Bumblebees are important pollinators of crops and wildflowers. Bumblebees generally produce one generation per year. One of the key stages for year-round rearing of bumblebees is breaking diapause. To evaluate the effects of a combination method of CO\textsubscript{2}-narcosis and cold treatment to break the diapause of *B. ignitus* and *B. terrestris* queens, we determined whether this method affected their ability to establish a colony after the diapause break. The diapause treatment regimes that were utilized were CO\textsubscript{2} (CO\textsubscript{2}-narcosis), CT-1M (cold treatment at 5°C for 1 mo), CT-1M-CO\textsubscript{2} (CO\textsubscript{2}-narcosis after cold treatment for 1 mo), CT-2M-CO\textsubscript{2} (CO\textsubscript{2}-narcosis after cold treatment for 2 mo), CT-2M-CO\textsubscript{2} (CO\textsubscript{2}-narcosis after cold treatment for 2.5 mo) and CT-2.5M-CO\textsubscript{2} (cold treatment at 5°C for 2.5 mo). In view of the effects on the colony developmental characteristics of *B. ignitus* queens, the most favorable diapause treatment was CT-1M-CO\textsubscript{2}. A combination method of CO\textsubscript{2}-narcosis and cold temperature treatment yielded better results than that of single CO\textsubscript{2}-narcosis or cold temperature treatment on the colony development of diapause-broken *B. ignitus* queens. In the case of *B. terrestris* queens, we concluded that a combination method of CO\textsubscript{2} and cold temperature treatment yielded better results than that of a single cold-temperature (up to 2 mo) treatment. In conclusion, the findings of the present study indicated that the combined application of CO\textsubscript{2} and cold temperature treatment was a favorable method for the colony development of diapause-broken *B. ignitus* and *B. terrestris* queens compared with only CO\textsubscript{2}-narcosis or cold temperature treatments. A combination method of CO\textsubscript{2} and cold treatment reduced the side effect of CO\textsubscript{2}-narcosis and shortened the duration of cold treatment by at least 1 mo.
Generally, Bombus species are eusocial insects with short-lived colonies that are found primarily in the temperate regions of the world. Queens are the only caste of Bombus to overwinter (enter diapause), whereas the workers and males perish in the late summer and early autumn, respectively. In the early spring, queens that have overwintered depart from their hibernation sites. The queen builds up a store of pollen and then lays her first batch of eggs into the pollen mass after finding a suitable site for the foundation of a colony. As soon as the workers of the first brood have emerged, they assume the foraging actives of the queen, after which she spends her time predominantly laying eggs. In the late summer, many males and new queens are produced. Only the mated queens hibernate and emerge in the spring (Heinrich, 1979; Duchateau and Velthusis, 1988).

Bumblebees generally produce one generation per year. One of the key steps in year-round rearing of bumblebees is breaking diapause. Diapause is defined as a stage in the development of certain animals during which morphological development may be suspended or markedly retarded (Andrewartha, 1952; Mansigh, 1971). The programming of diapause involves the development of specific behavioral, morphological, and physiological design features that uniquely prepare the diapause-destined insect for a period of developmental arrest (Denlinger, 2002). With the goal of inducing diapause break, several researchers attempted to first induce hibernation in bumblebee queens under controlled conditions, despite their lengthy ovarian diapause (Horber, 1961; Alford, 1969, 1975; Hoem, 1972; Beekman et al., 1998), or by CO₂ narcosis (Rösele and Rösele, 1984; van den Eijnde et al., 1991). Under natural conditions, the duration of the hibernation period ranges from 6 to 9 mo (Alford, 1969). Hoem (1972) maintained hibernating B. terrestris queens in mounds of soil in unheated greenhouses or in plastic containers with perlite as bedding; the bees were subsequently maintained in a refrigerator at 4-5°C for 8-9 mo. Rösele and Rösele (1984) demonstrated that prediapause B. terrestris queens that were narcotized using carbon dioxide (a 30-min narcosis treatment repeated twice) would start laying eggs within a week. However, this method produced many side effects. Pormeroy and Plowright (1979) found that this treatment induced the ejection of larvae by bumblebee workers in narcotized colonies. Rösele (1985) reported the emergence of some males among the first batch of workers in such colonies. Carbon dioxide-treated bumblebee queens sometimes produced males instead of workers and their nests could be of smaller size than those of overwintered queens (Tasei, 1994; Yoon et al., 2003). Although increased survival rates have been reported in some studies, few studies have attempted to evaluate the effects of different diapause methods, including a combination method of CO₂-narcosis and cold treatment, on the survival rates of diapaused queens and their subsequent ability to establish a colony.

