et al., 2005a), mating behavior (Walker, 1978), flight behavior (Rankin, 1980), maternal defensive behavior (Kight, 1998) and sexual behavior (Pathak and Bhandari, 2002; Ringo et al., 2005). In most cases the physiological, but not all the behavioral effects, were reversible by JH replacement therapy (Walker, 1978; Masner et al., 1979; Unnithan and Nair, 1979; Rankin, 1980; Li et al., 1993; Kight, 1998; Pathak and Bhandari, 2002; Chen et al., 2005a). However, despite the plethora of species tested and abundant studies that were conducted using precocenes, only few have directly tested the change in JH titer post treatment (Pratt and Bowers, 1977; Sohn et al., 1991; Chen et al., 2005a).

The studies above showed that precocenes affect multiple targets in insects, but little is known about either the site or the mode of their action. Particularly, it is still unclear whether the effects of precocenes are restricted to the CA (Unnithan et al., 1977; Unnithan and Nair, 1979; Haunerland and Bowers, 1985; Piulachs et al., 1989; Garcera et al., 1991; Hebbalkar and Sharma, 1991; Burks et al., 1992), or are more general due to their toxicity (Kelly and Fuchs, 1978; Farag and Varjas, 1981; Ergen, 2001). In species where precocenes have been shown to reduce JH levels (Pratt and Bowers, 1977; Sohn et al., 1991; Chen et al., 2005a), it has been suggested that they are activated by oxidation to form a highly reactive epoxide that destroys the parenchymal cells of CA by nucleophilic alkylation (Haunerland and Bowers, 1985). In addition to selectively damaging the CA cells, precocene treatment was found to: increase oxygen consumption rate by the ovaries that in turn remain inactivated (Garcera et al., 1989); cause hypertrophy of the fat body (Lee and Tan, 1981); decrease glycogen and protein contents in the fat body (Rup and Bangla, 1995; Amiri et al., 2010); and block the accumulation of fatty acids in the body of the pea aphid, *Acyrthosiphon pisum* (Chen et al., 2005b). However, it is still unclear whether these effects of precocene are direct, or indirect as consequence of the decreased in JH titers.

In contrast to the extensive study of the effects of precocene treatment in solitary insects, only a few studies of its effect have been conducted in social insect species, where JH exerts functions that are not limited to the context of reproduction. In the honey bee, where JH does not function as a gonadotropin but rather regulates behavioral maturation and division of labor (Hartfelder and Engels, 1998; Hartfelder, 2000), precocene-II treatment did not have any effects other than general toxicity and antifeedant activity in adults (Rembold et al., 1979; Fluri, 1983), but did cause atrophy of the CA in queen larvae (Goewie et al., 1978). In the termite *Coptotermes formosanus*, treatment with precocene-I (but not precocene-II) significantly delayed the formation of the first soldier and reduced the proportion of soldiers in the colony (Mao et al., 2010), and in the fire ant *Solenopsis invicta*, precocene-II inhibited dealation of alates in queenless colonies (Burns et al., 2002). To our knowledge, these are the only studies that have been conducted thus far with precocene in social insects.

In the present study we tested the effect of treatments with precocene-I and JH-III on queenless *B. terrestris* workers, examining titers of JH and methyl farnesoate (MF; a precursor to JH), reproduction, aggression and the production of the octyl ester components in Dufour's gland. These esters are present in subordinate, forager workers and advertise their sterility (Amsalem et al., 2009), their position in the hierarchy (Amsalem and Hefetz, 2010) and their foraging efforts (Amsalem et al., 2013). We further investigated whether the social rank of the worker modulates the effects of the treatment. We hypothesized that (1) precocene-I will affect reproduction, aggression and pheromone production by reducing the levels of JH/MF titers, and these effects will be fully or partially reversible after hormone replacement therapy; and (2) the effects of precocene-I will be worker-rank dependent.

RESULTS

Experiment 1: concentration-dependent effect of precocene-I on ovarian activation, mortality, and JH-III and MF titers in *B. terrestris* workers

The concentration-dependent effect of precocene-I on worker ovarian activation is presented in Fig. 1. Treatment had a significant effect on ovarian activation (nested ANOVA, treatment: $F_{3,105}$=67.7, P<0.001 followed by Tukey-type *post hoc* test P<0.02 for untreated and 1.2 mg precocene versus 3 and 6 mg precocene). Ovarian activation differed significantly between the untreated (2.24±0.25 mm, n=19 groups) and 1.2 mg precocene (2.39±0.6 mm, n=3 groups) groups relative to both the 3 mg (0.35±0.14 mm, n=12

