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Original Article

THE RELATIONSHIP OF WEIGHT AND OVARIAN DEVELOPMENT IN *BOMBUS TERRESTRIS* L. WORKERS UNDER DIFFERENT SOCIAL CONDITIONS

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Abstract

Egg-laying behaviour of bumblebee workers is highly flexible and influenced by the bees' social status and social environment. In this study, the dependence of the starting time of ovarian development in *Bombus terrestris* workers was determined under four different social conditions. The purpose was to test the effect of group stimulation and the presence of a queen on ovarian development. The relationship between weight and the commencement of ovarian development in workers was also investigated. We created four test groups: (1) 8 callow workers with a queen, (2) 8 callow workers without a queen, (3) 1 callow worker with a queen, and (4) 1 callow worker without a queen. Mated and hibernated (freshly awoken) *B. terrestris* queens made up Group 1 and 3. There was no significant difference between the starting times of ovarian development in the experimental groups composed of 8 callow workers in queenright (9.81 ± 2.44 days) and queenless (9.53 ± 2.53 days) conditions. However, ovaries of workers confined singly with a queen started to develop significantly earlier (11.77 ± 3.30 days) than workers confined singly without a queen (14.70 ± 3.56 days). The observations indicate that the presence of a queen does not inhibit the ovarian development of workers. The Point-Biserial Correlation Coefficient between the starting of ovarian development in workers and their weight was −0.013. However, there was a positive correlation between the weight and the ovarian developmental status of workers in groups containing 8 workers.

Keywords: *Bombus terrestris,* worker reproduction, ovarian development, weight of workers

INTRODUCTION

Because of their contribution to the pollination of crops, especially in greenhouses, there is worldwide scientific and commercial interest in bumblebees (Velthuis & van Doorn, 2006). Currently, more than 200 bumblebee species have been identified but the main species reared as a pollination agent is *Bombus terrestris* (Williams, 1998). Bumblebees are also model organisms for physiological and behavioural studies in social insects (Baer, 2003; Alaux et al., 2005).

B. terrestris colonies are annual and are headed by one singly-mated queen (Duchateau & Velthuis, 1988). There are three critical phases of colony development. The first phase, termed 'colony initiation', is the beginning of the social phase. The hibernated queen lays diploid eggs and produces the first workers in this first phase. The second phase is when the queen switches to laying haploid eggs concurrently with diploid eggs. However, this switch does not occur in colonies that produce no males (Gosterit, 2011). The 'competition point', which can occur in both early and late stages of colony life, initiates the third critical phase (Duchateau & Velthuis, 1988). This phase is characterised by oophagy by the founder queen, egg-robbing and attacks on the founder queen by

workers. Egg-laying by workers takes place in the third phase (Duchateau, 1991; Cnaani et al., 2000). Because workers are haplodiploid, they are capable of producing male offspring from unfertilised, haploid eggs (Lopez-Vaamonde et al., 2004). However, this process is limited. Although about 40% of workers eventually lay eggs, either during the competition phase or under queenless conditions, on average 95% of males are queen-produced (Alaux et al., 2004; Amsalem et al., 2015). These egg-laying workers, which also carry out in-nest tasks, inhibit oogenesis in younger workers and compete with the queen for male production. The conflict starts between the founder queen and workers, and also among workers (van Honk et al., 1981; Bloch & Hefetz, 1999; Foster at al., 2004; Alaux et al., 2006).

The competition phase, which can begin before the switch point, is correlated with the time of gyne production by colonies (Bloch, 1999; Gosterit, 2011). This conflict, which reduces colony efficiency, is affected by a number of factors. According to the classical hypothesis, pheromones produced by the founder queen inhibit ovarian development in the workers and prevent or delay their egg laying until the worker population reaches a certain size (van Honk et al., 1980; Roseler et al., 1981). However, it was shown that such pheromones are not spread by workers, although queenlessness does lead to earlier worker aggression (Lopez-Vaamonde et al., 2007). Nevertheless, according to Bloch & Hefetz (1999) the reproductive behavior of *B. terrestris* workers is inhibited by the queen, although the workers themselves also play an important role. Duchateau & Velthuis (1989) suggested that social interactions between individuals affect the ovarian development of workers. Furthermore, worker-size is a factor influencing the egg-laying behavior of workers that emerge from the first batch of eggs (van Honk et al., 1981). Ovarian development is related to the activity of the corpora allata. Older workers and the queen have inhibitory effects on the corpora allata in bumblebees (Roseler et al., 1981; Bloch et al., 1996; Cnaani et al., 2000). Females of *Psythirus vestalis* and *P. bohemicus*, which are the parasites of many bumblebee species, inhibit ovarian development and egg-laying of *B. terrestris* workers (Vergara et al., 2003). Together, the above studies show that many factors affect ovarian development and egg-laying in workers of *B. terrestris.* Therefore, further research is needed to understand the mechanisms underlying worker reproduction in *B. terrestris.*

