



No effect of juvenile hormone on task performance in a bumblebee (*Bombus terrestris*) supports an evolutionary link between endocrine signaling and social complexity

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ABSTRACT

A hallmark of insect societies is a division of labor among workers specializing in different tasks. In bumblebees the division of labor is related to body size; relatively small workers are more likely to stay inside the nest and tend (“nurse”) brood, whereas their larger sisters are more likely to forage. Despite their ecological and economic importance, very little is known about the endocrine regulation of division of labor in bumblebees. We studied the influence of juvenile hormone (JH) on task performance in the bumblebee *Bombus terrestris*. We first used a radioimmunoassay to measure circulating JH titers in workers specializing in nursing and foraging activities. Next, we developed new protocols for manipulating JH titers by combining a size-adjusted topical treatment with the allatotoxin Precocene-I and replacement therapy with JH-III. Finally, we used this protocol to test the influence of JH on task performance. JH levels were either similar for nurses and foragers (three colonies), or higher in nurses (two colonies). Nurses had better developed ovaries and JH levels were typically positively correlated with ovarian state. Manipulation of JH titers influenced ovarian development and wax secretion, consistent with earlier allatectomy studies. These manipulations however, did not affect nursing or foraging activity, or the likelihood to specialize in nursing or foraging activity. These findings contrast with honeybees in which JH influences age-related division of labor but not adult female fertility. Thus, the evolution of complex societies in bees was associated with modifications in the way JH influences social behavior.

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1. Introduction

Division of labor among workers specializing in different colony activities is a hallmark of insect societies. It is thought that worker task specialization improves colony efficiency and therefore, enhances colony fitness. Division of labor however, needs to be flexible enough to allow the colony to allocate workers to different tasks depending on changing conditions and perturbations (Beshers and Fewell, 2001; Oster and Wilson, 1978; Waibel et al., 2006). It is therefore important to understand the physiological mechanisms regulating task performance, their interactions with other physiological processes, and their environmental regulation.

Hormones, in particular, juvenile hormone (JH), have long been implicated as important modulators of task performance. Hormone actions during development regulate task differentiation between queens and workers as well as among morphologically distinct worker casts (reviewed in Bloch et al., 2009). JH is also important in species such as the honey bee in which morphologically similar workers perform

different tasks, but differ in their ages (known as ‘age-related division of labor’ or ‘age polyethism’). Young honeybee workers typically perform in-nest activities such as brood tending and nest cleaning, whereas older individuals perform outside activities such as nest defense and foraging. Honeybee foragers have the highest JH titers in the colony, and the age of first foraging is delayed in allatectomized bees (with severely reduced JH titers), and advanced in bees treated with JH-III (the natural JH homolog in bees and other hymenopteran insects, Bloch et al. 2000a) or JH mimics such as methoprene (Robinson, 1992; Sullivan et al., 2000). JH influences task performance by coordinated modulation of physiological and biochemical processes in tissues such as the brain, fat body and hypopharyngeal glands (HPG) that change their function along with worker task (Pandey and Bloch, 2015). The influence of JH on these tissues is mediated by differential gene expression such that young bees treated with JH or JH mimics typically show a more forager-like pattern of gene expression (Ament et al., 2012; Ueno et al., 2015; Whitfield et al., 2006). Although JH paces task-related behavioral development, it is not necessary for the performance of foraging behavior; bees for which the CA was removed can still successfully forage for nectar and pollen (Sullivan et al., 2000).

In contrast to age-related division of labor which has been extensively studied, mostly in the Western honey bee (*Apis mellifera*), very

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little is known on endocrine influences of size-related division of labor, which is common in wasps, ants, and bumblebees. The size and morphology of workers performing different tasks are determined by developmental processes during the larval and pupal stages. Nevertheless, there is profound plasticity during the adult stage in many species where individuals can switch between distinct activities such as nursing and foraging depending on colony needs. What regulates task performance in bumblebees and whether the behavioral modifications associated with task performance are mediated by the endocrine system is unknown.

In bumblebees and other solitary hymenoptera and “primitively” social insects (i.e., simple insect societies), JH seems to function as a gonadotropin regulating reproductive physiology and behavior (Smith et al., 2013; Wasielewski et al., 2011). In females, the gonadotropic function of JH is best manifested in the regulation of vitellogenesis and oocyte development (oogenesis) (De Loof et al., 2001). However, in honey bees and some species of ants and wasps with age-related division of labor, the queen, and egg-laying workers have low levels of JH compared to the sterile foragers (Corona et al., 2007; Dolezal et al., 2009; Pinto et al., 2000; Robinson et al., 1992; reviewed in Bloch et al., 2009). These observations led to the hypothesis that along the evolution of sociality in bees and wasps, JH has changed (or switched) its function from a gonadotropin into a regulator of task performance (Giray et al., 2005; Hartfelder and Engels, 1998; Robinson and Vargo, 1997; West-Eberhard and Turillazzi, 1996). In order to rigorously test this hypothesis however, it is necessary to go beyond correlation and study division of labor and reproduction in bees for which JH levels were both increased and decreased via manipulation (see below).

Bumblebees are specifically suitable for addressing this question because they are taxonomically related to honey bees, but the complexity of their sociality is intermediate between solitary and advanced eusocial insects. Thus, determining whether JH influences division of labor in bumblebees is essential for understanding the link between the evolution of sociality and JH signaling in bees. We studied *Bombus terrestris*, a species with well-documented size-related division of labor. Small workers typically perform in-nest activities such as brood tending and comb maintenance whereas their larger sisters are more likely to conduct foraging duties (Free, 1955; Goulson, 2003). The division of labor is less discrete than in honeybees because individuals commonly perform both foraging and brood care activities on the same day (Yerushalmi et al., 2006). We hypothesized that JH does not regulate division of labor in bumblebees because high JH levels in this species are typically measured for bees with active ovaries (Bloch et al., 2000a; Röseler, 1977), which are less likely to forage (van Doorn and Heringa, 1986). Moreover, earlier studies that investigated the influence of JH on bumblebee division of labor suggested that treatment with JH-analog or JH-I (which is not the natural JH homolog of bees, Bloch et al., 1996; Bloch et al., 2000a) did not affect the propensity to forage in workers of *Bombus impatiens* and *Bombus terrestris* (Cameron and Robinson, 1990; van Doorn, 1987). However, it is difficult to conclude based on these negative results that JH does not affect the division of labor in bumblebee because they did not include a treatment in which JH levels are reduced, and it was difficult to determine if the dose used to upregulate JH signaling was sufficient. On the other hand, the influence of JH on fertility has been thoroughly studied. Manipulation of JH signaling by means of JH-I treatment (Röseler, 1977; van Doorn, 1987), treatment with the allatoxin Precocene-I (Amsalem et al., 2014), and by a combination of allatectomy and JH-III replacement therapy (Shpigler et al., 2014), have unequivocally established that JH has gonadotropic functions in this species.