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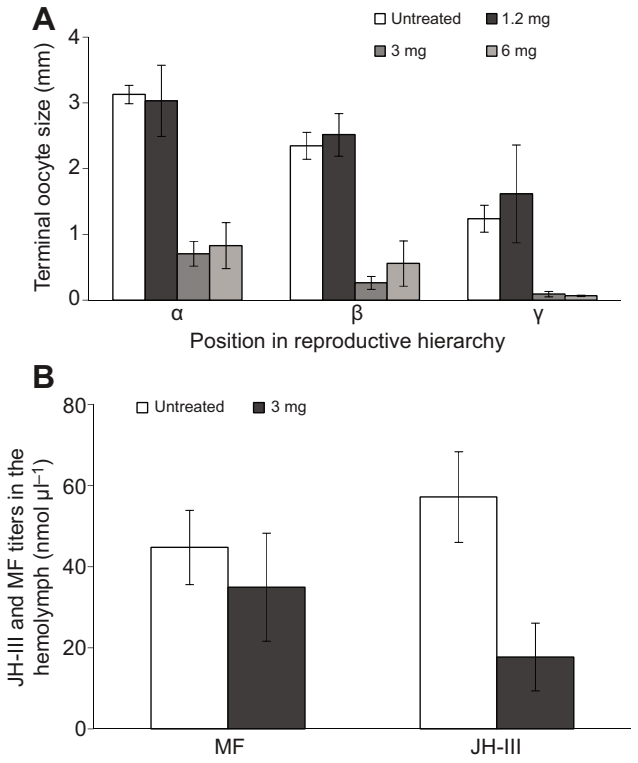

Fig. 1. The effect of different concentrations of precocene-I on ovarian activation, juvenile hormone (JH-III) and methyl farnesoate (MF) titers in *Bombus terrestris* workers. (A) Ovarian activation was measured in three-worker groups that were either untreated (n=57 workers, 19 groups) or fed with precocene-I in concentrations of 1.2 mg (n=9 workers, 3 groups), 3 mg (n=36, 12 groups) or 6 mg (n=15, 5 groups) diluted in their sugar water. Workers in each group were ranked according to their ovarian activation. (B) JH-III and MF titers were measured in the hemolymph of individual bees in three worker groups, either untreated (n=18 workers, 6 groups) or treated with 3 mg precocene-I (n=18 workers, 6 groups) diluted in sugar water per group. Groups were established using callow (<24 h) workers from mixed colonies and kept for 7 days. The mean of all individuals is presented as there was no difference in JH/MF levels according to rank. Data are presented as means ± s.e.m.

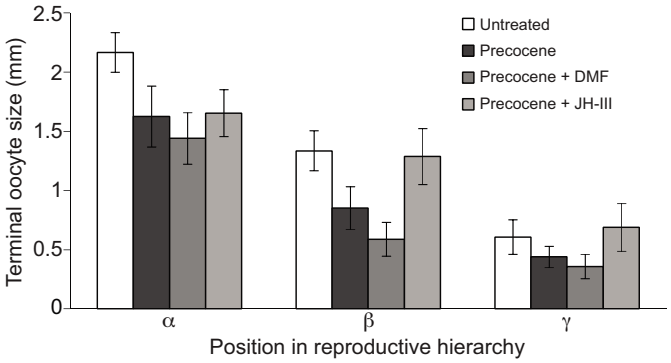

Fig. 2. The effect of precocene-I and JH-III on ovarian activation in *B. terrestris* workers. Ovarian activation was measured in individual bees kept in three-worker groups that were randomly assigned to one of the following treatments: untreated control groups (n=12 groups), fed with 3 mg precocene-I diluted in sugar water (n=12 groups), fed with 3 mg precocene-I and treated with a topical application of 5 µl dimethylformamide (DMF; n=14 groups), or fed with 3 mg precocene-I and treated with a topical application of 100 µg JH-III diluted in 5 µl DMF (n=11 groups). Groups were established using callow workers (<24 h) from mixed colonies and kept for 7 days. Workers in each group were ranked as α, β or γ according to their level of ovarian activation. Data are presented as means ± s.e.m.

groups) and 6 mg (0.48±0.3 mm, n=5 groups) precocene-treated groups. The effect of precocene-I on ovarian activation was equal for all ranks (rank nested in treatment: $F_{8,105}$=10.4, P<0.001 followed by Tukey-type *post hoc* test P<0.02 for α, β and γ in untreated and 1.2 mg versus α, β and γ in 3 and 6 mg treated groups) (Fig. 1A; statistics and sample size for all experiments are provided in supplementary material Table S1).

Because a precocene-I concentration of 1.2 mg did not affect worker ovarian activation, and mortality in the groups treated with 6 mg was very high (>50%, data not shown), we conducted the following experiment using a concentration of 3 mg per group of three workers.

