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 due to 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 was poorly studied in social insects. Here, we tested if precocene-I can effectively reduce JH levels in Bombus terrestris workers, and examined its effects on their physiology and behavior.

Precocene-I treatment of 3-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, affected mainly in 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 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.

Keywords: 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 through 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 contribute to our understanding of not only questions pertaining to the proximate mechanisms underlying reproduction, but also to shed light on the ultimate mechanisms leading to the transition from solitary to social life.

Juvenile hormone (JH) is a principle 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, 1998; Hartfelder, 2000). In social insects the hormone has acquired new roles such as regulating aggression and dominance in females of primitively eusocial insects like 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., 1974; 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 (Slama, 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 the JH levels is 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 on JH functions (Shpigler et al., 2014), the surgical trauma involved may in some cases (e.g., behavioral studies) renders the interpretation of the results more difficult (Sullivan et al., 2003).

As a primitively eusocial species, the bumblebee *Bombus terrestris* 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., 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 the honey bee where JH regulates division of labor and behavioral maturation rather than its traditional role as gonadotropin (Hartfelder and Engels, 1998; Robinson and Vargo, 1998; Hartfelder, 2000), in *B. terrestris* it retained its original role as 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, that is positively regulated by JH in most insects (but not in the honey bee, some termites and ants (Robinson and Vargo, 1998; 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 interrelation between JH and aggressive behavior in *B. terrestris* remain 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 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, which is seemingly less traumatic to surgical removal of the CA. 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) which 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, 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, Y. R. et al., 2005), 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, Y. R. et al., 2005). 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, Y. R. et al., 2005).

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 if 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, Y. R. et al., 2005), 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), to cause hypertrophy of the fat
body (Lee and Tan, 1980), to 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 pea aphid,
_Acrhythosiphon pisum_, body (Chen, Z. et al., 2005). 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
which are not limited to the context of reproduction. In the honey bee, where JH does not
function as 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 were
done thus far with precocene in social insects.

In the current 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 that these
effects will be fully or partially reversible after hormone replacement therapy. (2) The effects of
precocene-I will be worker-rank dependent.
RESULTS

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

The concentration-dependent effect of precocene-I on worker ovarian activation is presented in Figure 1. Treatment had a significant effect on ovarian activation (nested design ANOVA for “treatment” f3, 105=67.7, p<0.001 followed by Tukey-type post-hoc test p<0.02 for untreated and 1.2 mg precocene vs. 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, n=3 groups) groups relative to both the 3 mg (0.35±0.14, n=12 groups) and 6 mg (0.48±0.3, n=5 groups) precocene treated groups. The affect of precocene-I on ovarian activation was equal for all ranks (“rank” nested in “treatment”: f8,105=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) (Figure 1A; statistics and sample size for all experiments are provided in Supplementary Table S1).

Since 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 3 workers.

Overall, we established 53 groups of 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 1 worker during the experiment. Mortality in the 3 mg treated groups was 27.1% (26 out of 96 workers in 20 different groups) compare to 3.2% in the control groups (2 out of 63 workers in 2 different groups). Only groups where all 3 workers survived were included in the analyses. JH-III and MF titers in the hemolymph were tested in 12 groups (6 untreated and 6 groups treated with 3 mg precocene-I, Figure 1B). JH titers were significantly reduced in the treated groups compared to the untreated (nested design ANOVA for “treatment”: f1,30=6.6, p=0.015) and there were no significant differences between ranks (nested design ANOVA for “worker rank” nested in “treatment”: f4,30=1.4, p=0.25). With respect to MF hemolymph titers, there were no differences
between the groups or ranks (nested design ANOVA for “treatment”: $f_{1,30}=0.46$, $p=0.5$; for “rank” nested in “treatment”: $f_{4,30}=0.96$, $p=0.43$).

**Experiment 2: The 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 3 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 to untreated workers, and a single treatment with JH-III was able to increase ovarian activation in precocene-fed workers to be comparable to that of workers that were treated with the solvent (DMF) (nested design ANOVA for treatment: $f_{1,66}=7.72$, $p=0.007$ for untreated vs. precocene-I and $f_{1,69}=7.5$, $p=0.007$ for DMF vs. JH). Although the differences for “rank” nested in “treatment” were also significant (nested design ANOVA for “rank” nested in “treatment”: $F_{4,66}=15.78$, $p<0.001$ for untreated vs. precocene-I and $F_{4,69}=8.47$, $p<0.001$ for DMF vs. 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 (Figure 2, statistics and sample size on Table S1).