In this study we tested two key predictions of the hypothesis that JH does not influence division of labor in bumblebees. First, we tested whether circulating JH titers are correlated with task performance in field foraging colonies. Second, we developed a method for reducing circulating JH levels in bees by topical application of the allatoxin Precocene-I, and used this method in combination with replacement

therapy with JH-III (the natural JH of bumblebees, Bloch et al., 2000a) to unequivocally test the influence of JH on foraging and brood care activities. Taken together, our findings are consistent with the hypothesis that JH does not influence division of labor in bumblebees.

2. Material and methods

2.1. Bees

Incipient *Bombus terrestris* colonies were obtained from Polyam Pollination Services, Yad -Mordechai, Israel. Each colony contained a queen, 5–10 workers, and brood at various stages of development. Colonies at this stage are typically 2–4 days post-emergence of the first worker (Bloch et al., 1996; Bloch, 1999; Shpigler et al., 2013). Each colony was housed in a wooden nesting box (21 × 21 × 12 cm) in which the top and front walls were made of transparent Plexiglas panels. The nest boxes were placed in an environmental chamber (28 ± 1 °C; 45% ± 10% RH) in constant darkness at the bee research facility at the Edmond J. Safra campus of the Hebrew University of Jerusalem, Givat Ram, Jerusalem. Commercial sugar syrup obtained from Polyam Pollination Services and fresh honeybee collected pollen (mixed with sugar syrup) were provided ad libitum. Indoor observations were performed under dim red light, which the bees cannot see.

2.2. Experiment 1: JH hemolymph titer, division of labor and ovarian state in field foraging colonies

2.2.1. Experimental setup

For Trial 1 (July 2010) six incipient colonies were kept in an environmental chamber. Two focal colonies (marked as 1 and 2) were placed in observation nest boxes as described above. The nest boxes were connected to the outside, allowing the bees to forage for pollen and nectar. The other four colonies were placed in indoor nest boxes (21 × 21 × 12 cm) in the same room and were fed ad libitum with sugar syrup and pollen. Callow workers emerging in these four “donor colonies” were later introduced into the focal foraging colonies. The observation nest boxes were connected to the outside with a clear plastic tube (~1 m length, 2 cm diameter), and food provisioning was gradually tapered until the colony was self-supported at the age of about 10–12 days after first worker emergence. Three days later, when the experimental colonies contained about 30 workers, we started to introduce into them newly emerged workers. We collected the callow bees (0–24 h of age) from the donor colonies and tagged each with an individual colored number disk. In total we introduced 40 bees into colony 1 and 35 into colony 2 over two successive days. In order to keep the colony population at a size appropriate for their developmental stage, we also in parallel removed 30 bees from each experimental colony. This is important because worker number can affect the development and social organization of the bumblebee colony (Bloch, 1999; Woodard et al., 2013). In this setup, all the bees are age-matched but not genetically related. In a second trial (April 2011) we placed three unrelated focal colonies (marked 3, 4, and 5) in free foraging observation nest boxes as described above. This trial differed from the first in that the focal tagged bees emerged in the colony into which they were reintroduced after tagging. Thus, all the workers in each focal colony were full sisters (assuming single mating by the queen). When the colonies were about 10–12 days after first worker emergence, we collected newly emerged workers (0–24 h of age, clearly identified by their pale coloration), individually tagged them, and immediately returned to their home colony (the whole procedure took 60 min or less). We tagged and reintroduced focal bees over six days. Overall we tagged and reintroduced 26, 32 and 29 bees into colonies 3, 4, and 5, respectively. In this experimental setup, the bees are highly related but have a broader age range compared to the previous trial.

2.2.2. Behavioral observations

We observed the colonies in order to record the task performed by each focal bee. We focused on foraging and brood care (“nursing”) activities that are among the most ubiquitous, easiest to detect, and best characterized tasks in the colony. The observations started four days after the last callow bee was tagged and continued over three consecutive days. We observed the colonies for 2 h in the morning (between 07:00 and 10:00), and 2 h during late afternoon (between 16:00–19:00, before sunset), which are the most active foraging time of *B. terrestris* in this location (Yerushalmi et al., 2006). At each observation session we recorded foraging activity next to the nest entrance over one hour, and performed observations inside the chamber to record nursing activities for an additional session of one hour. Nest behavior observations were conducted using techniques to minimize disturbance to the focal colonies. The observation rooms were kept dark at all times. Nest observations were conducted using only dim red light which bees are unable to detect. The observers entered the rooms carefully and avoided touching the tables that held the colonies to avoid any movement or vibration of the colonies. The observations were conducted silently and there were no visible signs that bees were disturbed by the observations. During the observation in the chamber we also recorded bees carrying pollen loads or engaging in other conspicuous foraging related activities. A foraging trip was recorded as a bee flying out and returning to the hive. Larval feeding includes the opening of a brood cell, inserting the mouthparts into the cell, and a conspicuous abdominal contraction that takes a few seconds and during which the crop content of the nursing bees is squeezed out (Ribeiro, 1999; Shpigler et al., 2013; Woodard et al., 2013). A bee that performed three or more foraging trips in at least two of the observation days and was recorded tending brood fewer than three times during the whole observation period was classified as a ‘forager’. A bee that performed more than three feeding events in at least two days, and conducted fewer than three foraging trips during the whole observation period was classified as a ‘nurse’. A few bees that performed both behaviors with high frequency (over 3 times during the observation period) were classified as ‘intermediates’ (Yerushalmi et al., 2006), and not included in the analysis (see details in Table 1).

Shortly after accomplishing the last observation, we collected all the marked bees into a box (one for each colony) for further physiological analysis. The bees were collected at 9 or 10 days of age in Trial 1, and 8 to 14 days of age in Trial 2. In both trials the age of the bees was the same for foragers and nurses (t -test, $t_{(df = 9-23)}$, $t < 1.67$, $p > 0.05$).

2.2.3. JH titer measurements

We chilled each bee on ice for 2–5 min until cold anesthetized, and then fixed it with modeling clay on a wax base surgical plate with the dorsal side facing up. We made a small incision in the membrane connecting the head and the thorax and drew a hemolymph sample (1–7 μ l per bee) using a 10 μ l glass capillary tube (Drummond, Cat #: 5-000-1001). The collected hemolymph sample was immediately transferred into a 5 ml glass vial containing 500 μ l of HPLC grade acetonitrile (Bio lab, Cat # 01203501), and the vial was secured with a Teflon-lined cap. Vials and capillary tubes were baked at 500 °C for 3 h before use to eliminate any fat contamination of the samples. The hemolymph collection process took 1–2 min per bee. We kept the hemolymph samples

Table 1

Foraging and brood feeding behaviors during three days, recorded for bees collected for hemolymph JH titer analysis in Experiment 1. Shown are the mean and range (in brackets) for bees classified as nurses or foragers; N is the number of bees collected.