Worker reproduction in *B. terrestris* also affects colony developmental traits. Eggs laid by workers eventually lead to the increase in the numbers of males produced, and to a decrease in a colony's pollinator capacity. However, reproductive workers do not affect the gyne production (Lopez-Vaamonde et al., 2003). Placing a queen together with some callow workers is widely used to stimulate egg-laying (colony initiation) in the commercial rearing of *B. terrestris* (Gurel & Gosterit, 2008). These callow workers may start to lay eggs, and compete with the queen for the opportunity to lay eggs. Consequently, males may emerge in the first brood. This early emergence of males restricts the number of workers in the first brood and negatively affects colony development. Therefore, knowledge of the factors affecting egg-laying by workers is also important for the mass-rearing of *B. terrestris*.

In the present study, we modified the experiment of Duchateau & Velthuis (1989) to investigate the effect of group stimulation and the presence of a queen on ovarian development in workers. We measured the time that ovarian development commenced in *B. terrestris* workers and compared the ages when their ovaries started to develop in different social conditions (confined singly or in groups of 8 workers, in queenright and queenless conditions). We also investigated the association between body weight and the starting time of ovarian development of workers.

MATERIAL AND METHODS

Experimental design

Mated and hibernated *B. terrestris* queens were obtained from our laboratory-reared stocks.

The duration of diapause of all queens was about 8 weeks. To obtain callow workers (less than 12 hours old), old pupae were collected from laboratory-reared colonies and transferred to an incubator (30°C and 60% RH). Emerged workers were removed from the incubator, mixed, and placed into small starter boxes (8 × 8 × 6 cm) obtained from a company that rears *B. terrestris* commercially (Bio Group, Antalya-Turkey). To test the effects of different social conditions on worker ovarian development, we created four treatment groups. Group 1: 8 callow workers with 1 mated and hibernated queen (N= 24), Group 2: 8 callow workers without a queen (N= 24), Group 3: 1 callow worker with 1 mated and hibernated queen (N= 50), and Group 4: 1 callow worker without a queen (N= 50). Thus, in total, 484 callow workers and 74 mated and hibernated queens were used. The experiment was carried out in the dark in a climate-controlled room (28–29°C and 50% RH). During the experiment, all boxes were supplied with freshly thawed pollen collected by honeybees and sugar solution (50 Brix) ad libitum.

In controlled conditions, hibernated *B. terrestris* queens generally commence laying eggs of the first brood within about two weeks of being placed in the rearing box. After this stage, worker helpers are not required to stimulate the egg-laying of the queens. In Groups 1 and 2, composed of 8 callow workers in queenright and queenless conditions, the abdomens of workers from 2 of the 24 boxes were dissected and checked daily between the 2nd and the 13th day after placement in the starting boxes. This was done to determine whether their ovaries had started to develop. In Groups 3 and 4, from workers confined singly with and without a queen, the abdomens of workers from 10 of the 50 boxes were dissected and checked on day 4, 7, 10, 13, and 16. All workers were weighed individually on an electronic balance (sensitivity ±0.001 g) before the dissection. Dissection of the abdomen was performed under a microscope, cutting around and removing the dorsal surface with scissors, to determine ovary status. The time of commencement of ovarian development could easily be determined from the presence of unripe or ripe eggs.

