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Effect of Different Diapause Conditions on *Bombus terrestris* Colony Characteristics

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Key words
***Bombus terrestris*, Colony characteristics, Diapause, Duration, Temperature.**

ABSTRACT

Diapause is the very important stage in the artificial rearing of bumblebees that entails costs that become visible after diapause completion. In the present study different diapause temperature (-2-0 ºC), (1-3 ºC), (4-6 ºC) and duration (two and three months) showed the highest survival rate of the queens after diapause period of two months at 1-3 ºC. Highest colony initiation rate was observed at 1-3 ºC for three months duration. Early pre-oviposition period and early emergence of the first worker in a colony was also observed at three months diapause period at diapause temperature of 1-3 ºC. Larger colony size with the highest number of workers, males and progeny queens were also observed at 1-3 ºC for three months period which suggests the importance of diapause conditions on bumblebee colony characteristics.

INTRODUCTION

Bees belong to the Apidae family of large and exceedingly successful insect's order Hymenoptera. Currently, there are about 25,000-30,000 identified species of bees, belonging to more than 4,000 genera, and undoubtedly numerous other remaining to be discovered (Michener, 2000). The most important activity of bees is their pollination of natural vegetation and agricultural crops. Bees' pollination provides an ecosystem service and crop production practice to the farmers of the world. About 70% of the most valuable crops used directly for human consumption depend on bees to pollinate (Klein *et al*., 2007).

Among different pollinator bees, bumblebees are key pollinators in agricultural as well as natural ecosystems (Knight *et al*., 2009). These are social insects with one generation in a year and live in colonies comprised of different castes for their reproductive, foraging, defence and other tasks necessary for their survival (Goulson, 2010; Stelzer *et al*., 2010). Queens produce diploid or haploid egg on their own wish depending on the stage of colony development. These queens initiate the colony by producing a batch of workers which assist them in raising the subsequent brood (Jandt *et al*., 2009; Couvillon and Dornhaus, 2010). Toward the end of colony cycle, males and young queens are produced and these daughter queens go into the diapause stage after successful mating while rest of the colony finishes (Lopez *et al*., 2009).

Due to increasing food demand, many horticultural crops are grown in greenhouse and glasshouse systems which require pollinators throughout the year. Bumblebees serve this purpose effectively due to their morphological futures and maximize the crop yield (Corbet *et al*., 1991; Griffiths and Robberts, 1996; Sheikh *et al*., 2014). Highly commercial crop like tomato, pepper, strawberry, cucumber, brinjal, peach and apple are pollinated by bumblebee in most parts of the world (Aytekin *et al*., 2002; Goulson, 2003). They help the farmers to decrease their pollination labour cost and promise a good crop yield both in quantity and quality wise.

Crop pollinators comprising especially *Bombus* species value more than 14 billion euro industry of the world (Gallai *et al*., 2009). Approximately 250 known species of genus *Bombus* has been identified worldwide but only a few species are reared commercially under control condition (Cameron *et al*., 2007; Goulson, 2010). Due to their economic importance for pollination of greenhouse crops, European bumblebee, *Bombus terrestris L.* is reared on a large scale under controlled conditions since its introduction for greenhouse tomatoes production in the Netherlands and Belgium (Beekman and Stratum, 2000; Goulson and Hughes, 2015). This species produces large number of foraging workers and adapts quite well to artificial conditions (Velthuis and Doorn, 2006; Imran *et al*., 2015).

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Colony initiation, mating and diapause are the important stages in the artificial breeding of bumblebees. Although the development of production techniques has been rapid since its start in 1988, there still exist a problem in commercial rearing to maximize the quality and profitability of artificially reared colonies (Hughes, 1996). Commercial development of bumblebee rearing relies mainly to manage the diapause stage in their biological process to produce colonies of *B. terrestris* artificially. Diapause is the only stage that can be managed environmentally to increase the shelf life of colony and to produce the active colonies according to the farmer's demands (Eijnde *et al*., 1991).

Diapause is generally believed to entails costs that become visible after diapause completion and affect survival, development and reproduction of queen (Yoon *et al*., 2010). Diapause survival of the bumblebee queens is linked to their weight (Beekman *et al*., 1998; Vesterlund *et al*., 2014; Vesterlund, 2015). Difference in the length of diapause experienced by bumblebee queen may result in variance in post-diapause reproduction (Beekman and Stratum, 2000). In artificial hibernation of bumblebee queen, environmental conditions of low temperature with different durations of diapause affect the fat reserve of queen that seems to be largely responsible for queen survival during diapause and their post-diapause performance (Hahn and Denlinger, 2011; Kwon *et al*., 2013).