Colony	Nurses			Foragers		
	Feeding	Foraging	N	Feeding	Foraging	N
1	7.6 (3–12)	0.5 (0–3)	19	0.13 (0–2)	7.8 (3–20)	15
2	8.1 (4–15)	1.0 (0–3)	13	0.8 (0–3)	6.8 (3–13)	6
3	8.7 (5–14)	0.3 (0–1)	11	0.3 (0–1)	4.2 (3–7)	9
4	7.1 (3–15)	0.4 (0–1)	10	0.2 (0–2)	4.0 (3–7)	11
5	7.8 (3–13)	0.2 (0–1)	8	0.1 (0–1)	7.1 (4–11)	9

frozen (–20 °C) until they were shipped on dry ice to Michigan State University for radioimmunoassay analysis. We froze the body of the bee (–20 °C) for further analyses of ovarian development and body size as described below.

Measurements of JH titers were done using a radioimmunoassay (RIA) as described in (Jassim et al., 2000; Bloch et al., 2000a). Briefly, JH was extracted from the acetonitrile (0.5 ml) and 0.9% saline (1 ml) mixture by hexane (1 ml \times 2). The hexane phase, containing JH, was then dried and redissolved in methanol. An aliquot of the methanol was then dried and 200 μ l of phosphate buffer containing approximately 10,000 DPM of [10 -H 3 (N)]-JH III (NEN, 629 Gbq/mmol and a JH antibody (1:14,000 dilution) was added to half of each sample. Because in this experiment we used tritium-labeled JH III both for calculating recovery and for measuring unlabeled JH in the hemolymph, the percent binding in the RIA was adjusted by calculating a new, maximum binding. This was done by assuming that the molecules of additional radioactive JH bind to the JH-antibody in the same proportion as do the radioactive JH molecules in the JH-antibody mixture. The amount of JH in each sample was estimated using the new adjusted percent binding value. The samples were coded such that the individual performing the RIA was blind to the treatment. Of the 94 samples measured, 13 were contaminated with fat and 4 others had very high signal (over 3000 ng/ μ l) and were eliminated as outlier. The remaining 77 samples provided a sample size of 5–10 samples for each behavioral group/colony in Trials 1 and 2.

2.2.4. Assessment of ovarian state and size measurement

We fixed the bees on a wax filled dissecting plate under a stereo microscope (Nikon SMZ645). We used fine scissors to cut three incisions through the lateral and ventral abdominal cuticle and expose the internal organs. We immersed the tissue in honeybee saline (Huang et al., 1991) and gently removed the ovaries into a drop of saline on a microscope slide. We measured the length of all 8 terminal oocytes with an ocular ruler and used the average oocyte length as an index for ovarian state (Bloch et al., 2000b). We measured the length of the front wing marginal cell under a dissection microscope, \times 10 magnification (Nikon SMZ645) and used it as an index for body size. The length of the marginal cell is highly correlated with wing length and other indices for body size, and does not change with age or tear due to flight (Knee and Medler, 1965; Shpigler et al., 2013; Yerushalmi et al., 2006).

2.2.5. Statistical analyses

JH titers and size were compared by t -test, the length of the terminal oocyte was compared by Mann-Whitney test within each colony. We used the Spearman test to determine the correlation between JH-titers and oocyte length for bees in each colony. We used R for all the statistical analyses in this study.

2.3. Experiment 2: the influence of JH on brood care and foraging activities

To reduce JH titers in bumblebees we developed a new method using topical application of the allatotoxin Precocene-I (P-I, Sigma, cat. #195855), which is described below. Precocene I and II are natural extracts from the plant *Ageratum houstonianum*, which can cause atrophy of the corpora allata glands of insects. Precocene treatment reduced in vitro JH biosynthesis by corpora allata glands (Pratt and Bowers, 1977), as well as in many studies with live animals. Several methods have been used to apply precocene solutions to the insect body. These include spraying (Ayyanath et al., 2015), injection (Zhao and Zhu, 2013), topical application (Bowers et al., 1976; Unnithan et al., 1977) and feeding (Amsalem et al., 2014). Treatment with precocene was shown to affect JH regulated processes such as metamorphosis, oogenesis, and diapause induction, and was effective in both hemimetabolous and holometabolous insects (Bowers et al., 1976). Precocene-II treatment was reported to be ineffective in honey bees and, therefore, has not been used in JH research in bees (Fluri, 1983). Recently,

Amsalem et al. (2014) reported that oral treatment with P-I reduced JH hemolymph titers and slowed down ovarian development in queenless *Bombus terrestris* workers. However, while feeding is a convenient and noninvasive method it has the limitation that the exact amount of P-I consumed by each bee is unknown and the treatment takes a relatively long time. Moreover, the drug may affect the food palatability or consumption rate which in turn can affect the bee behavior or physiology. We therefore choose to develop a new noninvasive protocol in which precise, size adjusted amounts of P-I are topically applied to newly emerged bees. The protocol is easy to use and can be combined with replacement therapy with JH.

2.3.1. Reducing JH levels by topical Precocene-I treatment

We collected callow workers (<24 h after emergence from the pupa) and cold anesthetized them in 5 ml tubes immersed in ice (~2 °C) until immobile (5–10 min). The anesthetized bees were placed on ice chilled glass plates such that they were kept immobile during the treatment. Based on previous topical treatment protocols for bees, we applied the drugs in a 3.5 µl vehicle-solvent solution which was placed on the dorsal part of the thorax (Shpigler et al., 2014). We used castor oil as a vehicle for P-I. Castor oil is nontoxic and is used for delivering drugs to mammals (Gooren and Bunck, 2004; Kalepu et al., 2013; Shelke et al., 2007). The P-I and castor oil were mixed before application and vortexed at high speed for 45 s. In order to find the P-I amount that effectively reduced JH with minimal effect on survival we performed serial dilutions of P-I over a range of 0.1–1000 µg per bee (the final volume was always kept at 3.5 µl). Control bees were either treated with vehicle only, or were handled and chilled similarly to the other bees but not topically treated with any solution. Following treatment, the bees were left anesthetized for 10 additional minutes. During this time, it was possible to observe the delivered solution transition from a raised droplet on top of the thorax hairs to a spread out oily film below the thorax hairs. Thus, the waiting period minimized drug wiped off by the bee as it woke up. We then transferred groups of 3–5 workers of the same treatment into small cages (12 × 5 × 8 cm) supplemented with ad libitum food supply (pollen cake and 70% sugar syrup). After seven days we tested the survival and ovarian state (as proxy for JH levels, Shpigler et al., 2014). Following the first set of calibration experiments we narrowed down the tested P-I amounts to the range between 100 and 200 µg, for which survival rate was >75%.