Statistical analysis

For data analysis, SPSS Statistical Software was used. Groups 1 and 2 (referred to as Experiment A) and Groups 3 and 4 (Experiment B) were analysed separately because of the different numbers of workers (singly or in group) and observation days. We used Kaplan-Meier survival analysis to estimate the mean start time of ovarian development. The difference between the two groups in terms of the start time of ovarian development for Experiments A and B were compared using the Log Rank (Mantel-Cox) test. Ovarian development status at different worker ages was compared by Chi-Square tests in Experiments A and B. A t-test and calculation of the Point-Biserial Correlation Coefficient was used to determine the effect of the body weight of workers on their ovarian development.

RESULTS

The total numbers of workers that developed ovaries were: 103 in Group 1, 111 in Group 2, 36 in Group 3, and 30 in Group 4. In Experiment A, in the two groups composed of 8 callow workers, there were no significant differences between queenright and queenless conditions in the age at which workers' ovaries started to develop (Tab. 1). However, in Experiment B, the ovaries of workers confined singly with a queen (Group 3) started to develop significantly earlier than workers confined singly without queen (Group 4, $P<0.01$).

The percentages of workers in the four groups whose ovaries commenced development at different ages is shown on Tab. 2. In Experiment A (8 callow workers) the proportions were statistically different between the queenright and queenless groups at the ages of 5 days ($P=0.03$) and 7 days ($P=0.00$). This proportion was also significantly different between the queenright and queenless groups which included only 1 worker (Experiment B) at the age of 7 days ($P= 0.05$). At the end of the 13 days of Experiment A, the overall proportions of workers whose ovaries had started to develop were 57.5% (103 out of the 179 dissected workers) in Group 1 and 63.4% (111

Table 1
The age (days) at which ovarian development commenced in workers in different social conditions

Experiment A (8 workers)				Experiment B (single worker)			
	Mean ± S.D	95% Confidence Interval			Mean ± S.D	95% Confidence Interval	
Group 1	9.81 ± 2.44	9.35	10.29	Group 3	11.77 ± 3.30	10.70	12.84
Group 2	9.53 ± 2.53	9.06	9.99	Group 4	14.70 ± 3.56	13.48	15.92
Overall	9.67 ± 2.48	9.34	10.01	Overall	13.33 ± 3.65	12.46	14.19
Log Rank Chi-Square: 0.385; P=0.535				Log Rank Chi-Square: 13.679; P=0.000			

Groups 1 and 3, queenright; Groups 2 and 4, queenless.

out of the 175 dissected workers) in Group 2. In contrast, after the 16 days of Experiment B, these proportions were 73.5% (36 out of the 49 dissected workers) in Group 3 and 60.0% (30 out of the 50 dissected workers) in Group 4. However, the differences between the two groups in each experiment were not significant (Experiment A: P=0.278, $\chi^{2=}$ 1.283; Experiment B: P=0.072, $\chi^{2=}$ 3.568).

The Point-Biserial Correlation Coefficient between the commencement of ovarian development and the body weights of 453 workers that were examined throughout the study, was determined as −0.013. A significant difference was only found among the weight of workers which their ovaries started and did not start to develop in Experiment A which contained 8 workers (P<0.01), while this difference was not statistically important in Experiment B (Tab. 3).

Table 2
The proportions (%) of workers whose ovaries commenced development at different ages (days) in different social conditions (%)

Experiment A (8 workers)												
Days	2	3	4	5	6	7	8	9	10	11	12	13
Group 1	6.3	0.0	50.0	37.5	56.3	46.7	92.9	92.9	57.1	100.0	80	92.9
Group 2	0.0	0.0	26.7	75.0	81.3	100	61.5	100.0	73.3	91.7	92.9	78.6
Chi-Square	1.03	-	1.77	4.57	2.32	10.3	3.82	1.04	0.84	1.13	1.01	1.17
P	0.31	-	0.18	0.03	0.13	0.00	0.05	0.31	0.36	0.29	0.32	0.28

Experiment B (single worker)					
Days	4	7	10	13	16
Group 3	0	90.0	80.0	100.0	100.0
Group 4	0	50.0	60.0	70.0	80.0
Chi-Square	-	3.81	0.95	3.53	2.01
P	-	0.05	0.33	0.06	0.15

Groups 1 and 3, queenright; Groups 2 and 4, queenless.