Overall, we established 53 groups of workers either treated with 3 mg precocene-I (n=32 groups) or left untreated (n=21), from which we eliminated 22 groups because of mortality of at least one worker during the experiment. Mortality in the 3 mg treated groups was 27.1% (26 out of 96 workers in 20 different groups) compared with 3.2% in the control groups (two out of 63 workers in two different groups). Only groups where all three workers survived were included in the analyses. JH-III and MF titers in the hemolymph were tested in 12 groups (six untreated and six groups treated with 3 mg precocene-I; Fig. 1B). JH titers were significantly reduced in the treated groups compared with the untreated (nested ANOVA, treatment: $F_{1,30}$=6.6, P=0.015) and there were no significant differences between ranks (nested ANOVA, worker rank nested in treatment: $F_{4,30}$=1.4, P=0.25). With respect to MF hemolymph titers, there were no differences between the groups or ranks (nested ANOVA, for treatment: $F_{1,30}$=0.46, P=0.5; rank nested in treatment: $F_{4,30}$=0.96, P=0.43).

Experiment 2: effect of precocene-I and JH-III treatment on ovarian activation, aggression, Dufour's gland secretion, and JH-III and MF titers in *B. terrestris* workers

In this experiment we tested the effect of 3 mg precocene-I on ovarian activation, aggression, Dufour's gland secretion, and JH-III and MF titers in the hemolymph in workers that were kept for 7 days in three-worker groups. We also tested whether topical application of JH-III can remedy the effect of precocene-I.

Ovarian activation was significantly reduced in workers that were fed with 3 mg precocene-I compared with untreated workers, and a single treatment with JH-III was able to increase ovarian activation in precocene-fed workers to a level comparable to that of workers that were treated with the solvent dimethylformamide (DMF) (nested ANOVA, treatment: $F_{1,66}$=7.72, P=0.007 for untreated versus precocene-I and $F_{1,69}$=7.5, P=0.007 for DMF versus JH). Although the differences for rank nested in treatment were also significant (nested ANOVA: $F_{4,66}$=15.78, P<0.001 for untreated versus precocene-I and $F_{4,69}$=8.47, P<0.001 for DMF versus JH), none of the differences between workers of the same rank in different treatments were apparent in *post hoc* tests (P>0.05). Thus, the effects of both precocene-I and JH application on ovarian activation were equal for all ranks (Fig. 2; statistics and sample size are given in supplementary material Table S1).

Aggression was significantly reduced in workers that were fed with precocene-I compared with the untreated control groups. In this case, although a single treatment with JH-III significantly increased aggression compared with the treatment with DMF, it did not fully reverse this effect (nested ANOVA, treatment: $F_{1,72}$=14.23, P<0.001 for untreated versus precocene-I and $F_{1,69}$=4.65, P=0.03 for DMF versus JH). The difference between the treatments is attributed to

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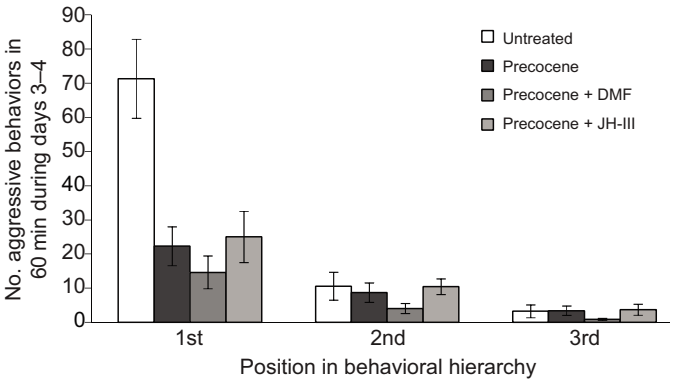

Fig. 3. The effect of precocene-I and JH-III on aggressive behavior in *B. terrestris* workers. Aggressive behavior was measured in individual bees kept in three-worker groups that were assigned to one of the following treatments: untreated control groups (n=12 groups), fed with 3 mg precocene-I diluted in sugar water (n=14 groups), fed with 3 mg precocene-I and treated with a topical application of 5 µl DMF (n=14 groups), or fed with 3 mg precocene-I and treated with a topical application of 100 µg JH-III diluted in 5 µl DMF (n=11 groups). Groups were established using callow workers (<24 h) from mixed colonies and kept for 7 days. Workers in each group were ranked as first, second or third according to their aggression index. Data are presented as means ± s.e.m.

the behavior of the most aggressive workers in each group (nested ANOVA, behavioral worker rank nested in treatment: $F_{4,72}$=23.2, P<0.001 followed by Tukey-type *post hoc* test P<0.001 for first/untreated versus all other groups and $F_{4,69}$=6.01, P<0.001 followed by Tukey-type *post hoc* test P<0.003 for first/JH versus all groups but second/JH and first/DMF; Fig. 3; supplementary material Table S1).