To more finely characterize the influence of different doses of P-I on physiological processes previously shown to be regulated by JH in bumble bees (Shpigler et al., 2014). We randomly assigned 1-day-old bees to 6 experimental groups: Control; P-I 140 µg; P-I 160 µg; P-I 180 µg; P-I 200 µg and vehicle only (castor oil) and placed them for seven days in small orphan ('queenless') groups as described above. Each experimental group included 20 bees in five cages (four bees per cage). The survival was 100%, 90%, 90%, 75%, 80%, 80% respectively. For each group we recorded the following indices: length of terminal oocyte in each ovariole for all bees, number of eggs laid, deposited wax weight, number of wax cells (including egg cups and nectar pots) for each cage. These traits were shown to be strongly influenced by JH in *B. terrestris* workers (Shpigler et al., 2014). P-I dosage were further refined by fine-tuning dosage for bees of different body size, as bumblebee workers can differ up to 10 fold in their body size (Goulson, 2003); marginal cell length was used as a size index (Yerushalmi et al., 2006, Shpigler et al., 2013). By correlating ovarian development and marginal cell length we were able to determine the lowest effective P-I dose for variably sized workers.

Based on these findings we developed a size/dosage protocol for P-I in treatment as describe in Table 2. These doses were later used in Experiment 3. This is to our knowledge the first size dependent drug treatment protocol developed for bumblebees and for insects in general.

2.3.2. JH replacement therapy

The JH replacement therapy treatment followed the protocol developed by Shpigler et al. (2014). One day old bees were treated with P-I

Table 2

Effective non-lethal size-matched Precocene-I amounts to deliver in castor oil for reducing JH levels in *B. terrestris*.

Marginal wing cell length	Precocene-I amount in castor oil
≤2.6 mm	160 µg
2.7–2.8 mm	180 µg
2.9–3.1 mm	200 µg
≥3.2 mm	220 µg

as described above. After one day of recovery from the first treatment we collected the bees and cold anesthetized them as described above. The chilled P-I treated bees were randomly divided into two groups. The first group was treated with 70 µg JH-III (Sigma, cat # J2000) dissolved in 3.5 µl of dimethylformamide (DMF, J.T Backer, cat # 7032) and topically applied to the dorsal part of the thorax. The second group was similarly treated with only DMF. The castor oil only group of the P-I treatment was also similarly treated with DMF. The handling control group was chilled on ice and handled in the same way but was not treated with solvent or JH. Bees were left anesthetized for ~10 min after treatment to minimize drug wiped off by the bee as it woke up (see above).

2.4. Experiment 3: the influence of JH on nursing and foraging activities

2.4.1. Preliminary experiment

Prior to developing the P-I/castor oil protocol described above, we first performed a preliminary experiment in which we used DMF as a vehicle to deliver precocene. One day old bees collected from "donor colonies" were randomly assigned to one of three treatments: 1) Precocene-I ("P-I") size adjusted treatment as described in Supplementary Table 1; 2) Vehicle only – bees treated with DMF as described above; 3) Control – the bees were handled, chilled, and tagged as the bees in the other groups, but were not treated with any drug or solvent. The size adjustment of Precocene-I dose in DMF was determined using the same methods as in developing the castor oil protocol. It is unknown why a different dosage was needed when using the different solvent. We individually marked the bees with colored number tags 1–2 h after the treatment, and allowed the glue to dry (30–60 min) and for the bees to fully recover from the chilling. The tagged bees from the three treatment groups were then introduced into four colonies. Treatments and introductions took place over three days. Each colony was housed in a wooden nesting box (21 × 21 × 12 cm) in which the top and front walls were made of transparent Plexiglas with a free access to outside. For each three bees introduced into a colony, we removed two local bees in order to balance colony population size (Bloch, 1999). Altogether we introduced 27, 26 and 27 bees from the P-I, vehicle only, and control treatment, respectively, into 4 "host" colonies. On the morning following the last introduction of treated bees, we started a series of detailed behavioral observations. The observations started when the focal bees were 2–4 days of age, and continued for five consecutive days. Morning and evening foraging observations were conducted daily. Each observation session lasted 30–90 min. Morning observations were performed between 06:30 to 09:30, and evening observations between 17:30 to 20:00. Bees were observed as they took off and landed on landing platforms. The unique tag ID was recorded for each departing and returning bee. Nursing observations were also conducted daily. Each in nest observation lasted 20–60 min per nest box. Nursing observations were performed at various times throughout the day (09:00–18:00). Nursing and foraging behaviors were defined as described for Experiment 1. Behavioral observations were conducted by AJS and an undergraduate assistant. The focal bees were collected after the last day of observation, when the bees were 7–9 days of age, we then determined their ovarian state as described above (Experiment 1). The results of this preliminary study can be found at Fig. S1.

2.4.2. Primary experiment

The experiment details are as in the preliminary experiment with the exceptions that the P-I was dissolved in castor oil (adjusted to size as describe in Table 2), and a replacement therapy group (P-I + JH) was added. Altogether we introduced 37, 34, 38 and 37 bees from the P-I, P-I + JH, vehicle only, and control treatment, respectively, divided evenly between three “host” colonies that were housed in observation nests. On the morning following the last introduction of treated bees, we started a series of detailed behavioral observations. At the first day of observations the focal bees were 3–5 days of age. Behavioral observations were conducted by AJS and an undergraduate assistant as described for the preliminary experiment. Bees were classified as a ‘forager’; or a ‘nurse’ based on the criteria described for Experiment 1. Table 3 summarizes brood feeding and foraging activities for bees classified as ‘nurses’ and ‘foragers’ in the main experiment.

The focal bees were collected, after the last observation day at the age of 7–9 days, and their ovarian state was determined as described for Experiment 1. The survival of the bees was 62%, 61%, 73% and 75% for P-I, P-I + JH, vehicle only, and control, respectively.

2.5. Statistical analysis

The distribution of the number of nurses and foragers in each experimental group was tested for deviation from a random distribution using χ^2 test for independence. The rate (number of acts per hour) of nursing and foraging activities between the experimental groups was compared using one-way ANOVA followed by a Tukey post hoc test. We used Kruskal-Wallis test followed by Nemenyi post hoc test for pair wise comparisons to test the influence of treatment on ovarian state (length of terminal oocyte does not fit normal distribution).