Aggression was significantly reduced in workers that were fed with precocene-I compared to the untreated control groups. In this case, a single treatment with JH-III, although significantly increased aggression compared to the treatment with DMF, did not fully reverse this effect (nested design ANOVA for “treatment”: $f_{1,72}=14.23$, $p<0.001$ for untreated vs. precocene-I and $f_{1,69}=4.65$, $p=0.03$ for DMF vs. JH). The difference between the treatments is attributed to the behavior of the most aggressive workers in each group (nested design ANOVA for “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 1st/untreated vs. all other groups and $f_{4,69}=6.01$, $p<0.001$ followed by Tukey-type post-hoc test $p<0.003$ for 1st/JH vs. all groups but 2nd/JH/ and 1st/DMF) (Figure 3, 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 design ANOVA for treatment: $f_{1,60}=1.91$, $p=0.17$ for untreated. vs. precocene-I and $f_{1,48}=0.59$, $p=0.44$ for DMF vs. JH; for “rank” nested in “treatment”: $f_{4,60}=1.3$, $p=0.28$ for untreated vs. precocene-I and $f_{4,48}=0.5$, $p=0.73$ for DMF vs. JH) (data are not shown). However, the proportion of esters was significantly higher in the precocene-I treated groups compared to the untreated control (nested design ANOVA for “treatment”: $f_{1,60}=4.21$, $p=0.04$) with the differences attributed mostly to the gamma workers (for “rank” nested in “treatment”: $F_{4,60}=2.93$, $p=0.02$; post hoc: $p=0.02$ for $\gamma$/Prec. vs. all groups but $\alpha$/Un. and $\alpha$/Prec). The treatment with JH-III did not fully recover the increase in ester production caused by precocene (and $f_{1,48}=0.37$, $p=0.54$ for DMF vs. JH). However, although JH-III did not reduce ester production compared to the DMF treatment, there were significant differences between groups when “rank” was nested in “treatment” with the effect being attributed to the gamma workers (nested design ANOVA $f_{4,60}=2.93$, $p=0.02$ followed by Tukey-type post-hoc test $p=0.02$ for $\gamma$/Prec. vs. all groups but $\alpha$/untreated and $\alpha$/precocene and $f_{4,48}=2.51$, $p=0.05$ for DMF vs. JH followed by Tukey-type post-hoc test $p>0.05$ for all groups (Figure 4, Table S1).

The titer of JH, but not that of MF, was reduced after feeding the workers with precocene-I regardless to their social rank (JH: nested design ANOVA for treatment: $f_{1,18}=4.84$, $p=0.04$, for “rank” nested in “treatment”: $f_{4,18}=1.03$, $p=0.41$; MF: nested design ANOVA for treatment $f_{1,18}=3.53$, $p=0.076$, for “rank” nested in “treatment”: $f_{4,18}=1.04$, $p=0.411$). A single topical application of JH-III increased the JH-III, but not the MF titers in the hemolymph (JH: nested design ANOVA for treatment: $f_{1,20}=9.73$, $p=0.005$, for “rank” nested in “treatment”: $f_{4,20}=3.09$, $p=0.03$ followed by Tukey-type post-hoc test $p<0.05$ for alpha worker vs. all the other groups; MF: nested design ANOVA for treatment: $f_{1,20}=0.86$, $p=0.36$; for “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 alpha workers (Figure 5, 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 gonadotropin, in some highly eusocial insects its role has shifted to regulate social behavior (Barth et al., 1974; 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 current 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 the findings in other solitary insects (Pratt and Bowers, 1977; Sohn et al., 1991; Chen, Y. R. et al., 2005), and can be used to reduce JH titer in *B. terrestris*. However, precocene-I did not reduced MF (the precursor of JH-III) in statistically significant manner (p=0.07, 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 since 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 accumulate in the CA nor 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 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 to 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 so far 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 a direct evidence for the necessity of JH for oocyte development and egg laying in queenless *B. terrestris* workers.