The total secretion of Dufour's gland [hydrocarbons, esters and fatty acid (Amsalem et al., 2009)] did not differ between either treatments or ranks (nested ANOVA, treatment: $F_{1,60}$=1.91, P=0.17 for untreated versus precocene-I and $F_{1,48}$=0.59, P=0.44 for DMF versus JH; rank nested in treatment: $F_{4,69}$=1.3, P=0.28 for untreated

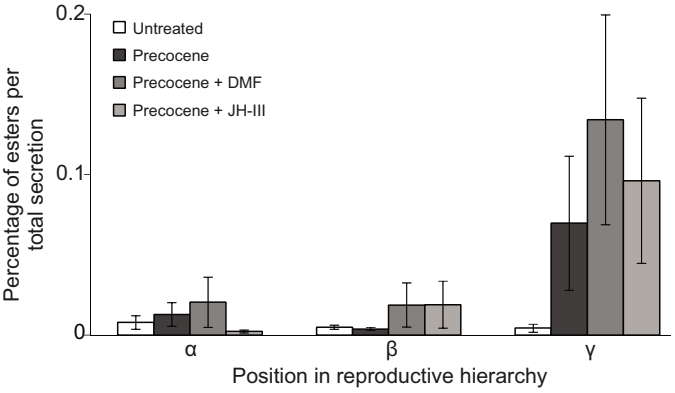

Fig. 4. The effect of precocene-I and JH-III on ester-sterility signal production in Dufour's gland of *B. terrestris* workers. Chemical secretion was analyzed in individual bees kept in three-worker groups that were assigned to one of the following treatments: untreated control groups (n=11 groups), fed with 3 mg precocene-I diluted in sugar water (n=11 groups), fed with 3 mg precocene-I and treated with a topical application of 5 µl DMF (n=10 groups), or fed with 3 mg precocene-I and treated with a topical application of 100 µg JH-III diluted in 5 µl DMF (n=8 groups). Groups were established using callow workers (<24 h) from mixed colonies and kept for 7 days. Workers in each group were ranked as α, β or γ according to their level of ovarian activation. Data are presented as means ± s.e.m.

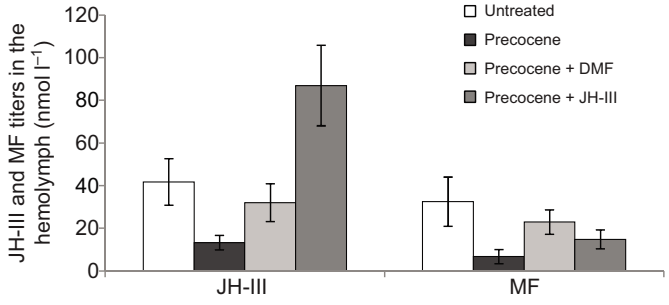

Fig. 5. The effect of precocene-I and treatment with JH-III on JH-III and MF titers in the hemolymph of *B. terrestris* workers. JH-III and MF titers were measured in individual bees that were kept in three-worker groups and were assigned to one of the following treatments: untreated control workers (n=13 workers), fed with 3 mg precocene-I diluted in sugar water (n=11 workers), fed with 3 mg precocene-I and treated with a topical application of 5 µl DMF (n=14 workers), or fed with 3 mg precocene-I and treated with a topical application of 100 µg JH-III diluted in 5 µl DMF (n=12 workers). Groups were established using callow workers (<24 h) from mixed colonies and kept for 7 days. Data are presented as means ± s.e.m.

versus precocene-I and $F_{4,48}$=0.5, P=0.73 for DMF versus JH; data not shown). However, the proportion of esters was significantly higher in the precocene-I treated groups compared with the untreated control (nested ANOVA, treatment: $F_{1,60}$=4.21, P=0.04), with the differences attributed mostly to the γ workers (rank nested in treatment: $F_{4,60}$=2.93, P=0.02; *post hoc*: P=0.02 for γ/precocene versus all groups but α/untreated and α/precocene). The treatment with JH-III did not fully recover the increase in ester production caused by precocene ($F_{1,48}$=0.37, P=0.54 for DMF versus JH). However, although JH-III did not reduce ester production compared with the DMF treatment, there were significant differences between groups when rank was nested in treatment, with the effect being attributed to the γ workers (nested ANOVA, $F_{4,60}$=2.93, P=0.02 followed by Tukey-type *post hoc* test P=0.02 for γ/precocene versus all groups but α/untreated and α/precocene and $F_{4,48}$=2.51, P=0.05 for DMF versus JH followed by Tukey-type *post hoc* test P>0.05 for all groups (Fig. 4; supplementary material Table S1).