3. Results

3.1. Experiment 1: JH hemolymph titer, task performance and ovarian state in field foraging colonies

Hemolymph JH titers were similar for nurses and foragers in three of five colonies (Fig. 1A; colonies 2, 3, 5; two-tailed t -test, $t_{(df > 9)} \leq 1.66$, $p > 0.11$, $d < 0.90$). In the two other colonies, JH titers were significantly higher for the nurses (Fig. 1A; colonies 1, 4; two tails t -test: $t_{(df = 15)} = 4.03$, $p = 0.001$ $d = 2.01$; $t_{(df = 15)} = 2.79$, $p = 0.014$, $d = 1.40$, respectively). The ovaries of nurses and foragers were similarly developed in the three colonies in which JH titers were similar (Fig. 1B; colonies 2, 3, 5; Mann-Whitney test, $Z < 1.25$, $p > 0.2$, $r < 0.31$), and significantly larger in nurses in the two other colonies, in which the nurses also had significantly higher JH titers (Fig. 1B; colonies 1, 4; Mann-Whitney test: $Z = 3.68$, $p < 0.001$, $r = 0.75$; $Z = 2.03$, $p = 0.04$, $r = 0.49$; respectively). Given that JH levels vary significantly among colonies, we tested the correlation between JH titers and terminal oocyte length separately for each colony. We found that bees in colonies 1 and 4 in which JH levels and ovarian development were different between nurses and foragers also had a significant linear correlation between the two variables (Fig. 1C; Spearman rank correlation: colony 1: $\rho = 0.83$, $p < 0.001$; colony 4: $\rho = 0.84$, $p < 0.001$). In bees from

colonies 2, 3 and 5 we did not find a significant correlation between JH titer and terminal oocyte length (Fig. 1C). Foragers were significantly larger than nurses in four of the five colonies (Fig. 1D; colonies 1, 2, 3, 5; two-tailed t -test, $t_{(df > 9)} \geq 2.26$, $p < 0.041$, $d > 1.23$). This finding is consistent with the size-related division of labor of bumblebees and suggests that our behavioral observations and collection protocol adequately classified bees as nurses and foragers. These results suggest that specialization in nursing or foraging activities is not associated with differences in JH titers. The higher JH hemolymph titers in nurses in a few of the colonies appears to be related to their better developed ovaries rather than to the nursing tasks they perform.

3.2. Experiment 2: reducing JH levels by topical treatment with Precocene-I

Given that it has already been shown that oral treatment with P-I reduces JH titers as well as affecting JH regulated physiology (Amsalem et al., 2014), here we limited ourselves to measuring indices of JH controlled physiology. We found a significant dose-dependent effect of P-I on ovarian development (Fig. 2A, Kruskal-Wallis, $\chi^2_{(df = 5)} = 43.0$, $p < 0.001$, $\eta^2 = 0.42$), egg laying (Fig. 2B; Kruskal-Wallis, $\chi^2_{(df = 5)} = 17.2$, $p = 0.004$, $\eta^2 = 0.59$), the construction of wax cells (Fig. 2C; Kruskal-Wallis, $\chi^2_{(df = 5)} = 23.8$, $p < 0.001$, $\eta^2 = 0.82$) and the amount of wax deposited in the cage (Fig. 2D; Kruskal-Wallis, $\chi^2_{(df = 5)} = 25.4$, $p < 0.001$, $\eta^2 = 0.87$). The castor oil treated bees were similar to the control bees for all measurements (Fig. 1A and Fig. 2, Nemenyi post hoc test, $p > 0.05$). This is important as some of the components of castor oil influenced ovary development in ticks (Sampieri et al., 2012; Sampieri et al., 2013). The bees in the different treatment groups did not differ in size (data not shown, ANOVA, $F_{(df = 5)} = 0.35$, $p = 0.87$, $\eta^2 = 0.02$). Following the dose-response experiment we used the doses summarized in Table 2 for the following experiments. Given that *B. terrestris* workers show broad body size range we further refined our protocol by adjusting the P-I dose to the bee body size (see Table 2 and Material and Methods section).

3.3. Experiment 3: the influence of JH on brood care and foraging activities

We first performed a preliminary experiment that included three treatments: handling control, vehicle only (DMF), and P-I in DMF. We found that the three groups differed in ovarian development (Fig. S1; Kruskal-Wallis, $\chi^2_{(df = 2)} = 6.35$, $p = 0.042$, $\eta^2 = 0.15$). The P-I treatment however, did not affect foraging or nursing activity (Fig. S1; one-way ANOVA, foraging: $F_{(df = 2)} = 0.63$, $p = 0.53$, $\eta^2 = 0.03$; nursing: $F_{(df = 2)} = 0.39$, $p = 0.68$, $\eta^2 = 0.02$).

In the main experiment we used castor oil rather than DMF as a vehicle for the P-I treatment, and added a fourth treatment of replacement therapy with JH-III. The frequency of both nursing and foraging activities did not differ among bees from the four treatment groups (Fig. 3A and B; one way ANOVA: nursing, $F_{(df = 3)} = 1.02$, $p = 0.39$, $\eta^2 = 0.04$; foraging, $F_{(df = 3)} = 0.37$, $p = 0.77$, $\eta^2 = 0.04$). In a complementary analysis, we classified the bees as a nurse or forager (as described in the methods for Exp. 1). Using this approach, we found that the proportion of nurses and foragers was similar across the four treatment groups (Fig. 3C; $\chi^2_{(3)} = 0.46$; $p = 0.92$). We did find a significant influence of precocene treatment on ovarian state (Fig. 3D, Kruskal-Wallis test, $\chi^2_{(df = 3)} = 9.6$; $p = 0.022$, $\eta^2 = 0.13$). Bees treated with P-I had the smallest terminal oocytes in their ovaries which were significantly smaller than in the control group (Nemenyi post hoc test, $p = 0.014$). Replacement therapy recovered oocyte growth to the level observed for control bees. However the observed increase in terminal oocyte length compared to the P-I treated bees was not statistically significant (Nemenyi post hoc test, $p = 0.23$). The vehicle only group did not differ statistically from the other three groups. Body size cannot account for the observed differences in ovarian state as the marginal cell length did not differ between treatment groups (data not shown; ANOVA: $F_{(df = 3)} = 1.21$; $p = 0.3$, $\eta^2 = 0.05$). The influence of the treatment

Table 3

Foraging and brood feeding activities, recorded for bees with manipulated JH levels in Experiment 3. Shown are the mean and range (in brackets) for bees classified as nurses or foragers; N is the sample size.