Different concepts regarding artificial diapause of *B. terrestris* queen exists and need to be studied due to variable responses affecting optimum efficiency and causing detrimental side effect.

MATERIALS AND METHODS

Origin of bumblebee colonies and their rearing

Colonies of bumblebees (*Bombus terrestris delmatinus* (*Dalla Torre*) were imported from Biobest®, Belgium and reared in Non-Apis Bee Laboratory, Department of Entomology, Pir Mehr Ali Shah, Arid Agriculture University Rawalpindi under controlled conditions (27±2 ºC and 60±10% relative humidity) to multiply the colonies for research study. Colonies were provided with pollen and sugar solution (1.5:1, w/v) as diet source. Experimental queens were obtained from the mass rearing system and reared separately for mating. After successful mating queens were weighed before artificial diapause and only those queens having a weight equal to 0.7 g or more were used in this study.

Diapause treatments

Three different diapause temperatures (-2-0 ºC, 1-3 ºC and 4-6 ºC) and two different diapause durations of 2 and 3 months were used during artificial hibernation. Queens were kept in plastic containers half filled with wood dust. 25 queens were kept in one container. Sugar solution and pollen were also provided in the plastic container. These queens were artificially hibernated in a refrigerator for a duration of 2 and 3 months at 3 different diapause temperatures (-2-0 ºC, 1-3 ºC and 4-6 ºC) under complete darkness and relative humidity of 60±10%. After one-week sugar solution and pollen were removed from the container because at that time queens were physiologically adapted to diapause otherwise got fungus attack.

Colony initiation and development

After successful artificial hibernation period survived queens were given CO_2 treatment and transferred into flight cage under white light in climate room of 28 ºC and relative humidity of 60±10% (Yoon *et al*., 2004). After activation period of 01 week, queens were transferred into starter box (16×11×7 cm) for colony initiation under complete darkness. Ground of plastic boxes was covered with cardboard material that provides an effective surface for initiation of egg lying. Honeybee collected frozen pollen grains from different flowers and sugar solution was also provided to the queens in the starter boxes. Queens in the plastic box were also provided with artificial pupa and two small sized workers of *B. terrestris* that helped in the initiation of oviposition (Gurel and Gosterit, 2008). After the emergence of first brood workers colonies were shifted to the larger boxes for colony development and maturation. Larger boxes of bumblebees colonies were connected with sugar tank that provide a source of nectar to the reared colonies. Frozen pollen was also provided to the colonies on daily basis. Illuminated red light with minimum light intensity at which observation was possible to observe different life history parameters of colonies.

Observation of parameters

Different developmental parameters that were observed include: (i) Survival rate and colony initiation rate of queens. (ii) Pre-oviposition period. (iii) Number of egg cells and workers produced in first brood. (iv) Emergence timing of first worker, males and progeny queen in a colony. (v) Switch point and Competition point timing. (vi) Number of workers, progeny queens and males produced in a colony, and (vii) Lifespan of founding queen.

Data analysis and statistic

Means of different life parameters were subjected to statistical methods using means ± SE and compared using t-test or DMRT at 5% probability for comparison of percentage values. Chi-squares analysis was performed using SPSS programs (Norus, 2006).