The titer of JH, but not that of MF, was reduced after feeding the workers with precocene-I regardless of their social rank (JH: nested ANOVA, treatment: $F_{1,18}$=4.84, P=0.04; rank nested in treatment: $F_{4,18}$=1.03, P=0.41; MF: nested ANOVA, treatment: $F_{1,18}$=3.53, P=0.076, rank nested in treatment: $F_{4,18}$=1.04, P=0.411). A single topical application of JH-III increased the JH-III levels, but not the MF titers in the hemolymph (JH: nested ANOVA, treatment: $F_{1,20}$=9.73, P=0.005; rank nested in treatment: $F_{4,20}$=3.09, P=0.03 followed by Tukey-type *post hoc* test P<0.05 for α workers versus all other groups; MF: nested ANOVA, treatment: $F_{1,20}$=0.86, P=0.36; rank nested in treatment: $F_{4,20}$=0.09, P=0.98). While the effect of precocene-I in reducing JH titer was equal for all ranks, the JH replacement therapy effect was particularly large in the α workers (Fig. 5; supplementary material Table S1).

DISCUSSION

JH is an important regulator of insect development, physiology and behavior. While in many insect species JH functions in the adult as a gonadotropin, in some highly eusocial insects its role has shifted to regulate social behavior (Barth et al., 1975; Hartfelder and Engels, 1998; Bloch et al., 2000b; Hartfelder, 2000; Giray et al., 2005; Amsalem et al., 2014). In bumble bees, which are considered to be primitively eusocial (Michener, 1974), JH seemed to retain its role in reproduction but was also suggested to mediate aggression and

dominance behavior (Roseler, 1977; Van Doorn, 1986; Bloch et al., 2000b). However, little is known about the association between JH, aggression and ovary activation and the effect of rank within the social structure on these parameters. The present study investigated the effect of the JH inhibitor precocene-I on JH titer and consequently on reproduction, aggression and pheromone production in queenless workers of the bumble bee *B. terrestris*.

Our results clearly show that precocene-I affects JH titer, similarly to findings in other solitary insects (Pratt and Bowers, 1977; Sohn et al., 1991; Chen et al., 2005a), and can be used to reduce JH titer in *B. terrestris*. However, precocene-I did not reduce MF (the precursor of JH-III) in a statistically significant manner (P=0.07; supplementary material Table S1), and a topical application of JH-III increased JH, but not MF titers. The occurrence of MF titers in the hemolymph is puzzling as it is assumed to be converted to JH-III in the CA via epoxidase during the last step of JH biosynthesis (Pratt and Bowers, 1977; Haunerland and Bowers, 1985), and neither accumulates in the CA nor is secreted to the hemolymph. However, a recent study demonstrated the occurrence of MF in the hemolymph of several insect orders, often at concentrations greatly exceeding those of JH-III, and was suggested to function as a circulating hormone in insects (Teal et al., 2014). Supporting this hypothesis are the selective effects of precocene-I on MF and JH titers. However, MF was measured in the hemolymph and not in the CA, and the enzyme that was identified as the epoxidase (CYP15A1) was not inhibited by precocenes in a previous study (Helvig et al., 2004).

Precocene-I reduced ovarian activation and JH titer in workers irrespective of their social rank, and its effects were remedied by JH-III replacement therapy. This is, again, in line with the effects of precocene in other solitary insects (Lee and Tan, 1980; Samaranayaka-Ramasamy and Chaudhury, 1981; Deb and Chakravorty, 1982; Socha and Hodkova, 1983; Li et al., 1993; Kumar and Khan, 2004). This also confirms the gonadotropic function of JH in *B. terrestris* that was until now based on correlations (Roseler, 1977; Van Doorn, 1989; Bloch et al., 1996; Bloch et al., 2000b; Bortolotti et al., 2001; Shpigler et al., 2010), and provides direct evidence for the necessity of JH for oocyte development and egg laying in queenless *B. terrestris* workers. Similar conclusions were reached by a recent study performing allatectomy in *B. terrestris* workers (Shpigler et al., 2014), supporting the effect of precocene. However, although precocene-I altered aggressive behavior and sterility signal production in workers, these effects could not be fully remedied by JH-III replacement therapy and were rank-dependent. Most of the aggression within *B. terrestris* queenless groups is performed by the most reproductive worker, while the ester-sterility signal is commonly produced by the subordinate workers (Amsalem et al., 2009; Amsalem and Hefetz, 2010; Amsalem and Hefetz, 2011; Amsalem et al., 2013). Accordingly, the main effect of precocene-I was to reduce aggression in the most dominant workers (first in the behavioral hierarchy) and increase pheromone production in the least productive workers (γ in the reproductive hierarchy), while no specific changes were recorded for the other workers in the dominance hierarchy (Figs 3, 4). Although reproduction in small queenless groups is dominated by one worker that tends to be both the most aggressive and the most productive (Amsalem and Hefetz, 2011), subordinate workers can also eventually fully activate their ovaries and lay eggs. Thus, JH activates ovaries in all ranks, but the rate at which they do so depends on the social rank.