Table 3
The weight of workers affecting whether ovarian development commenced in different social conditions

	Ovaries started to develop	n	Mean weight ± S.D	P
Experiment A (8 workers)	Yes	214	0.256 ± 0.058	0.003
	No	140	0.236 ± 0.047	
Experiment B (single worker)	Yes	66	0.239 ± 0.049	0.603
	No	33	0.245 ± 0.060	

Queenright and queenless groups were not distinguished; n values indicate the total numbers of workers dissected in Experiments A and B.

DISCUSSION

In bumblebees, the dominance rank and reproductive capacity of workers are determined by morphological and physiological characteristics, including age, body size, and task, and also by external factors, including colony demography and the spatial organisation of workers in the nest (Amsalem et al., 2015). Bloch & Hefetz (1999) reported that oviposition of *B. terrestris* workers occurs at a wide range of ages, between 6 and 81 days. In this study, the mean starting time of ovarian development among all groups varied from 9–15 days. This result is consistent with the conclusion of Duchateau & Velthuis (1989).

There was no significant difference between queenright and queenless conditions in the starting time of ovarian development in the groups composed of 8 callow workers. But interestingly, ovaries of workers confined singly with a queen started to develop significantly earlier than workers confined singly without a queen. The presence of a queen induced earlier ovarian development of workers in these groups. Our results do not support the classical hypothesis that worker reproduction is inhibited by a queen pheromone (Roseler et al., 1981; Duchateau & Velthuis, 1988). It was reported by Lopez-Vaamonde et al. (2007), that queenlessness causes earlier worker aggression. We do not fully understand the meaning of this difference but it could be explained by reproductive competition in relation to the mutual interests of the queen and workers.

There are no published data on the association between weight and the commencement of ovarian development in workers. But, there have been several studies of egg-laying behavior in relation to worker size. van Doorn (1989) found a positive correlation between body size and dominance rank in small groups of four workers of unequal body size under queenless conditions. van Honk et al. (1981) found that the association between worker size and egg-laying behaviour only appeared in the first batch of eggs under queenright conditions. In the present study, considering all groups (a total of 453 workers) we found that there was no relationship between weight and the commencement of ovarian development of workers. However, the groups containing 8 workers (Groups 1 and 2) showed a positive correlation between weight and ovarian development. Furthermore, the ovaries of workers housed in groups of 8 started to develop earlier (9.67 ± 2.48 days) than the workers confined singly (13.33 ± 3.65 days) (combining groups with and without a queen; Tab. 1). These findings could reflect the behavioural hierarchy and dominance among the workers within groups.

Previous research has shown that the addition of a single callow worker of *B. terrestris* (as in

Group 3 in the present study) represents the best ratio for egg-laying and colony production in mass rearing operations (Gurel & Gosterit, 2008). However, these helper workers start to lay eggs and males emerge in the first brood. Production of males at the early stages of colony life may reduce the worker force and negatively affect colony ergonomics and growth (Keller & Nonacs, 1993). Therefore, knowledge of the timing of the critical stage when the ovarian development of workers starts would help to improve the colony production. We show that, at the age of 7 days, almost all (90%) workers had developed ovaries in Group 3 (consisting of 1 callow worker with a queen). Therefore, removing the old helper worker and adding a young worker weekly could be an effective means to stimulate egg-laying (colony initiation) in the commercial rearing of *B. terrestris.*

In conclusion, the egg-laying behaviour of workers affects the colony life of *B. terrestris*. Previous studies showed that this behaviour depends on numerous factors. However, it remains unclear why *B. terrestris* workers lay eggs and which factors affect their egg-laying behaviour. The present study shows that 1 week can be accepted as the critical age for the commencement of ovarian development in *B. terrestris* workers. The presence of a queen did not inhibit ovarian development in groups containing 8 workers. The pheromonal status of a queen can affect the ovarian development of workers. Egg cells were observed in Group 1. However, eggs laid by queens could not be discriminated from those laid by workers. Because the queens used in the experiment were freshly awoken after diapause, their pheromonal impact on workers was probably low. In contrast to groups containing 8 workers, the ovaries of workers confined singly with a queen started to develop significantly earlier than the ovaries of workers confined singly without a queen. In addition, there was a positive correlation between body weight and the commencement of ovarian development in workers in groups containing 8 workers.

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