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Chilling Temperature and Humidity to Break Diapause of the Bumblebee Queen *Bombus terrestris*

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Bumblebees are widely used to pollinate various crops, especially tomato, in greenhouses and fields. An artificial hibernation is essential for year-round rearing of the bumblebee, which passes through one generation per year. Here, we investigated whether a chilling temperature and humidity affect artificial hibernation of the bumblebee queen *Bombus terrestris*. In chilling temperature regimes of 0°C, 2.5°C, 5°C, 7.5°C or 12.5°C under constant humidity >70%, the queens stored at 2.5°C exhibited the highest rate of survival, which was 74.0% at one month, 67.0% at two months, 60.0% at three months, 46.0% at 4 months, 33.0% at 5 months, and 24.0% at 6 months. Rates of survival decreased at the following temperatures: 0°C, 5°C, 7.5°C and 12.5°C. Colony developmental characteristics after diapause were 1.2- to 1.5-fold higher than those of queens stored at 5°C. In terms of chilling humidity, the queens hibernated at 70% under 2.5°C exhibited the highest rate of survival, which was 93.3 ± 3.4% at one month, 83.3 ± 0.0% at two months, 76.7 ± 0.0% at 3 months and 36.7 ± 12.1% at 5 months. The rates of oviposition, colony foundation and progeny-queen production of queens hibernated at 70% were 80.8%, 30.8% and 30.8%, respectively. These values correspond to 1.7- to 3.3-fold increases in comparison to queens stored at 50% humidity. Therefore, 2.5°C and 70% R.H. were the favorable chilling temperature and humidity conditions for diapause break of *B. terrestris* queens.

Key words: Bumblebee, *Bombus terrestris,* Hibernation, Chilling temperature, Chilling humidity, Survival rate, Oviposition, Colony development

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Introduction

Bumblebees are an important pollinator of various greenhouse crops and are particularly effective in pollinating tomatoes (Buchmann and Hurley, 1978; Banda and Paxton, 1991; Free, 1993). The value of bumblebee-pollinated tomato crops is estimated to be € 12,000 million per year (Velthus and Doorn, 2006). In general, *Bombus* species are annual eusocial insects with short-lived colonies that are found mainly in temperate regions of the world. Queens are the only caste to overwinter (enter diapause), and workers and males die during late summer and early autumn, respectively. In early spring, queens that overwintered leave their hibernation sites. The queen builds up a store of pollen and lays her first batch of eggs into the pollen mass after searching for a suitable site to found a colony. As soon as the workers of the first brood have emerged, they take over the foraging activities of the queen, who from that point on spends most of her time laying eggs. In late summer, many males and new queens are produced. Only mated queens hibernate and emerge in the spring (Heinrich, 1979, Duchateau and Velthusis, 1988).

Diapause is an important adaptation in insects that occurs in many species, enabling them to persist in regions that would otherwise be unfavorable for permanent habitation and to maintain high numbers in an environment that might otherwise support only a small population. Frequently diapause occurs in the lifecycle stage that is highly adapted to resist the rigors of the climate. Diapause is defined as a stage in the development of certain animals during which morphological development may also be suspended or greatly retarded (Andrewartha, 1952; Mansigh, 1971). The programming of diapauses channels development along a specific pathway characterized by behavioral, morphological, and physiological design features that uniquely prepare the diapauses-destined insect for a period of developmental arrest (Denlinger, 2002). Diapause most likely causes the variation among queens that might lead to differences in the ability to produce offspring (Beekmand and Vanstratum, 2000).