Treatment	Nurses			Foragers		
	Feeding	Foraging	N	Feeding	Foraging	N
Control	6.9 (3–13)	0.2 (0–2)	15	1.2 (0–3)	8.2 (3–12)	5
Castor oil	6.8 (3–15)	0.4 (0–2)	13	2.1 (0–3)	6.6 (3–20)	7
P-I	6.8 (3–13)	0.2 (0–1)	12	0.8 (0–2)	4.4 (3–7)	5
P-I + JH	6.7 (3–11)	0.2 (0–2)	13	1.3 (0–3)	4.3 (3–7)	6

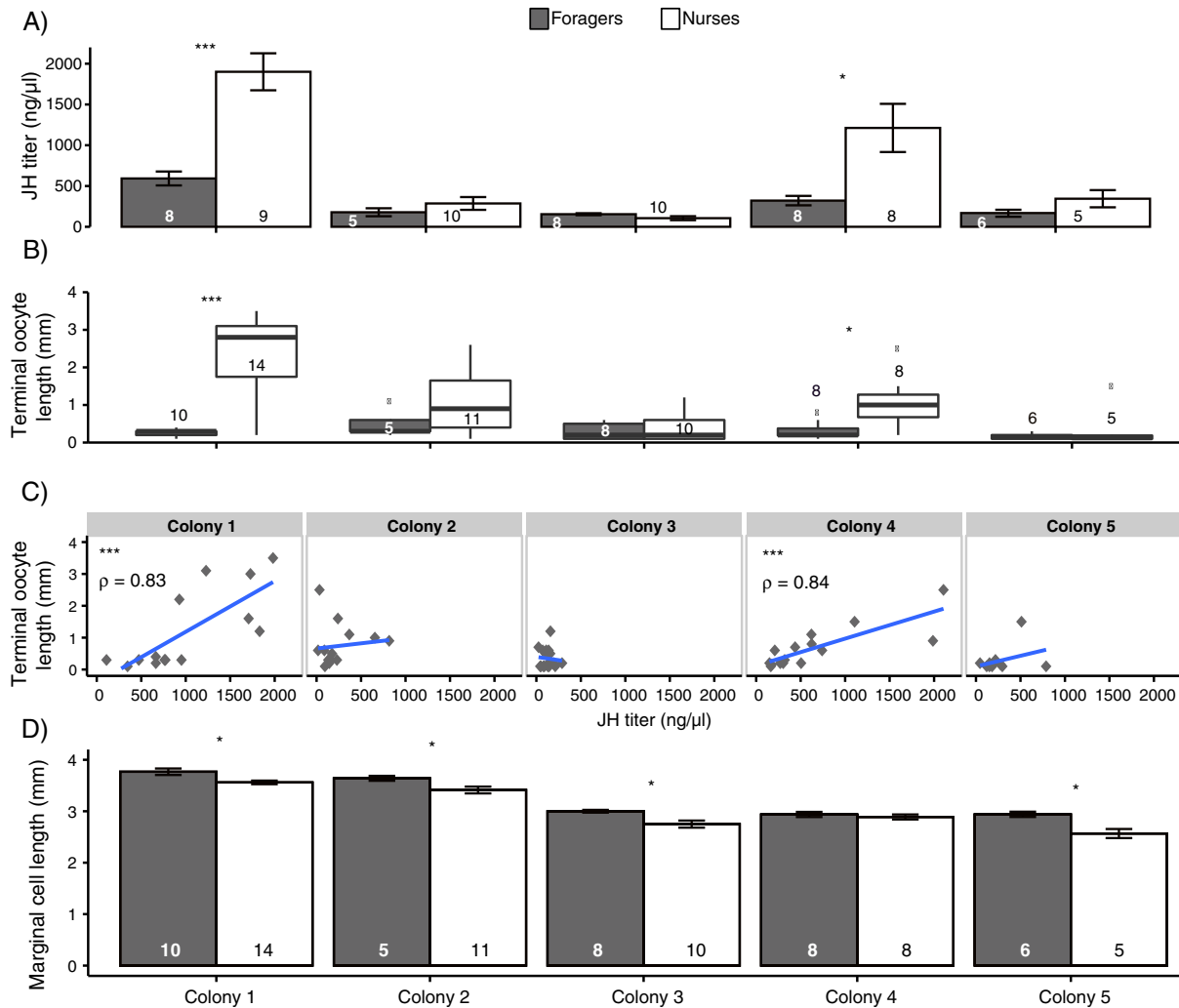


Fig. 1. The relationship between JH titers, task performance, and ovarian state in *Bombus terrestris* colonies. (A) Hemolymph JH titers, (B) terminal oocyte length, (C) the correlation between JH titers and terminal oocyte length, and (D) body size. Foragers – filled boxes, nurses – open boxes. We repeated this experiment with 5 different colonies. For graphs A, D the bars represent average \pm SE. For graph B the boxes shows the median (black middle line) and the upper and lower lines of the box show the interquartile range (IQR) between the first quartile (Q1) and the third quartile (Q3). The whiskers are the upper and lower adjacent values, which are the most extreme values within $Q3 + 1.5 * IQR$ and $Q1 - 1.5 * IQR$, respectively, outliers are indicated with a dot. Significant p-value (* $p < 0.05$; *** $p < 0.001$) were obtained from *t*-tests for JH titer (A) and body size (D), and Mann-Whitney tests for terminal oocyte length (B). Sample sizes are in the boxes/bars of each graph. For graph C, each diamond represents a single bee; the blue line depicts the linear regression model for the relationship between JH titer and terminal oocyte length. ρ and *p*-value (***) were obtained from Spearman's rank correlation analyses.

on ovarian development serves as a positive control indicating that our manipulations were effective. The modest differences in ovarian state in this experiment compared to Experiment 2 are probably explained by the fact that the bees in this experiment were in queen-right conditions under which the ovaries develop slowly (Bloch et al., 1996; Duchateau and Velthuis, 1989; Röseler, 1974), and therefore the ovaries of the control groups were also not well developed (compare ovary size in Fig. 3 and Fig. S1 to Figs. 1 and 2).

4. Discussion

Our measurements of JH titers in bees performing nursing and foraging activities and the results of the manipulation experiments are consistent with the hypothesis that JH does not influence task performance and thus, the division of labor among bumblebee workers (Cameron and Robinson, 1990). These findings contrast with studies in honeybees in which foraging behavior is associated with high JH titers, and manipulations of JH titers influence the time of transition from nursing to foraging activities (Robinson, 1985, 1987; Sullivan et al., 2000). In fact, our study suggests that the situation in the bumblebee

is almost opposite to the honeybee because foragers had lower or similar JH titers compared to nurses. Taken together with the strong evidence that JH functions as a gonadotropin in *B. terrestris* (Amsalem et al., 2014; Shpigler et al., 2014; Fig. 2, Fig. 3), our study provides strong support to the hypothesis that JH influences different aspects of social organization (regulation of reproduction vs division of labor among workers) in bumblebees and honeybees.