To evaluate the effects of a combination method of CO₂-narcosis and cold treatment for diapause break in B. ignitus and B. terrestris queens, we determined whether this method affected their ability to establish a colony after diapause break. This is the first study describing a combination method of CO₂-narcosis and cold-treatment application for breaking the diapause of B. ignitus and B. terrestris queens.

Materials and Methods

Origin of experimental insects

The insects used in the experiment were second and sixth-generation queens acquired from B. ignitus and B. terrestris colonies that were reared year-round in a climate-controlled room (27°C, 65% relative humidity, and continuous darkness) at the Division of Applied Entomology, Department of Agricultural Biology, National Academy of Agricultural Science, Republic of Korea.

Indoor rearing

The basic colony-rearing technique used in the experiment was described previously by Yoon et al. (2002). The queens were reared in three different types of cardboard and plastic boxes for nest initiation (10.5 × 14.5 × 6.5 cm), colony foundation (21.0 × 21.0 × 15.0 cm), and colony maturation (24.0 × 27.0 × 18.0 cm).
cm). Queens were first confined individually in small boxes for colony initiation and remained there until oviposition. After the adults from the first brood had emerged, the nest was transferred to a medium-sized box for colony foundation and was stored there until the number of workers reached 50. The nest was subsequently moved to a large box for further colony development. A 50% sugar solution and pollen dough were provided ad libitum. The pollen dough was composed of a sugar solution and pollen (v:v 1:1).

**Colony development by *B. ignitus* queens given different diapause-breaking treatments**

To evaluate the effects of different diapause-breaking methods on the colony development of *B. ignitus* queens, the following diapause-breaking treatments were used: CO₂ (CO₂–narcosis), CT-1M (cold treatment at 5°C for 1 mo), CT-1M-CO₂ (CO₂–narcosis after cold treatment at 5°C for 1 mo), CT-2M (cold treatment at 5°C for 2 mo), or CT-2M-CO₂ (CO₂–narcosis after cold treatment at 5°C for 2 mo). CO₂–narcosis involved exposure to 99% CO₂ for 30 min daily during two consecutive days beginning the second day after mating (Yoon et al., 2003). The cold-temperature-treated queens were individually maintained in bottles filled with perlite in a perforated plastic box containing perlite to prevent mold growth under a constant temperature of 5°C and 80% humidity. At the end of treatment, the queens were transferred to flight cages for three days (Yoon et al., 2004b). Each queen was reared in a climate-controlled room (27±1°C, 65% R.H. and continuous darkness). To stimulate egg-laying, two narcotized *B. ignitus* workers aged 10-20 d (after emergence) were placed with each queen. The number of *B. terrestris* queens tested in this study using CO₂, CT-1M, CT-1M-CO₂, CT-2M, or CT-2M-CO₂ was 32, 18, 23, 48 and 50, respectively.

The ability of a queen to develop a colony was based on the preoviposition period, the rates of oviposition, colony foundation, and progeny-queen production, the period until the emergence of the first adult, and the number of adults. The colony foundation period was defined as the period necessary for more than 50 workers to emerge from a colony. The period until the emergence of the first adult represents the duration from the first oviposition to the emergence of the first adult. The queens that did not evidence oviposition within 60 d were excluded from the analysis of the number of oviposited colonies.

**Colony development by *B. terrestris* queens given different diapause-breaking treatments**

To examine the effects of different diapause-breaking treatments on the colony development of *B. terrestris* queens, the following treatments were used: CO₂ (CO₂–narcosis), CT-1M (cold treatment at 5°C for 1 mo), CT-1M-CO₂ (CO₂–narcosis after cold treatment at 5°C for 1 mo), CT-2M-CO₂ (CO₂–narcosis after cold treatment at 5°C for 2 mo), CT-2M (cold treatment at 5°C for 2 mo), CT-2.5M-CO₂ (CO₂–narcosis after cold treatment at 5°C for 2.5 mo), and CT-2.5M (cold treatment at 5°C for 2.5 mo). The conditions used for CO₂–narcosis and cold treatment of *B. terrestris* queens were identical to those used for *B. ignitus* queens. Each queen was reared in a climate-controlled room (28±1°C, 65% R.H. and continuous darkness). To stimulate egg-laying, two narcotized *B. terrestris* workers aged 10-20 d (after emergence) were placed with each queen. The number of *B. terrestris* queens tested in the CO₂, CT-1M, CT-1M-CO₂, CT-2M, CT-2M-CO₂, CT-2.5M, and CT-2.5M-CO₂ experiments was 35, 44, 54, 44, 100 and 72, respectively.