In year-round rearing of bumblebees, one of the key stages is diapauses break. To break diapause, several authors have tried to induce hibernation of bumblebee queens under controlled conditions, despite the long ovarian diapause in bumblebees (Horber, 1961; Alford, 1969, 1975; Hoem, 1972; Beekman *et al.,* 1998). Under natural conditions, the period of hibernation may last anywhere from 6 to 9 months. Additionally, queens have been found hibernating in the soil in spherical or oval chambers at a depth of 8 cm in north-facing banks or slopes in southern England, but the depths at which queens occurred varied considerably depending on the ground conditions (Alford, 1969a). Hoem (1972) maintained hibernating *B. terrestris* queens in mounds of soil in unheated greenhouses or in plastic containers with perlite as bedding; bees were then placed into a refrigerator at 4~5°C for 8~9 months. Asada (2004) reported that chilling in a refrigerator at 5°C for 4 months was effective to induce nest initiation by *B. hypocrite hypocrite* queens. Diapausing bumblebee queens require large fat reserves, and these fat reserves are used during diapauses; the amount of metabolic reserves remaining after completion of diapause depends on diapause length (Holm, 1972). Beekman *et al.* (1998) showed that weight prior to entering diapause has an important effect on the diapause survival of bumblebee queens: queens with a wet weight below 0.6 g were unable to survive diapause, irrespective of diapause length. Although some attempts have yielded high survival rates, few attempts have been made to assess the effect of different diapause conditions, such as chilling temperature, humidity, duration and time on diapause survival of queens and their subsequent ability to start a colony.

To examine the effects of chilling temperature and humidity on diapause break of *B. terrestris* queens, we investigated whether chilling temperature and humidity affect artificial hibernation of the bumblebee queen *B. terrestris.* Here, we describe the favorable chilling temperature and humidity for diapause break of *B. terrestris* queens.

Materials and Methods

Origin of experimental insects

Experimental insects were 5th-6th generation queens obtained from *B. terrestris* colonies that were reared year-round in a controlled climate room (28°C, 65% relative humidity, and continuous darkness) at the Department of Agricultural Biology, National Academy of Agricultural Science, Republic of Korea.

Indoor rearing

The basic colony-rearing technique followed that described in Yoon *et al*. (2002). The queens were reared in three types of plastic boxes for nest initiation ($10.5\times14.5\times6.5$ cm), colony foundation ($21.0\times21.0\times15.0$ cm), and colony maturation ($24.0\times27.0\times18.0$ cm). Queens were first confined individually to small boxes for colony initiation and remained there until oviposition. To stimulate egg-laying, two narcotized *B. terrestris* workers, aged 10~20 days after emergence, were added to each box with a queen (Yoon and Kim, 2002). When the adults emerged from the first brood, the nest was transferred to a medium box for colony foundation and stored there until the number of workers reached 50. The nest was then moved to a large box for further colony development. A 40% sugar solution with 0.3% sorbic acid and pollen dough were provided ad libitum (Yoon *et al*., 2005). The pollen dough was made from a sugar solution and pollen (v : v = 1 : 1).

Chilling temperature favorable for artificial hibernation of *B. terrestris* queen

To examine the effects of chilling temperature on diapause break of *B. terrestris* queens, the following environmental conditions were provided. The chilling temperature regimes were defined as 0°C, 2.5°C, 5°C, 7.5°C or 12.5°C under constant humidity >70%. After a mating period lasting 7 days (Yoon *et al.,* 2008), mated *B. terrestris* queens (15 days after emergence) were weighed before the experiment was initiated. The queens were individually preserved in a bottle filled with perlite in a perforated plastic box with perlite to avoid the growth of mold and stored in a different chilling chamber (in continuous darkness) for 6 months. The survival rate of queens was surveyed each month. A total of 100 *B. terrestris* queens were used in this experiment. At the end of treatment, surviving queens were weighed again. The *B. terrestris* queens were stored for 12 weeks at 2.5°C or 5°C, which yielded higher survival rates than other chilling temperatures. Queens were then placed in flight cages for three days (Yoon *et al*., 2004b). Finally, each queen was reared in a climate-controlled room (27 ± 1°C, 65% R.H. and continuous darkness). The developmental ability of each colony was estimated by preoviposition period, rate of oviposition, colony foundation and progeny-queen production. The queens that did not oviposit within 40 days were excluded from analysis of the number of oviposited colonies (Yoon *et al*., 2004a). Colony foundation was defined as the time period necessary for more than 50 workers to emerge from a colony.