The differences in the precocene-I effects on reproduction, aggression and pheromone production may suggest disparate effects of JH: it may affect ovarian activation directly and aggression or pheromone production indirectly. A similar effect was reported for cockroaches (Chen et al., 2005a), where precocene-II reduced aggression in males but treatment with JH-III did not increase aggression. In the same study, it was suggested that while ovarian activation is directly regulated by JH, neither aggression nor pheromone production are regulated by CA or JH. Such regulation requires complex coordination between multiple factors such as JH biosynthesis and its hemolymph titer, aggressive behavior, ovarian activation, vg levels, pheromone production and perhaps also signaling molecules such as the brain biogenic amines octopamine and dopamine.

Aggression precedes ovarian activation in queenless *B. terrestris* workers and the production of ester-sterility signal is strongly related to individual reproductive status (Amsalem et al., 2009) and the level of aggression in the group (Amsalem and Hefetz, 2010; Amsalem and Hefetz, 2011). In a recent study (Amsalem et al., 2014), we showed that treatment with JH-III did not increase aggression in queenless workers, which is consistent with lack of increased aggression in the JH-III replacement group in the present study. The finding that aggression was correlated with vg mRNA levels (which are also not regulated by JH) (Amsalem et al., 2014), along with previous studies, led us to propose that JH/ovarian activation and vg/aggression are interlinked in *B. terrestris*, but are likely to be regulated separately by a third player. The brain biogenic amines octopamine and dopamine were also investigated in *B. terrestris* workers and have been shown to be correlated with dominance and egg maturation, respectively (Bloch et al., 2000a). Taking all of these studies together with the findings of the present study suggests that high JH levels increase brain octopamine levels that in turn increase aggression levels and consequently vg production and ovarian activation. Such a scenario explains the findings pertaining to the interrelationship between ovarian activation, vg levels and aggression. Activated ovaries may then send a positive feedback to the brain. Such a signal may result in elevated dopamine levels [that are needed for egg maturation (Bloch et al., 2000a)] and egg laying. The fact that hormone replacement therapy did not reconstitute the effects on aggression and pheromone production can be explained by a direct effect of external JH-III on JH titer without inducing its biosynthesis in the CA. By bypassing the CA, the external JH operates directly on the ovaries, leading to elevated uptake of vg by the ovaries, thus activating them. The activated ovaries consequently send a positive feedback to the brain, signaling to elevate dopamine levels that are translated into egg maturation, but do not affect aggressive behavior or pheromone production.

Although this model is highly speculative, it explains well all the currently known findings on the regulation of ovarian activation and aggression by JH in *B. terrestris*. Yet, we cannot completely exclude the possibility that JH directly regulated both aggression and ovarian activation and that the application of JH-III did resume aggression in the precocene-I treated bees; because treatment with JH-III was performed only on day 2, there was a delay in the onset of aggression beyond days 3–4, in which we made the observations (Amsalem and Hefetz, 2010). Other possibilities are that aggression (and, likewise, ester production) was irreversible because of a toxicity effect of precocene-I, and that changes in aggression and ovarian activation were due to a change in the workers' feeding behavior, as was previously suggested (Szczepanik et al., 2005). Although we did not collect the precise data, control bees in almost all cases consumed the initial amount of sugar water faster than the precocene-fed workers. Such an antifeedant activity may possibly interfere with JH production via the insulin-signaling pathway. In this case, the effect caused by precocene-I may have actually been

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an indirect effect resulting from a change in feeding behavior. Examining direct effects of precocene on CA morphology and size will allow us to distinguish between an antifeedant and an allatocidal effect of precocene in *B. terrestris*.

The strong negative correlation between the terminal oocyte size and the amount of esters produced by workers (Amsalem et al., 2009), the high amounts in subordinate workers that were exposed to less aggression (Amsalem and Hefetz, 2010) and in foragers compared with house bees (Amsalem et al., 2013) all indicate that workers signal their status as non-reproductives in the highly competitive *B. terrestris* colony. Reduction of the JH titer by precocene-I reduced aggression and ovarian activation, and accordingly also increased ester production in the most subordinate worker within each group. Ester levels remain high although the level of aggression within the group decreased. Thus, ester production responded primarily to the changes in the ovaries, strengthening their role as an honest signal reflecting worker reproductive status. According to our model, ester production cannot be subjected to a negative feedback by the ovaries because it will predict reduction in esters in JH-III-treated workers, which we did not observe. It is therefore assumed that ester production is regulated directly by the brain, presumably by the brain octopamine levels.

The present study demonstrates the role of JH in reproduction and social behavior in *B. terrestris* workers and highlights the importance of the different phenotypes JH promotes as function of its levels in different ranks within the social group. The finding that precocene-I can inhibit JH in a bumble bee species provides us with a new tool to manipulate endocrine pathways in social insects. As these pathways underlie key social behaviors, they help us to better understand the evolution of these behaviors as well as the relationship between aggression, endocrine pathways and ovary activation.