The ability of a queen to develop a colony was based on the preoviposition period, the rates of oviposition, colony foundation, and progeny-queen production, the period until the emergence of the first adult, and the number of adults. The colony foundation period was defined as the period necessary for more than 50 workers to emerge from a colony. The period until the emergence of the first adult represents the duration from the first oviposition to the emergence of the first adult. The queens that did not evidence oviposition within 60 d were excluded from the analysis of the number of oviposited colonies.

**Statistical analysis**

The statistical analyses were conducted using the Chi-squared test and Tukey’s pairwise comparison test (MINITAB Release 13 for Windows, 2000). The Chi-squared test was used to compare the colony development of *B. ignitus* and *B. terrestris* queens. Tukey’s pairwise comparison test was used to examine the durations until colony initiation and first adult emergence, as well as the number of adults produced.
the preoviposition period among the groups given different diapause treatments (Tukey’s pairwise comparison test: F=0.56, df=4, 81, p=0.693) (Fig. 1). Among the diapause-treatment groups, *B. ignitus* queens given the CO₂ treatment exhibited the best egg-laying characteristics. Insect metabolism is influenced in various ways by carbon-dioxide exposure and other environmental factors. Röseler and Röseler (1984) demonstrated that narcotizing prediapausing queens using carbon dioxide (a 30-min narcosis repeated twice) inhibited the formation of fat reserves, increased the content of juvenile hormone *in vitro*, and induced oogenesis. Histological studies of the honeybee revealed evidence of CO₂ effects on the neurosecretory cells of brain, the corpora allata; additionally, the titer of juvenile hormone was increased (Nicolas, 1989).

The colony development of *B. ignitus* queens given different diapause treatments was investigated (Fig. 2). Regarding the rate of colony foundation, which is one of the main criteria of colony quality in commercial rearing, the CT-1M-CO₂-treated queens showed the best performance, 26.1%, which was 1.6-26.1 times higher than that of queens given the other diapause methods, which were 16.0%, 8.6%, 3.1% and 0%, respectively. The CT-1M (cold treatment for 1 mo) queens did not form a colony. There were significant differences in the colony foundation rate of *B. ignitus* queens given different diapause treatments (Chi-squared test: X²=9.450, df=4, p=0.051) (Fig. 1). Among the diapause-treatment groups, *B. ignitus* queens given the CO₂ treatment exhibited the best egg-laying characteristics. Insect metabolism is influenced in various ways by carbon-dioxide exposure and other environmental factors. Röseler and Röseler (1984) demonstrated that narcotizing prediapausing queens using carbon dioxide (a 30-min narcosis repeated twice) inhibited the formation of fat reserves, increased the content of juvenile hormone *in vitro*, and induced oogenesis. Histological studies of the honeybee revealed evidence of CO₂ effects on the neurosecretory cells of brain, the corpora allata; additionally, the titer of juvenile hormone was increased (Nicolas, 1989).

**Results and Discussion**

**Colony development by *B. ignitus* queens given different diapause-breaking treatments**

To evaluate the effects of different methods for breaking diapause on the egg-laying characteristics of *B. ignitus* queens, we investigated their oviposition rates (Fig. 1). Among the queens given these diapause treatments, the oviposition rate of CO₂-narcotized queens showed the best performance, at 68.8%, which was 1.2-2.5 times higher than that of queens given the other diapause methods, which were 56.5%, 44.0%, 43.8% and 27.8% for the CT-1 M-CO₂-, CT-2 M-CO₂-, CT-2 M-, and CT-1 M-treated queens, respectively. There was no significant difference among the values for the treatment groups (Chi-squared test: X²=9.450, df=4, p=0.051) (Fig. 1). The preoviposition periods of queens given different diapause treatments, except for CT-2M-CO₂ and CT-2M queens, were 25.0-25.7 d. The average preoviposition period of queens that were treated using CT-1M was 25.0±14.1 d, followed by the CO₂-, CT-1M-CO₂-, CT-2M-CO₂- and CT-2M-treated queens. There were no significant differences in
Table 1. Period to adult emergence in the colonies of *B. ignitus* queens given different diapause treatments