Chilling humidity favorable for artificial hibernation of *B. terrestris* queens

To determine the chilling humidity that was favorable for artificial hibernation of *B. terrestris* queens, the chilling humidity regimes were defined as 50%, 70% or 90% under a constant temperature of 2.5°C. Mated *B. terrestris* queens (15 days after emergence) were weighed and stored in a bottle filled with perlite in a perforated plastic box with perlite in a different chilling chamber for 5 months. A total of 30 *B. terrestris* queens were used in this experiment, which involved three replicates. At the end of treatment, surviving queens were weighed again. The surviving *B. terrestris* queens that had been stored for 5 months at 50% or 70% were placed in flight cages for three days, and then each queen was reared in a climate-controlled room (27±1°C, 65% R.H. and continuous darkness). The developmental ability of each colony was estimated based on rate of oviposition, colony foundation and progeny-queen production.

Statistical analysis

Statistical analyses were conducted using Chi-square tests and Tukey's pair-wise comparison tests (one-way ANOVA) (MINITAB Release 13 for Windows, 2000).

Results and Discussion

Chilling temperature favorable for artificial hibernation of *B. terrestris* queens

To examine the effects of chilling temperature on diapause break of *B. terrestris* queens, we investigated the survival rate of queens stored at different chilling temperatures under a constant humidity >70% (Fig. 1). At chilling temperature regimes of 0°C, 2.5°C, 5°C, 7.5°C or 12.5°C, the queens stored at 2.5°C exhibited the highest survival rate, which was 74.0% at one month, 67.0% at two months, 60.0% at three months, 46.0% at 4 months, 33.0% at 5 months, and 24.0% at 6 months. The survival rate of *B. terrestris* queens was significantly affected by chilling temperature (Chi-square test: x^2=18.133, DF=4, p=0.001 at one month; x^2=10.047, p=0.040 at 2 months; x^2=23.552, p=0.0001 at 3 months; x^2=33.474, p=0.001 at 4 months; x^2=43.112, p=0.0001 at 5 months; and x^2=42.712, p=0.0001 at 6 months).

Table 1 shows the change in weight of *B. terrestris* queens after artificial hibernation at different chilling temperatures. The rate of weight loss after artificial hibernation over the course of 12 weeks was 11.3~13.2%. A high chilling temperature was associated with a high rate of weight loss. Horber (1961) found that the average weight loss during hibernation was 151.3 mg. Furthermore, he showed that surviving queens were heavier than queens that died during hibernation. Alford (1969 a, b) also found that body weight was 57% water in bumblebee queens following hibernation and that both live weight and dry weight was reduced by about 50%. Fat makes up an average of 34% of the total dry weight of the bumblebee queens prior to hibernation, and 80% of this fat is absorbed during hibernation. A well-developed fat body

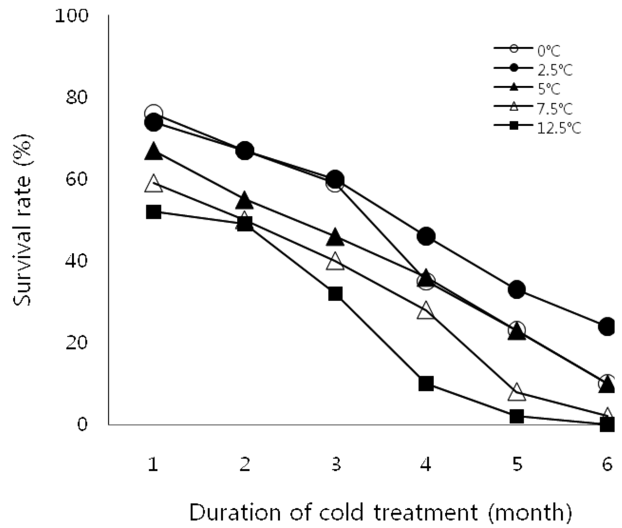

Fig. 1. Survival rate of *B. terrestris* queens at different artificial hibernation chilling temperatures. A total of 100 queens were exposed to chilling temperature regimes. Cold treatment was initiated within 15 days after emergence and was performed at over 70% humidity. There was a significantly difference in survival rate at different chilling temperatures at $p < 0.05$ and $p < 0.001$ using the Chi-square test, except at two months after cold treatment.