Similar conclusions were reached by the a recent study performing allatectomy in *B. terrestris* workers (Shpigler et al., 2014), supporting the effect of precocene in performing chemical allatectomy. 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 ester sterility signal is commonly produced by the subordinate workers (Amsalem et al., 2009; Amsalem and Hefetz, 2010, 2011; Amsalem et al., 2013). Accordingly, the main effect of precocene-I was to reduce aggression in the most dominant workers (1st in the behavioral hierarchy) and increase pheromone production in the least productive workers (gamma in the reproductive hierarchy), while no specific changes were recorded for the other workers in the dominance hierarchy (Figures 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), also subordinate workers can 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, Y. R. et al., 2005) where precocene-II reduced aggression in males but treatment with JH-III did not increase aggression. In this case as well, it was suggested that while ovarian activation is directly regulated by JH, neither aggression nor pheromone production are regulated by the CA/JH. Such a regulation requires a complex coordination between multiple factors such as JH biosynthesis and its hemolymph titer, aggressive behavior, ovarian activation, vitellogenin 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 esters sterility signal is strongly related to the individual reproductive status (Amsalem et al., 2009) and to the level of aggression in the group (Amsalem and Hefetz, 2010, 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 current 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 correlate with dominance and egg maturation, respectively (Bloch et al., 2000a). Taking all of these studies together with the findings of the current study suggests that high JH levels increase brain octopamine levels that in turn increase aggression levels and consequently vg production and ovarian activation. Such scenario explains the findings pertaining to the interrelation 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 the hormone replacement therapy did not reconstitute the effects on aggression and pheromone production can be explained by a direct effect of an 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 elevated 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 JH of ovarian activation and aggression 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, but since 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, also ester production) was irreversible due to 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 it 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 been actually 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 effects 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), their high amounts in subordinate workers that
were exposed to less aggression (Amsalem and Hefetz, 2010) and in foragers compared to 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, esters 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 since 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 current 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. Since 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.

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AUTHOR CONTRIBUTION

EA carried out the behavioral physiological and chemical analyses, designed the study and wrote the paper. PT performed the analyses of juvenile hormone and methyl farnesoate hemolymph titers. CMG and AH designed the study and wrote the paper along with EA. All authors read and approved the final manuscript.

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 hours old), individually tagged and kept for 7 days in small wooden boxed in groups of 3 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). Preliminary study we conducted showed that delivering precocene was effective by feeding and in order to combine between 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 a chronic treatment (Barron et al., 2007). A preliminary study we conducted showed that applying high concentration of precocene-I at once results in high mortality compared to a feeding period lasting 24 h.
Workers were fed with precocene-I (Sigma, cat 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 3 bees consumed within 24 hours after 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 (JH-III, Sigma, cat J2000, purity ≥ 65%) we used dimethylformamide (DMF, J.T Backer cat: 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 totally consumed the precocene-sugar water. Treatments were done 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 whole experiment. Details of the different experiments are provided below.

**Aggressive behavior**

Groups were observed for 10 minutes each, 3 times a day (morning: 9:00-11:00 am; noon: 12:00-2:00 pm; evening: 5:00-7:00 pm) during days 3 and 4 after group establishment (a total of 60 minutes per group). During these days aggression reaches a peak of activity and 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 (Duchateau, 1989; Amsalem and Hefetz, 2010, 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 1<sup>st</sup>, 2<sup>nd</sup> or 3<sup>rd</sup>, 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 1 ovariole per ovary; workers possess 4 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, 2011). In each group workers were ranked as \( \alpha, \beta \) or \( \gamma \), 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 \( \mu \)l pentane containing 1 \( \mu \)g eicosane as internal standard. The samples were kept at -20°C until analysis. Chemical analyses were performed by gas chromatography (Varian CP 3800) using DB-1 fused silica capillary-column (30m x 0.25mm ID) under a temperature program from 170°C to 300°C at 4°C/min. Compound identity was ascertained by GC/MS and retention times as compared to synthetic compounds (Amsalem et al., 2009). Compound quantification was achieved by GC peak integration compared to the internal standard under the above chromatographic conditions.