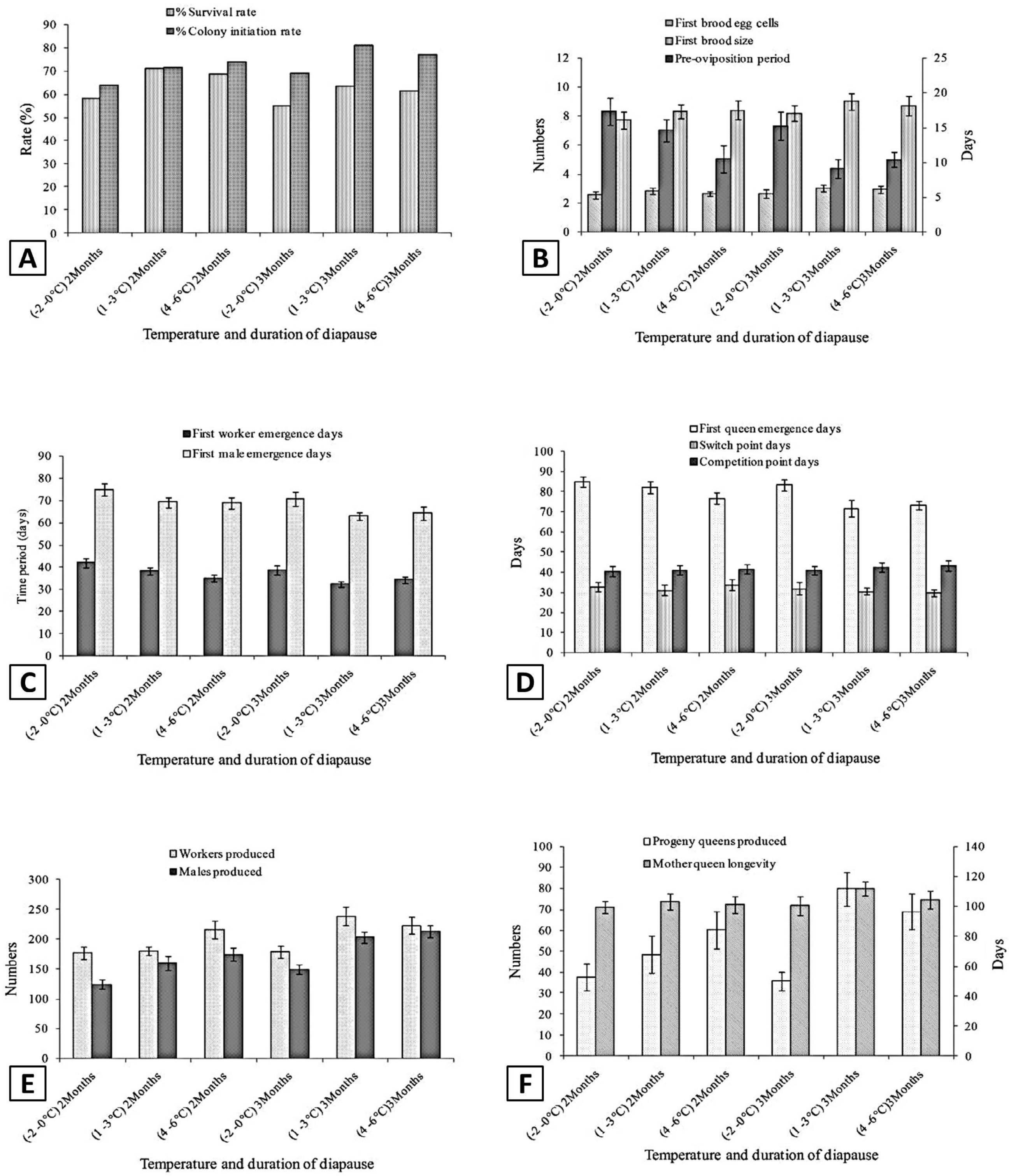

Fig. 1. Effect of diapause temperature and duration on: survival and colony initiation rate of queens (A), pre-oviposition period, first brood egg cells number and size (B), first worker and male emergence in the colony (C), first queen emergence and switch and competition point timing (D), total workers and males produced (E), and total progeny queens produced and mother queen longevity (F) of *Bombus terrestris* under laboratory conditions.

RESULTS

Survival and colony initiation rate of queens

Among all the six diapause treatments, highest survival rate (71%) was observed at the two months diapause duration when queens were stored at the temperature of 1-3 ºC. Queen survival rate was 68.66% at 4-6 ºC under the same diapause duration. Lowest survival rate of 55% was observed for the three months diapause duration when queens were stored at -2-0 ºC (Fig. 1A). It was observed that queens stored at 1-3 ºC for three months diapause duration initiated highest percentage of the colony (81.05%). Lowest colony initiation percentage of 63.85 % was observed at -2-0 ºC for two month diapause duration (Fig. 1A).

Pre-oviposition period

Significantly shortest pre-oviposition period (9.1±1.37 days) was observed for queens those survived three months diapause duration at a temperature of 1-3 ºC. In comparison, longest pre-oviposition period (17.3±1.94 days) was observed at -2-0 ºC at diapause duration of two months ($F_{(5, 59)}$ = 3.73, P= 0.0057) (Fig. 1B).

Egg cells in first brood

Statistical results showed no significant effect of diapause treatments on the production of egg cells in first brood. However, maximum number of egg cells (3.0±0.21) produced by the queen who experienced three months diapause duration at a temperature of 1-3 ºC. Minimum number of the egg cells (2.5±0.26) produced by the queen who survived the two months diapause duration at -2-0 ºC ($F_{(5, 59)}$ = 0.66, P= 0.6578) (Fig. 1B).