MATERIALS AND METHODS

Bees

Colonies of *B. terrestris* (Yad Mordechai Apiary, Israel) were obtained 3–5 days after the first worker had emerged. They were maintained in the laboratory in nest boxes (23×23×10 cm) at a constant temperature of 30°C and 50–60% relative humidity, and supplied *ad libitum* with a sugar solution and fresh pollen collected from honey bee colonies. Newly emerged workers of approximately the same size were collected from mixed colonies as callow (<24 h old), individually tagged and kept for 7 days in small wooden boxes in groups of three workers. In all analyses described below, workers were 7 days old.

Precocene-I feeding and JH-III application

A successful topical administration of JH-III to *B. terrestris* workers has been demonstrated in previous studies (Shpigler et al., 2010; Amsalem et al., 2014). A preliminary study we conducted showed that delivering precocene by feeding was effective, and in order to combine two consecutive treatments and to minimize disturbance to the treated workers, we decided to deliver precocene-I orally and JH-III topically. Oral administration is particularly useful for chronic treatments (Barron et al., 2007). A preliminary study we conducted showed that applying a high concentration of precocene-I all at once results in high mortality compared with a feeding period lasting 24 h.

Workers were fed with precocene-I (Sigma-Aldrich, catalog no. 195855-1G, purity 99%) that was directly mixed into their sugar water. The desired amount of precocene-I was added to a 1 ml sugar water solution (1:1 w:v), which the three bees consumed within 24 h of group establishment. See description of concentrations below (Experiment 1). Once the entire amount of sugar water was consumed, workers were provided with unlimited, untreated sugar water for the remaining of the experiment.

In order to dissolve JH-III (Sigma-Aldrich, catalog no. J2000, purity ≥65%) we used dimethylformamide (DMF; J. T. Backer, catalog no. 7032-1L) as a solvent. Therefore, we added another control where workers were fed with precocene-I and then received a topical application of DMF in addition to the treatment where workers were fed with precocene-I and then received a topical application of JH-III diluted with DMF. Applications of JH-III or DMF were performed on the second day post group establishment and after workers had completely consumed the precocene–sugar water. Treatments were carried out by topical application to the dorsal part of the thorax of either 5 µl of DMF or 100 µg JH-III diluted in 5 µl of DMF. These doses were previously shown to be effective in modulating behavior and physiology of worker bumble bees (Shpigler et al., 2010; Amsalem et al., 2014). Workers were provided with unlimited pollen during the entire experiment. Details of the different experiments are provided below.

Aggressive behavior

Groups were observed for 10 min each, three times a day (morning: 09:00–11:00 h; noon: 12:00–14:00 h; evening: 17:00–19:00 h) during days 3 and 4 after group establishment (a total of 60 min per group). During these days, aggression reaches a peak of activity and the level of aggression between subordinate and dominant workers is significantly different (Amsalem and Hefetz, 2010). Three antagonistic behaviors were monitored: humming, darting and attack [for definitions, see previous publications (Duchateau, 1989; Amsalem and Hefetz, 2010; Amsalem and Hefetz, 2011)]. An 'aggression index' was calculated for each bee by summing the total aggressive behaviors the bee performed during 60 min of observations. In each group, workers were ranked as first, second or third, according to their aggression index ('behavioral hierarchy').

Ovarian activation

Individual bees were dissected under a stereo-microscope in double-distilled water. The length of the terminal oocyte in the three largest ovarioles (at least one ovariole per ovary; workers possess four ovarioles per ovary) was measured with a scaled ocular. Mean terminal oocyte length for each bee was used as an index of ovarian activation (Amsalem and Hefetz, 2010; Amsalem and Hefetz, 2011). In each group, workers were ranked as α, β or γ, according to their index of ovarian activation ('reproductive hierarchy').

Chemical analysis of Dufour's gland secretion

During ovary dissection, Dufour's gland was cleanly separated from the sting apparatus and extracted in 50 µl pentane containing 1 µg eicosane as internal standard. The samples were kept at −20°C until analysis. Chemical analyses were performed by gas chromatography (Varian CP 3800) using a DB-1 fused silica capillary column (30 m×0.25 mm ID) under a temperature program from 170 to 300°C at 4°C min^{-1}. Compound identity was ascertained by GC/MS and retention times were compared with synthetic compounds (Amsalem et al., 2009). Compound quantification was achieved by GC peak integration compared with the internal standard under the above chromatographic conditions.