To our knowledge this is the first study measuring JH titers for nurses and foragers in a bumblebee species. It is notable that in two of five colonies JH levels were significantly higher for nurses compared to foragers. A similar trend was seen in two additional colonies in which the differences were not statistically significant (Fig. 1A, colonies 2 and 5). These findings are consistent with an earlier study showing that in two out of three colonies nurses had higher levels of *Krüppel homolog-1* (*Kr-h1*) (Shpigler et al., 2010). The transcription factor *Kr-h1* is a major readout of JH in insects (Cui et al., 2014; Pandey and Bloch, 2015; Song et al., 2014), and was shown to be upregulated by JH also in *B. terrestris* (Shpigler et al., 2010). Given the higher levels in nurses, perhaps JH influences division of labor, but in a different way than in honeybees? For example, JH may stimulate brood care activities,

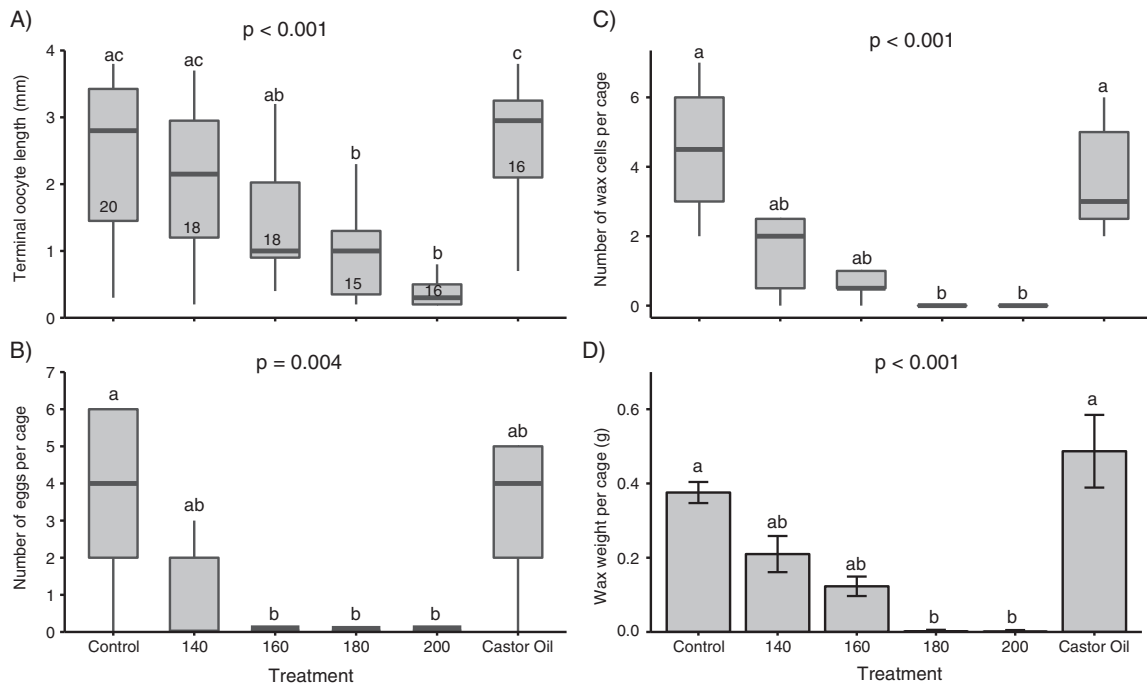


Fig. 2. Dose dependent effect of Precocene-I on reproduction, wax secretion, and cup building in small queenless groups. (A) Length of terminal oocytes; (B) number of eggs per cage; (C) number of wax cells per cage (egg cells and sugar pots); (D) the weight of wax deposited. The values on the X axis are μg Precocene-I. For graphs A, B and C; the boxes details as in Fig. 1B. For graph D the bar represents average \pm SE. Sample sizes are inside the boxes in A; sample size was 5 cages (groups) for each treatment in B, C and D. The p-values were obtained by Kruskal-Wallis tests; groups with different letters are significantly different in Nemenyi post hoc test ($p < 0.05$).

or inhibit foraging behavior. We believe that this is not the case, but rather that the high JH and *Kr-h1* levels in nurses relates to their better developed ovaries, rather to causative relationships between JH and brood tending behavior. The first support for this premise is the high correlation between JH and ovarian state in Experiment 1. In colonies 1 and 4 (Fig. 1) in which nurses had significantly higher JH titers, their

ovaries were also better developed compared to foragers, and the patterns for JH and ovary state appear similar also in the three other colonies (compare Fig. 1A and B). Moreover, in the colonies in which the range of ovarian development was broad enough JH titers were positively correlated with the length of the terminal oocyte (an index for ovarian state; Fig. 1C). The reason for the differences in JH levels