First brood size

Insignificant difference in the production of first brood workers was observed in different diapause treatment. However, maximum number of workers was produced at diapause duration of three months with diapause temperature of 1-3 ºC and minimum number of workers were produced at -2-0 ºC at two months diapause duration ($F_{(5, 59)}$ = 0.57, P= 0.7249) (Fig. 1B).

Emergence timing of first worker, male and daughter queen

Shortest time of first worker emergence was observed at a diapause temperature of 1-3 ºC for three months diapause duration (32.30±1.3 days) that overlap the values at 4-6 ºC diapause temperature for a duration of two and three months. Statistically different result with the longest time of first worker emergence (42.0±2.13 days) was observed at diapause duration of two months at a temperature of -2-0 ºC ($F_{(5, 59)}$=4.40, P=0.0020) (Fig. 1C).

It was observed that emergence of males was significantly earliest (62.80±1.69 days) in colonies at diapause temperature of 1-3 ºC for a duration of three months. Emergence of male taken longer time (74.90±2.79 days) for two months diapause duration at a temperature of -2-0 ºC ($F_{(5, 59)}$ = 2.77, P= 0.0269) (Fig. 1C). Significantly shortest time for the production of progeny queens was observed in three months diapause duration at a temperature of 1-3 ºC and 4-6 ºC (71.70±4.14 and 73.2±2.14 days). In comparison, the longest time for the emergence of queens was observed at -2-0 ºC diapause temperature for two months duration (85.10±2.55 days) ($F_{(5, 59)}$ = 3.55, P= 0.0076) (Fig. 1D).

Switch and competition point days

There existed insignificant difference in the timing of switch point event however it was earliest (29.8±1.94 days) when founding queen was stored for three months diapause period, at 4-6 ºC. Delayed switch point event (33.8±2.64 days) was observed at two months diapause period under same temperature range ($F_{(5, 59)}$ = 0.37, P= 0.8661) (Fig. 1D).

Insignificant difference was observed in the timing of competition point event. However, it was earlier (40.6±2.65 days) for two months diapause period at -2 to 0 ºC. Late occurrence of competition point (43.2±2.48 days) was observed at 4-6 ºC diapause temperature for three months diapause period ($F_{(5, 59)}$=0.18, P=0.9703) (Fig. 1D).

Total number of workers, males and progeny queens produced

Production of workers was significantly highest (238.90±15.78) in colonies of those queens who survived the three months diapause duration at 1-3 ºC. Lowest production of workers (176.90±9.88) was observed under two months diapause period at -2-0 ºC ($F_{(5, 59)}$ = 4.67, P= 0.0013) (Fig. 1E). Significantly highest production of males was observed at diapause temperature of 1-3 ºC and 4-6 ºC for the duration of three months. Lowest production of males was observed at -2-0 ºC for diapause duration of two months ($F_{(5, 59)}$ = 11.9, P= 0.0000) (Fig. 1E). We observed the highest production of progeny queens (79.70±7.85) at 1-3 ºC for diapause duration of three months. Lowest production of progeny queens (35.90± 4.47) was observed at -2-0 ºC for diapause duration of three months ($F_{(5, 59)}$ = 5.20, P= 0.0006) (Fig. 1F).

Mother queen longevity

Results from the statistical analysis showed no significant difference in the lifespan of founding queen at different diapause treatments. However, it was observed longest at 1-3 ºC for diapause duration of three months.

In comparison, shortest lifespan of founding queen was observed at -2 to 0 ºC for the diapause duration of two and three months ($F_{(5, 59)}$ = 0.72, P= 0.6110) (Fig. 1F).

DISCUSSION

During the lifecycle of bumblebee it spends about 6-9 months in diapause in soil and has one generation in a year. In the indoor rearing, bumblebee queens were artificially hibernated in a refrigerator to mimic their diapause in nature. In the commercial scale rearing of *B. terrestris*, we cannot keep queens in hibernation for such a long duration because it increase the cost of production and produce only one generation. The only way to get the multiple generations is to reduce the duration of diapause. In the present study effect of different diapause duration and temperature on various parameters of colony development was studied.