Determination of JH and MF hemolymph titers

Samples for determining JH-III and methyl farnesoate (MF) hemolymph titers were taken on day 7. For hemolymph extraction, the bees were anesthetized with ice and under a stereoscope binocular. Blood was sucked by capillary action into a pulled glass needle that was inserted under the fourth abdominal segment. We pooled 7–10 µl from each individual that was mixed with nine times the volume of HPLC grade methanol. Samples were stored at −20°C until shipping. Hemolymph was sent to the USDA-ARS laboratory in Gainesville, Florida, where it was processed according to a described protocol (Teal et al., 2000; Teal and Proveaux, 2006; Jones et al., 2010; Niño et al., 2012). Immediately after hemolymph extraction, the bees were killed by freezing on dry ice and stored at −20°C until dissections and analyses.

Experiment 1: concentration-dependent effect of precocene-I on ovarian activation, mortality, and JH-III and MF titers in B. terrestris workers

In this experiment, we investigated the concentration-dependent effect of precocene-I on ovarian activation using three different concentrations that were applied to *B. terrestris* workers by feeding (as described above). Groups were established with three callow workers (<24 h) of approximately

the same size taken from different source colonies. The groups were assigned to one of four treatments: 1.2, 3.0 or 6.0 mg precocene-I, or untreated control (preliminary results showed that lower concentrations did not have any effect on ovarian activation). Workers were kept for 7 days and killed by freezing on dry ice. Thus, they were 7 days old by the time of the analysis. Overall, we established 53 groups. Among these, we collected data on ovarian activation in 31 full groups (groups in which one or more bees died were eliminated). Using the bees from the 3 mg precocene-I groups, we also determined the effect of precocene-I on bee mortality rate and on JH-III and MF hemolymph titers, compared with untreated worker groups. Mortality was documented as it occurred during the 7 days of the experiment, after which the groups were either killed for further analysis or eliminated because of high mortality. Mortality usually occurred during days 1–3, indicating it was due to a direct toxic effect of precocene. For JH-III and MF hemolymph titers, we sampled 36 workers taken from 12 different groups. Furthermore, because we assessed the ovarian activation for individual workers within the groups, we could test whether worker rank in the social hierarchy modulated the effects of precocene-I and JH-III.

Experiment 2: effect of precocene-I and JH-III treatment on ovarian activation, aggression, Dufour's gland secretion, and JH-III and MF titers in *B. terrestris* workers

In this experiment, we investigated the effect of precocene-I on aggressive behavior, ovarian activation, Dufour's gland secretion and the hemolymph titers of JH-III and MF in *B. terrestris* workers. We further tested whether a topical application of JH-III can compensate for these effects. Furthermore, because we assessed the aggression index and ovarian activation for individual workers within the groups, we could test whether worker rank in the social hierarchy modulated the effects of precocene-I and JH-III. Groups were established with three callow workers (<24 h) of approximately the same size taken from different source colonies. Three worker groups were assigned to one of four treatments: (1) precocene-I-treated groups: workers were fed with 3 mg precocene-I per group (its optimal concentration, see Results); (2) precocene–DMF-treated groups: workers were fed with 3 mg precocene-I per group and were treated with a topical application of 5 µl DMF; (3) precocene–JH-treated groups: workers were fed with 3 mg precocene-I per group and were treated with a topical application of 100 µg JH-III diluted in 5 µl of DMF; and (4) untreated groups: workers received untreated sugar water at the same volume as the experimental groups during the first 24 h and were then provided with untreated, unlimited sugar water, as were the experimental groups. Workers were kept for 7 days and euthanized on dry ice. Overall, we collected behavioral data from 51 groups. Among these groups, we collected data on ovarian activation of all three workers in 49 groups and chemical data on Dufour's gland secretion in 40 groups. For JH-III and MF hemolymph titers we randomly sampled 50 workers taken from 22 different groups.

Statistics

The effects of precocene-I on ovarian activation, aggression, Dufour's gland secretion and JH-III and MF titers in the hemolymph were tested using a nested design ANOVA where 'worker rank' (α, β or γ for reproductive hierarchy or first, second or third for behavioral hierarchy) was nested in 'treatment' (different concentrations/treatment with precocene-I/DMF/JH-III). Workers were included in the analysis only if ranking was available (data on o either varian activation or aggression index) for all group mates. Ranking the workers was necessary because workers from the same group may be considered as dependent samples. By using this test we controlled for any impact caused by the dominance status of bees in each group. When data are presented as proportions (ester-sterility signal), they were arcsin transformed before performing parametric tests. Data are presented as means ± s.e.m. Significant differences were accepted at α=0.05.

Competing interests

The authors declare no competing financial interests.

Author contributions

E.A. carried out the behavioral physiological and chemical analyses, designed the study and wrote the paper. P.T. performed the analyses of juvenile hormone and methyl farnesoate hemolymph titers. C.M.G. and A.H. designed the study and wrote the paper along with E.A. All authors read and approved the final manuscript.

Funding

The work was supported by a grant from The Israel Science Foundation founded by the Israel Academy of Sciences (ISF grant no. 535/08 to A.H.).

Supplementary material

Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.107250/-/DC1

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