<table>
<thead>
<tr>
<th>Diapause treatments</th>
<th>n</th>
<th>First adult emergence</th>
<th>n</th>
<th>Worker</th>
<th>n</th>
<th>Male</th>
<th>n</th>
<th>Queen</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO2</td>
<td>3</td>
<td>25.3 ± 5.5</td>
<td>11</td>
<td>53.5 ± 19.9</td>
<td>1</td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT-1M</td>
<td>1</td>
<td>50</td>
<td>0</td>
<td>61</td>
<td>0</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT-1M-CO2</td>
<td>9</td>
<td>27.9 ± 5.2</td>
<td>9</td>
<td>68.6 ± 13.8</td>
<td>7</td>
<td>86.3 ± 17.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT-2M</td>
<td>6</td>
<td>23.5 ± 6.4</td>
<td>4</td>
<td>64.5 ± 23.0</td>
<td>2</td>
<td>81.5 ± 2.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT-2M-CO2</td>
<td>8</td>
<td>24.1 ± 3.9</td>
<td>9</td>
<td>57.6 ± 18.6</td>
<td>2</td>
<td>71.5 ± 0.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

No significant difference in the period to the emergence of the first adult was detected among the diapause treatment groups at p < 0.05 using the Tukey’s pairwise test.

squared test: \( \chi^2 = 12.032, df = 4, p = 0.017 \). As shown in Fig. 2, the rate of progeny-queen production of the CT-1M-CO2: queens was 26.1%; this value was 4.4-26.1-fold higher than that of the CT-2M-CO2, CT-2M, CO2, or CT-1M queens. The progeny-queen production rate of *B. ignitus* queens was significantly affected by the diapause treatment at the level of p < 0.05, determined using the Chi-squared test (\( \chi^2 = 15.597, df = 4, p = 0.004 \)).

Table 1 shows period to adult emergence in the colonies of *B. ignitus* queens given different diapause treatments. The period to the emergence of the first worker for the CT-2M queens was the shortest among the queens given any of the diapause treatments (23.5±6.4 d), followed by the CT-2M-CO2, CT-2M-CO2, CO2, CT-1M-CO2 and CT-1M queens. No significant difference in the period to the emergence of the first worker was detected among the diapause treatment groups (Tukey’s pairwise comparison test, \( F = 1.08, df = 4, 37, p = 0.379 \)). The period to the emergence of first the male, which was 53.5-68.6 d, was not significantly different between the diapause treatment groups (\( F = 0.15, df = 4, 41, p = 0.960 \)). Similarly, the period to the emergence of the first queen, which was 71.5-86.3 d, was also not affected by the diapause treatment (\( F = 0.29, df = 4, 23, p = 0.884 \)). The period until the first emergence of workers, males and queens of post-hibernating *B. ignitus* queens was 25.7-31.2 d, 67.7-79.0 d and 78.9-103.0 d, respectively and those of the CO2-treated *B. ignitus* queens was 23.7-25.0 d, 39.1-44.9 d and 65.3-82.7 d, respectively (Yoon et al., 2002; Yoon et al., 2003).

The relationship between the number of adults produced and the diapause treatment was investigated (Table 2). The numbers of workers reared in the colonies of the queens given CT-1M-CO2 or CT-2M-CO2 treatment was 111.4±34.8 and 108.8±34.9, which was 1.7-1.8 times that of the queens given CO2 or CT-2M treatments; nevertheless, there was no significant difference among the diapause treatment groups (Tukey’s pairwise comparison test, \( F = 2.82, df = 4, 18, p = 0.056 \)). Worker production is important in evaluating the potential pollination efficiency of an insect pollinator. Asada and Ono (2002) found that the first oviposition day and the date of the emergence of the first worker had significant effects on the number of workers and queen progeny produced in laboratory-reared *B. ignitus* and *B. hypocrite* colonies because earlier oviposition by foundresses would result in earlier colony development. The number of males produced by queens given different diapause treatments, which was 251.5-364.8, was not significantly different among the diapause treatment groups (\( F = 0.35, df = 4, 21, p = 0.841 \)).

Regarding the number of queens produced, which is an important factor in the year-round rearing of bumblebees, the CT-1M-CO2 and CT-2M-CO2 treatment groups produced 6.7±6.1 and 7.3±9.3, respectively. These numbers were 2.2-7.3-fold higher than those for queens that were given the other treatments. However, the number of queens produced by *B. ignitus* queens was not significantly different among the diapause treatment groups (\( F = 0.38, df = 3, 9, p = 0.773 \)). Diapause most likely underlies the variation among queens that may lead to differences in their ability to produce offspring (Beekmand and Vanstratum, 2000).