**Determination of juvenile hormone and methyl farnesoate 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 cold ice and under a stereoscope binocular. Blood was sucked by capillary action into a pulled glass needle that was inserted under the 4th abdominal segment. We pooled 7-10 \( \mu \)l from each individual that were mixed with 9 times the volume of HPLC grade methanol. Samples were stored at -20°C until shipping. Hemolymph was sent to the USDA-ARS lab in Gainesville, Florida, where it was processed according to a described protocol (Teal et al., 2000; Teal and Proveaux, 2006; Jones et al., 2010; Nino et al., 2012). Immediately after hemolymph extraction, the bees were sacrificed 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, 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 3 different concentrations which were applied to *B. terrestris* workers by feeding (as described above). Groups were established with 3 callow workers (<24 hours) of approximately the same size taken from different source colonies. The groups were assigned to one of 4 treatments: 1.2, 3.0, 6.0 mg precocene-I or untreated control groups (preliminary results showed that lower concentrations did not have any effect on ovarian activation). Workers were kept for 7 days and sacrificed on dry ice. Thus, 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 its effect on bee mortality rate and on JH-III and MF hemolymph titers, compared to untreated worker groups. Mortality was documented as it occurred during the 7 days of the experiment, after which the groups were either sacrificed 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: The 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 3 callow workers (<24 hours) of approximately the same size taken from different source colonies. Three worker groups were assigned to one of 4 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 (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 did the experimental groups. Workers were kept for 7 days and sacrificed on dry ice. Overall we collected behavioral data in 51 groups. Among these groups, we collected data on ovarian activation of all 3 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 / γ for reproductive hierarchy or 1st, 2nd and 3rd 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 (either data on ovarian activation or aggression index) for all group mates. Ranking the workers was necessary since 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 transformed using arcsin before performing parametric tests. Data are presented as mean ± SE. Significant differences were accepted at α=0.05.
Captions to figures

Figure 1: The effect of different concentrations of precocene-I on ovarian activation. juvenile hormone and methyl farnesoate titers in B. terrestris workers. A. Ovarian activation was measured in 3-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 3 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 individual is presented since there was no difference in JH/MF levels according to rank. Data are presented as means ± SE.

Figure 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 3-worker groups that were randomly assigned to one of the following treatments: (a) untreated control groups (n=12 groups), (b) fed with 3 mg precocene-I diluted in sugar water (n=12 groups), (c) fed with 3 mg precocene-I and treated with a topical application of 5 µl DMF (n=14 groups), (d) 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 and γ according to their level of ovarian activation. Data are presented as means ± SE.

Figure 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 3-worker groups that were assigned to one of the following treatments: (a) untreated control groups (n=12 groups), (b) fed with 3 mg precocene-I diluted in sugar water (n=14 groups), (c) fed with 3 mg precocene-I and treated with a topical application of 5 µl DMF (n=14 groups), (d) 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 1st, 2nd or 3rd according to their aggression index. Data are presented as means ± SE.

**Figure 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 3-worker groups that were assigned to one of the following treatments: (a) untreated control groups (n=11 groups), (b) fed with 3 mg precocene-I diluted in sugar water (n=11 groups), (c) fed with 3 mg precocene-I and treated with a topical application of 5 µl DMF (n=10 groups), (d) 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 and γ according to their level of ovarian activation. Data are presented as means ± SE.

**Figure 5:** The effect of precocene-I and treatment with JH-III on juvenile hormone and methyl farnesoate titers in the hemolymph of *B. terrestris* workers. JH-III and MF titers were measured in individual bees that were kept in 3-worker groups and were assigned to one of the following treatments: (a) untreated control workers (n=13 workers), (b) fed with 3 mg precocene-I diluted in sugar water (n=11 workers), (c) fed with 3 mg precocene-I and treated with a topical application of 5 µl DMF (n=14 workers), (d) 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 ± SE.
References


Piulachs, M. D., Cassier, P. and Belles, X. (1989). Ultrastructural changes induced by precocene II and 3,4-dihydroprecocene II in the corpora allata of *Blattella germanica* Cell and Tissue research **258**, 91-99.


Figure 1

A

Average terminal oocyte size (mm)

Position in reproductive hierarchy

- Alpha
- Beta
- Gamma

Untreated
1.2 mg
3 mg
6 mg

B

JH-III and MF titers in the hemolymph (pMol/µl)

- Untreated
- 3 mg

Methyl farnesoate
Juvenile hormone III
Figure 2

Average terminal oocyte size (mm)

Position in reproductive hierarchy

Untreated
Precocene
Precocene + DMF
Precocene + JH
Figure 3

The figure shows the number of aggressive behaviors performed in 60 min during days 3-4. The y-axis represents the number of behaviors, while the x-axis shows the position in the behavioral hierarchy (1st, 2nd, 3rd). Four treatment groups are compared: Untreated, Precocene, Precocene + DMF, and Precocene + JH.
Figure 4

Percentage of esters per total secretion

Position in reproductive hierarchy

- Untreated
- Precocene
- Precocene + DMF
- Precocene + JH

Alpha | Beta | Gamma
Figure 5

Juvenile hormone Methyl fernasoate (pMol)

- Untreated
- Precocene
- Precocene + DMF
- Precocene + JH

JH-III and MF titters in the hemolymph (pMol)