Table 1. Change in weight of *B. terrestris* queens after artificial hibernation at different chilling temperatures

Chilling temperatures	n	Change in weight after hibernation (12 weeks)		
		Before (g)	After (g)	Rate of weight loss (%)
0.0°C	26	0.80±0.10	0.71±0.10	11.3
2.5°C	55	0.81±0.12	0.73±0.10	12.0
5.0°C	54	0.82±0.10	0.73±0.09	13.2
7.5°C	47	0.83±0.12	0.73±0.09	12.2

n = the number of queens surveyed.

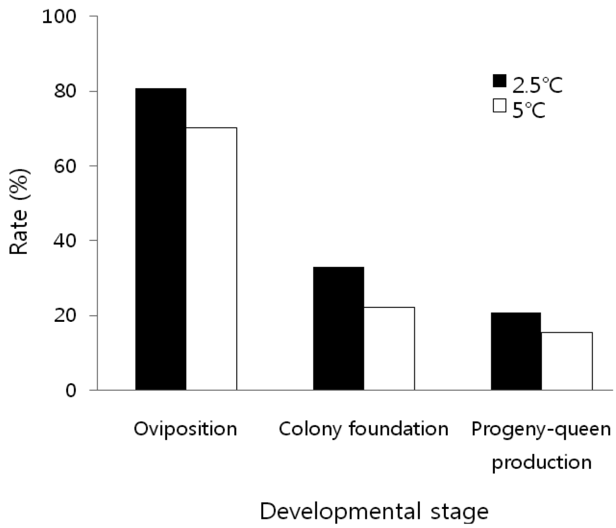

Fig. 2. Colony development of *B. terrestris* reared after artificial hibernation at 2.5°C or 5°C. One hundred queens were submitted to chilling temperature regimes. Cold treatment was initiated within 15 days after emergence and performed at >70% relative humidity. There were no significant differences in rate of oviposition, colony foundation or progeny-queen production among *B. terrestris* reared after artificial hibernation at 2.5°C or 5°C, at $p < 0.05$ using the Chi-square test.

in a queen bumblebee will thus be important for safe hibernation.

We surveyed the developmental characteristics of *B. terrestris* queens stored for 12 weeks at 2.5°C or 5°C, which resulted in higher survival rates than other chilling temperatures (Fig. 2). The rates of oviposition, colony foundation and progeny-queen production of queens stored at 2.5°C were 80.9%, 33.0% and 20.9%, respectively. These values correspond to 1.2- to 1.5-fold increases in comparison to queens stored at 5°C; however, there were no significant differences in the developmental rates of *B. terrestris* reared post-diapause in artificial hibernation at 2.5°C as compared to 5°C (Chi-square test: x^2=3.394, DF=1, p=0.065 for oviposition rate; x^2=3.245, p=0.072 for colony foundation rate; and x^2=1.101, p=0.294 for progeny-queen foundation). Table 2 shows the time necessary for preoviposition, colony foundation and first adult emergence among *B. terrestris* reared after artificial hibernation at 2.5°C or 5°C. The time to preoviposition among queens stored at 2.5°C was 8.5±4.8 days, which was about one day shorter than for queens stored at 5°C, but there was no significant difference (Tukey's pairwise comparison test: F=1.18, DF=1, 159, p=0.279). Similarly, the time necessary for colony foundation (50.4~50.7 days) was not affected by chilling temperatures during diapause (F=0.05, DF=1, 56, p=0.816). No significant difference was detected in the time to emergence of the first worker for queens stored at 2.5°C or 5°C (F=0.01, DF=1, 90, p=0.904). The time to emergence of the first male and queen among queens stored at 2.5°C was 56.8 days and 77.1 days, respectively, which was 4.6~5.7 days longer than among queens stored at 5°C; nevertheless, there was no significant difference (F=1.55, DF=1, 60, p=0.218, for time to emergence of a male; F=1.77, DF=1, 37, p=0.191, for time to emergence of a queen).

The relationship between the numbers of progeny produced and chilling temperatures during diapause was investigated by comparing the time it took for more than 50 workers to emerge from each colony (Table 3). The numbers of adults produced by queens stored at 2.5°C amounted to 130.5±36.9 workers, 197.6±47.0 males, and 50.8±44.7 queens, which was 3.5- to 21.7 numbers more than that observed for queens stored at 5×C. However, this difference was not significant (F=0.13, DF=1, 46, p=0.718, for the number of workers; F=1.91, DF=1, 38, p=0.175, among males; F=0.06, DF=1, 38, p=0.811 among queens).