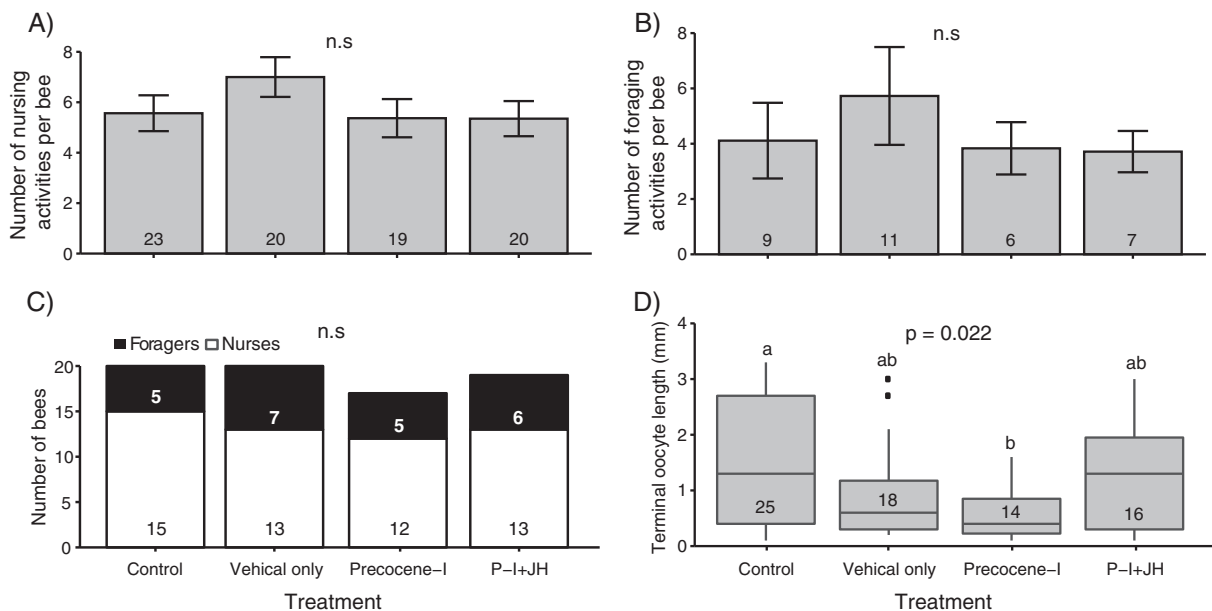


Fig. 3. The influence of JH on nursing and foraging activities. To manipulate JH levels we performed four treatments: 'Control': bees handled and chilled but not treated with a drug or vehicle; 'Vehicle only': bees treated with castor oil and DMF; 'Precocene-I': bees were treated with Precocene-I (in castor oil); 'P-I + JH': replacement therapy, bees treated with both Precocene-I (in castor oil) and JH-III (in DMF). (A) Nursing activity (number of larval feeding events per bee during the whole observation period). (B) Foraging activity (number of foraging trips during the whole observation period). Foraging and nursing activities were similar across treatment groups (one way ANOVA: Nursing $p = 0.39$, Foraging: $p = 0.77$). The bar represents average \pm SE. Sample sizes are inside the boxes. (C) The number of bees classified as nurses (white) and foragers (black) in the experimental groups (χ^2 test: $p = 0.92$). (D) Ovarian state. Additional details are in Fig. 1B. The p-values were obtained from Kruskal-Wallis tests; groups with different letters are significantly different in Nemenyi post hoc test ($p < 0.05$).

between colonies are unknown but may reflect both developmental and genetic colony variation (Giray et al., 1999). The manipulation of JH titers by P-I and JH-III treatments further corroborated the premise that JH does not affect nursing (or foraging) activity. In both the preliminary experiment and in Experiment 3, our manipulations affected ovarian development (a positive control), but not task performance (Fig. 3 and Fig. S1).

The finding that JH does not influence division of labor in *B. terrestris* leaves the question of endocrine regulation of division of labor in bumblebees (and other species with size related division of labor) open. The lack of JH influence stresses the need to test the influence of additional endocrine and neuroendocrine signals on task performance in bumblebees. It is also possible that hormonal regulation is less important for the regulation of task performance in bumblebee workers, many of which (specifically medium and large individuals) perform both foraging and nursing activity on the same day (Yerushalmi et al., 2006). Perhaps hormone action is too slow for regulating task switching at this time scale, or that the influence of endocrine functions on division of labor is limited to priming some individuals to be more likely than others to perform a certain task (e.g., foraging). In honeybees, nurses and foragers represent two distinct behavioral/physiological states, differing in physiology, brain neuroanatomy, and the pattern of gene expression in various tissues. For example, JH coordinates many of this task related changes by acting on various tissue such as the brain (Whitfield et al., 2006), hypopharyngeal glands (Ueno et al., 2015), and fat body (Ament et al., 2012). By contrast little is known on physiological or molecular correlates of task performance in bumblebee workers, and whether task performance is associated with coordinated modifications in various tissues.

Our manipulation experiments (Experiments 2 and 3) provide additional experimental support for the hypothesis that JH functions as a gonadotropin in bumblebees (Amsalem et al., 2014; Shpigler et al., 2014). This strong evidence for JH influence on fertility, together with the lack of JH influence on task performance in the current study, underscore the enigma concerning the evolution of JH signaling in the Hymenoptera, and its possible association with the evolution of advanced sociality. The regulation of fertility is considered the ancestral function of JH in insects (Riddiford, 2008; Riddiford, 2012). The evidence suggesting that JH functions as a gonadotropin in solitary bees (Smith et al., 2013) and wasps (Tibbetts et al., 2013), as well as in “primitively” social *Polistes* wasps (Giray et al., 2005), and bumblebees (this study, Amsalem et al., 2014; Shpigler et al., 2014; Bloch et al., 2000a) is consistent with an ancestral gonadotropic function in the Hymenoptera. But the situation is different in the honeybee *A. mellifera* in which JH does not affect adult female reproduction (Pinto et al., 2000; Robinson et al., 1992), but rather paces the transition from nursing to foraging activities (Sullivan et al., 2000).

How can JH regulate apparently two different functions in two related species? Two main hypotheses have been proposed for addressing this evolutionary enigma. The ‘novel- or single-function hypothesis’ proposes that the role of JH has changed from reproductive functions in solitary and “primitively” eusocial species (those with small and simple societies which commonly lack morphologically distinct queen and worker castes), to an exclusively behavioral function in highly eusocial societies. The split-function hypothesis on the other hand, proposes that JH originally functioned in the regulation of both reproduction and behaviors such as foraging or guarding in ancestral solitary species. Later in evolution, when reproductive and brood-care tasks were divided between queens and workers, the effects of JH were also divided, with JH involved in regulating reproductive physiology in queens, and behavioral maturation, manifested as age-correlated changes, in worker tasks (Giray et al., 2005; Robinson and Vargo, 1997; West-Eberhard, 1969). A major difficulty of the novel function hypothesis is understanding how a hormone can end regulating an essential processes such as reproduction and at about the same time take on a different essential processes such as regulating age-related division of labor. A key

prediction of the split function hypothesis is that there should be species showing an intermediate stage in which JH influences both reproduction and behavior. Studies in wasps suggesting that in some species JH influences both ovarian activity and age-related changes in task lend credence to the split function hypothesis (Giray et al., 2005; Shorter and Tibbetts, 2009). However, recent studies show no consistent relationships between JH, social organization, task performance and reproduction, suggesting that the influence of JH on social behavior is much more complex than suggested by the split function hypothesis (Kelstrup et al., 2014; Tibbetts et al., 2013; Tibbetts and Sheehan, 2012). Moreover, even if there was a stronger support for one of these hypotheses in wasps, it should be acknowledged that the interplay between JH signaling and social evolution is not necessarily the same in bees and wasps.

Bumblebees provide an excellent model system with which to study the interplay between JH signaling and social evolution because the complexity of their social organization seems intermediate between solitary and facultatively social bees on one side, and advanced eusocial species such as honeybees and stingless bees on the other. In contrast to honeybees, in *B. terrestris* it is clear that JH regulates fertility. Thus, according to the split function hypothesis it could be expected that in bumblebees JH influences both task performance and reproduction, but our study shows that this is not the case. If JH indeed influenced foraging behavior in ancestral solitary bees, then this influence may have been lost in bumblebees. It should be also noted that in honeybees JH influences the development of flight behavior and the gonads in a similar way in workers, queens, and drones (de Oliveira Tozetto et al., 1997; Engels and Ramamurthy, 1976; Giray and Robinson, 1996; Harano, 2013; Wegener et al., 2013) which is also not consistent with predictions of the split function hypothesis. Studies on the functions of JH in additional bee species showing broad levels of social organization (including solitary species) are needed for clarifying this point. In addition, it is important to understand the molecular and physiological processes that are regulated by JH in the brain and other tissues of bees representing various degrees of social complexity.

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