Considering the above-described colony developmental characteristics, the most favorable diapause treatment was CT-1M-CO2 (CO2–narcosis after cold treatment for 1 mo). A combination method of CO2- and cold-temperature treatment provided better results than did single CO2–narcosis or cold temperature treatment in terms of the colony development of...
diapause-broken *B. ignitus* queens. Although the *B. ignitus* queens given the CO₂–narcosis treatment demonstrated the best egg-laying characteristics, the colony developmental ability of these queens was 1.4–8.4 times lower than that of CT-1M-CO₂, CT-2M-CO₂ and CT-2M queens. Carbon-dioxide-treated bumblebee queens sometimes produced males instead of workers and their nests could be of smaller size than those of overwintered queens (Tasei, 1994; Yoon et al., 2003). Among the cold-treated *B. ignitus* queens, the CT-1M queens did not form a colony. Therefore, the necessary cold period for *B. ignitus* queens was determined to be at least 2 mo. Yoon et al. (2013) reported that a cold period of at least 2 mo applied 12 d after emergence was found to be the most favorable condition for diapause break in *B. ignitus* queens.

**Colony development by *B. terrestris* queens given different diapause-breaking treatments**

We investigated the oviposition rate of *B. ignitus* queens to determine the effects of different methods for diapause break on their egg-laying characteristics (Fig. 3). Among the those given the seven diapauses treatments, the oviposition rate of CO₂–narcotized queens was highest, at 85.7%, which was 1.0–1.5 times higher than that of queens given the other diapause methods, which decreased in the order of CT-2.5M-CO₂ (84.7%), CT-2.5M (83.0%), CT-2M-CO₂ (80.0%), CT-2M (79.5%), CT-1M-CO₂ (74.1%), and CT-1M (59.1%). There were significant differences among the treatment groups (Chi-squared test: $\chi^2=15.751$, df=6, $p=0.015$) (Fig. 3). The preoviposition period of the CT-2.5M-CO₂ queens was the shortest among the diapause treatment groups, at 10.8±6.9 d. This value was 1.5–2.5 times shorter than that of the other diapause treated queens, which followed in order of CT-2.5M (15.9±0.1 d), CT-2M (17.1±11.5 d), CO₂ (18.3±9.9 d), CT-2M-CO₂ (18.4±9.5 d), CT-1M-CO₂ (22.6±9.3 d) and CT-1M (27.4±18.0 d). There were significant differences in the preoviposition period of the different diapause treatment groups (Tukey’s pairwise comparison test: $F=9.95$, df=6, 296, $p=0.0001$) (Fig. 3). In the case of cold treatment, the longer the cold duration is, the shorter the preoviposition period of the queen appears to be. Considering the egg-laying characteristics of the diapause treatment groups, the *B. terrestris* queens given the CO₂ treatment had the best rate of oviposition and those given the CT-2.5 M-CO₂ treatment exhibited the best (shortest) previposition period. In *B. terrestris*, Larrere et al. (1993) observed that the neurosecretory cells of diapausing queens resumed activity at 72 h after CO₂–narcosis. Although egg-laying is only one small step in the process of successfully rearing bumblebees, it is a key factor in the large-scale production of commercial colonies and can be manipulated to reduce production costs. Yoon et al. (2004a) reported that the queen that had the earliest first oviposition day could make a stronger colony and could shorten the colony-formation period.

The colony development of *B. terrestris* queens given different diapause treatments was investigated (Fig. 4). Regarding the rate of colony foundation, the CO₂-treated queens showed the best performance, 31.4%, which was 1.0–2.3 times higher than that of queens given the other diapause treatments, which followed in order of CT-2.5M, CT-2M, CT-2.5M-CO₂, CT-1M-CO₂ and CT-1M queens, at 31.0%, 29.5%, 27.8%, 26.7%, 20.4% and 13.6%, respectively. The CT-1M *B. ignitus* queens did not form a colony, whereas the CT-1M *B. terrestris* queens did form a colony. There were no significant differences in the colony foundation rate of *B. terrestris* queens given different diapause treatments (Chi-squared test: $\chi^2=6.541$, df=6, $p=0.365$). As shown in Fig. 4, the rate of progeny-queen production of the CT-2.5M queens was 44.0%; this
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