The queens stored at 2.5°C exhibited the highest rate of survival among chilling temperature regimes of 0°C, 2.5°C, 5°C, 7.5°C or 12.5°C. Furthermore, colony developmental characteristics after diapause were increased 1.2- to 1.5-fold compared to those of queens stored at 5°C. Therefore, our results show that 2.5°C is the favorable chilling temperature for diapause break of *B. terrestris* queens. Beekman *et al.* (1998) stated that duration is more

Table 2. Time necessary for preoviposition, colony foundation and first adult emergence of *B. terrestris* reared after artificial hibernation at 2.5°C or 5°C

Chilling temperature (°C)	Preoviposition (days)	n	colony foundation (days)	n	First adult emergence (days)					
					Worker	n	Male	n	Queen	n
2.5	8.5±4.8	41	50.4±6.0	35	24.4±3.4	56	56.8±16.8	42	77.1±8.5	24
5	9.3±5.0	39	50.7±5.6	23	24.5±3.5	36	51.1±17.6	20	72.5±12.9	15

1) n = the number of colonies surveyed
2) There were no significant differences in time to preoviposition, colony foundation or adult emergence of *B. terrestris* reared after diapause at 2.5°C or 5°C, at $p < 0.05$ using Tukey's pair-wise comparison test.

Table 3. Number of adults produced by *B. terrestris* queens reared after artificial hibernation at 2.5°C or 5°C

Chilling temperature (°C)	Number of adults produced					
	Worker	n	Male	n	Queen	n
2.5	130.5±36.9	32	197.6±47.0	24	50.8±44.7	24
5	126.3±38.3	16	175.9±49.7	16	47.3±45.3	16

1) n = the number of colonies surveyed
2) There were no significant differences in the number of adults produced by *B. terrestris* queens after artificial hibernation at 2.5°C or 5°C, at $p < 0.05$ using Tukey's pair-wise comparison test.

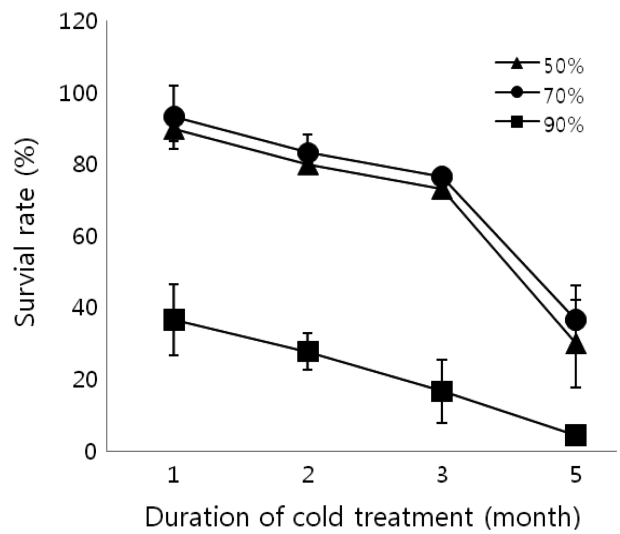

Fig. 3. Survival rate of *B. terrestris* following hibernation at different humidities. Thirty queens were submitted to chilling humidity regimes, with three replicates. Cold treatment was initiated within 15 days after emergence and performed at 2.5°C. There were significant differences in survival rate at different chilling humidities ($p < 0.05$ and $p < 0.001$ using Tukey's pair-wise comparison test).

important than temperature in determining diapause survival; temperature has no effect on the survival of queens. In contrast, the preoviposition period is affected by temperature.