Our results showed highest survival rate at diapause duration of two months as compared to three months under a temperature range of 1-3 ºC. Related studies also confirm an inverse relationship between diapause duration and queen survival (Gosterit and Gurel, 2009; Yoon *et al*., 2010; Kwon *et al*., 2013). Survival rate of the queen during diapause depends on the amount of food reserves in the form of fat bodies mostly lipids and glycogen (Fliszkiewicz and Wilkaniec, 2007). Increase in diapause temperature and duration, increases the cellular metabolic activity and more fat reserves are used consequently survival rate of the queen is decreased (Hodek and Hodkova, 1988; Gretenkord and Drescher, 1997; Hahn and Denlinger, 2011; Duchateau *et al*., 2004; Hiiesaar *et al*., 2006). These statements confirm our results and previous studies concerning decrease trend in queen survival with increase in diapause duration.

Optimum conditions during diapause stage could result in better colony initiation and development of bumblebee queen (Beekman *et al*., 1998). Our results showed significantly shortest pre-oviposition period at diapause duration of three months at 1-3 ºC. Other related studies on *B. terrestris, B ignites* and *B. hypocrite hypocrite* also confirm positive effect of temperature and extended duration of diapause on pre-oviposition period of founding queen (Asada, 2004; Amin *et al*., 2008; Gosterit and Gurel, 2009; Yoon *et al*., 2013; Kwon *et al*., 2013). However, Yoon *et al*. (2010) observed no significant effect of various diapause temperatures on the pre-oviposition period of *B. terrestris* queen. Contradiction in results might be due to the difference in the CO_2 treatment, which play important role in the activation of ovary and start of oogenesis.

In the case of a total number of workers produced in first brood our results showed insignificant effect. The results are in agreement with Beekman and Stratum (2000) and Gosterit and Gurel (2009) who also observed no significant effect of diapause experience on the production of first batch of workers. However, Kwon *et al*. (2013) observed significant effect of diapause temperature and duration on the number of workers produced in first batch. Difference in the diet sources (pollen and sugar concentration) and rearing boxes (size and material) might have caused the variation in results.

Since production of sexuals is critical for colony success, the correct timing of this event is of key importance (Oster and Wilson, 1978; Poitrineau *et al*., 2009; Holland *et al*., 2013). In our study, we observed significant effect of diapause temperature and duration on the emergence timing of first worker, male and progeny queen. Amin and Kwin (2011) also observed a significant difference in emergence timing of progeny queens following different diapause duration of the founding queen. Yoon *et al*. (2010) and (2013) observed different result which showed no significant effect of diapause temperature on the emergence timing of worker, male and progeny queen in *B. terrestris* and *B. ignitus* colonies. Variation in the results might be due to the difference in the CO_2 necrosis treatment to the post-hibernated queens and difference in the initiation methods of the colonies.

It has been documented that greater number of workers were capable of increasing the number of sexuals produced (Oster and Wilson, 1978; Poitrineau *et al*., 2009). Results from our experiment showed significantly the highest production of workers and sexuals (male, progeny queen) for those queens who survived the three months diapause duration at a temperature of 1-3ºC. Related studies also confirm considerable effect of artificial diapause conditions on the population of workers, males and progeny queens in bumblebee colonies (Wang *et al*., 2006; Amin *et al*., 2008, 2011; Lopez-Vaamonde *et al*., 2009; Kwon *et al*., 2013). However, some of the previous studies have also documented the insignificant effect of diapause duration and temperature on the adult's population (Beekman and Stratum, 2000; Gosterit and Gurel, 2009; Yoon *et al*., 2010, 2013). Conflicting results might be due to variation in diet sources, the age of mating sexuals and size of founding queens which might act as a mechanism regulating population level outcomes.

During diapause period of insect development, environmental conditions of temperature, humidity and photoperiod cause some sort of biochemical changes even at a lowest metabolic rate (Danks, 1987; Kostal, 2006) that can affect the insect's fitness and their subsequent reproductive performance (Chang *et al*., 1996). Thus, diapause conditions not only affect the successful diapause survival but also play important role in post-diapause performances that play a key role in natural selection (Kroon and Veenendaal, 1998). In a number of insects species a trade-off between diapause conditions and their reproductive behaviour have been documented (Irwin and Lee, 2000; Musolin and Numata, 2003). In the case of B. *lucorum* queens susceptibility to pathogens increase at higher diapause temperature because it affects the phenoloxidase enzyme activity and concentration of hemolymph protein which subsequently affect the post-diapause performance of queens (Vesterlund *et al*., 2014).

CONCLUSIONS

This study suggests that diapause temperature and duration play important role in the survival and post-diapause performance of the queen. Highest survival rate was observed at two months diapause duration, however, better colony development and reproductive performance was observed at 1-3 ºC for three months duration. Commercial bumblebee breeders can manipulate diapause condition for the provision of colonies according to the farmer's demands.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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