Chilling humidity favorable for artificial hibernation of *B. terrestris* queens

We investigated the effects of chilling temperature on diapause break of *B. terrestris* queens (Fig. 3). In chilling humidity of 50%, 70%, or 90% under a constant temperature of 2.5°C, the queens stored at 70% exhibited the highest rate of survival: 93.3±3.4% at one month, 83.3±0.0% at two months, 76.7±0.0% at 3 months and

Table 4. Change in weight of *B. terrestris* queens after artificial hibernation at different chilling humidities

Chilling periods	Chilling humidities	n	Change in weight of after hibernation		
			Before (g)	After (g)	Rate of weight loss (%)
3 months	50%	73	0.82±0.11	0.75±0.07	9.3
	70%	66	0.74±0.10	0.68±0.08	8.8
	90%	15	0.82±0.08	0.76±0.07	7.9
5 months	50%	31	0.83±0.10	0.62±0.07	33.9
	70%	29	0.73±0.07	0.58±0.05	25.9
	90%	3	0.91±0.02	0.67±0.02	35.8

n = the number of queens surveyed.

36.7±12.1% at 5 months. The survival rate of *B. terrestris* queens was statistically affected by chilling humidity (Tukey's pair-wise comparison test: F=48.06, DF=2, 6, p=0.001 at one month; F=171.86, p=0.001 at 2 months; F=104.71, p=0.001 at 3 months; and F=10.96, p=0.015 at 5 months).

Table 4 shows the change in weight of *B. terrestris* queens after artificial hibernation at different chilling humidities. The rate of weight loss after artificial hibernation during 3 months was 7.9~9.3%, and after 5 months, it was 25.9~35.8%. The longer the chilling duration is, the higher the rate of weight loss is. Hoem (1972) reported that the weight losses occurred during the first half of the hibernation period after which time the body weight increased. Significant, positive correlations were found between the body weight of the queens and the length of the period of survival during and after hibernation, respectively. The weight of *B. terrestris* queens prior to hibernation varied from 400 mg to 1000 mg and after hibernation from 300 mg to 900 mg. Similarly, the weight of *B. lapidarius* queens prior to hibernation was 250~850 mg and 250~750 mg after hibernation.

We surveyed the colony developmental characteristics of *B. terrestris* queens stored for 5 months at 50% or 70%, which showed higher survival rates than queens stored at 90% chilling humidity (Fig. 4). The rates of oviposition, colony foundation and progeny-queen production of queens hibernated at 70% were 80.8%, 30.8% and 30.8%, respectively. These values correspond to 1.7- to 3.3-fold increases in comparison to queens stored at 50% humidity. The colony development of *B. terrestris* reared after diapause was significantly affected by chilling humidity during diapause (Chi-square test: x^2=7.239, DF=1, p=0.007 for oviposition rate; x^2=2.690, p=0.101 for colony foundation rate; and x^2=3.990, p=0.046 for progeny-

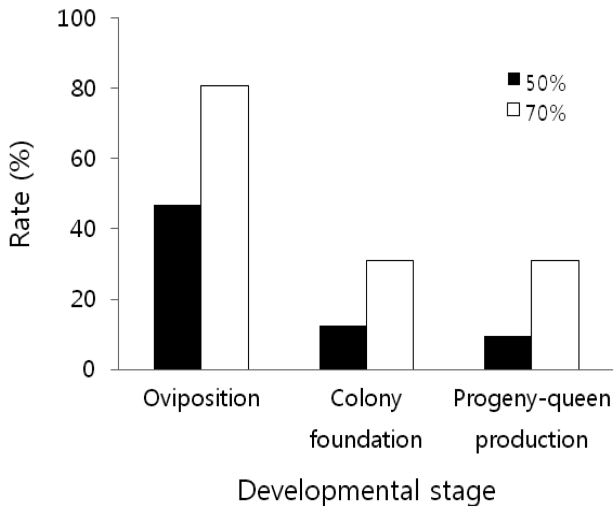

Fig. 4. Colony development of *B. terrestris* reared after artificial hibernation at 50% or 70% humidity. Cold treatment was initiated within 15 days after emergence and performed at 2.5°C. There were statistically significant differences in the rate of oviposition and progeny-queen production among *B. terrestris* reared after artificial hibernation at 50% and 70% ($p < 0.05$ using the Chi-square test).

queen foundation). In view of this survival rate and the colony developmental characteristics after artificial hibernation, the most favorable chilling humidity was determined to be 70%.

The present study shows the favorable chilling temperature and humidity for diapause break of *B. terrestris* queens. Taken together, the present results indicate that 2.5°C and 70% R.H. were the favorable chilling temperature and humidity conditions for diapause break of *B. terrestris* queens.

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