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**DETERMINATION OF BUMBLEBEE QUEEN BODY WEIGHT AND
NUTRIENT LOSS DURING DIFFERENT DIAPAUSE DURATION**

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**THE DEGREE OF MASTER OF SCIENCE
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APPROVAL OF THE THESIS

"Determination of Bumblebee Queen Body Weight and Nutrient Loss During Different Diapause Duration" submitted by Ali Hassan AL-SAMARRAIE in partial fulfillment of the requirements for the degree of Master of Science in Department of Animal Science, Graduate School of Natural and Applied Science, Süleyman Demirel University by,

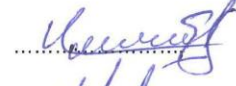
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COMMITMENT

This thesis to be written in accordance with the academic and ethical rules and showing all the literature information reference used is located in the thesis statement.

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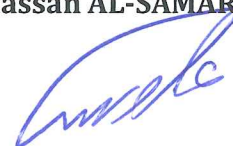
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ABSTRACT

Master of Science

DETERMINATION OF BUMBLEBEE QUEEN BODY WEIGHT AND NUTRIENT LOSS DURING DIFFERENT DIAPAUSE DURATION

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Bumblebees are known as the natural plant pollinators and *Bombus terrestris* is one of the five major types of Bumblebees. Bumblebees are not like honey bees. Bumblebee queen who forms the main colony hibernates during winter time. When the diapause time extends, survival rate and colony forming decreased for the bumblebee queens. There are some studies on weight loss and survival rate during the diapause time, but there are not enough studies on nutrient losses of bumblebees during diapause. The aim of this project was to determine the weight and nutrient loss occurring in the body of bumblebee queen in the diapause duration. Mated Bumblebee queens were collected from regular breeding colony of bumblebee farm at Agricultural Research and Application Center in Süleyman Demirel University. Mated Bumblebee queens were allowed to diapause for 0, 15, 30, 45, 60, 75 and 90 days. Each group had three replicates. Survival rates were determined at end of the diapause and they were frozen immediately at -20 °C for euthanasia. The samples were dried at 55 °C in hot oven and the dried samples lipid, soluble protein, glycogen, total sugar, dry matter and ash were determined. The data were analyzed with one way ANOVA and Tukey test was used to determine the differences between the averages as a result of analysis of variance. Diapause significantly affected body weight loss, protein level, glycogen level and total sugar level ($P < 0.05$) but it did not affect lipid level. Body weight losses were increased when the diapause time increased. Mean body weight losses (15.79 %) were highest at the 90 day diapause. During different diapauses period, soluble protein levels were (493.13±8.01 mg/g) before diapauses and then went down to (307.9±6.19 mg/g) at day 30, after that it went up to (461.36±4.6 mg/g) at day 75. Diapause duration had no effect on the lipids levels during different diapause duration. Diapause duration had a significant effect on the glycogen levels. Glycogen levels were very low (174.52±2.81 mg\g) before diapause and queens started to reserve the sugar as glycogen until 30 days. Then, queens gradually use glycogen reserves. Diapause duration had a significant effect on the sugar levels. Total sugar levels were (143.87±7.73 mg\g) before diapause and gradually changed (107.9±4.56 mg\g) at 90 day. Diapause duration had no effect on the

ash percentage levels. Protein levels were negatively correlated with glycogen levels ($r=-0.805$, $P<0.001$). The changes of the soluble protein level are important during 30 and 70 day diapause duration. This result should be further investigated to understand reason of increase in the protein metabolism during diapause.

Keywords: *Bombus terrestris*, Hibernation, Body Weight, Nutrient loss

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ÖZET

Yüksek Lisans

FARKLI DİYAPOZ SÜRELERİNDE BOMBUS ANA ARILARININ VÜCUT AĞIRLIĞI VE BESİN MADDE KAYBININ BELİRLENMESİ

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Bombus arıları diğer arılar gibi doğal ortamda bitki tozlayıcı olarak bilinir ve *Bombus terrestris* bombus arıları içerisinde bulunan beş önemli türden biridir. Bombus arıları bal arılarından farklıdır. Bombus arıları kış uykusuna (diyapoz) yatmakta ve ana arıdan tekrar koloni oluşturmaktadır. Diyapoz süresine bağlı olarak yaşama ve koloni oluşturma güçleri zamanla ters orantılı olarak azalmaktadır. Bombus arılarının diyapoz döneminde vücut ağırlığı kayıpları ile ilgili çalışmalar vardır fakat bombus arılarında diyapoz döneminde besin kayıpları ile ilgili yeterli çalışma bulunmamaktadır. Genellikle diğer hayvanlarda yapılan çalışmalarda diyapoz döneminde hayvanların vücut yağlarını kullandıkları tespit edilmiştir. Bu projenin amacı diyapoz döneminde Bombus arılarında vücutlarında oluşan ağırlık ve besin kaybının tespit edilmesidir. Bombus ana arıları SDÜ TARUM Bombus arı yetiştirme merkezinde temin edilmiştir. Normal yetiştiriciliği yapılan kolonilerden çiftleşmiş ana arılar alınıp daha sonra tek seferde 0, 15, 30, 45, 60, 75, 90 gün olmak üzere diyapozda bırakılmıştır. Her bir grup üç paralel olarak çalışılmıştır. Her bir grubu en az 50x3 arı temsil edecek şekilde oluşturulmuştur. Diyapoz süresi sonunda hayatta kalanların oranları belirlendikten sonra -20 °C dondurularak ötenazi uygulanmıştır. Daha sonra arılar 55 °C sıcaklıktaki etüvde kurutulup kuru madde, yağ, çözünebilir protein, glikojen, toplam şeker ve kül analizler yapıldı. Veriler üzerinde tek yönlü varyans analizi yapıp varyans analizi sonucunda ortalamalar arasındaki farklılıkların belirlenmesinde Tukey testi kullanılmıştır. Diyapoz süresi vücut ağırlığı kaybına, çözünebilir protein seviyesine, glikojen, toplam şeker seviyesine etki etmiştir ($P < 0.05$) fakat vücut yağ ve kül miktarına etkisi olmamıştır. Vücut ağırlığı kaybı zamana bağlı olarak artmıştır. Diyapozun 90 gününde %15.79 a ulaşmıştır. Diyapoz başlamadan önce (493.13 ± 8.01 mg/g) olan çözünebilir protein düzeyi 30 günde (307.9 ± 6.19 mg/g) düşmüş, daha sonra 75 günde (461.36 ± 4.6 mg/g) en yüksek seviyeye ulaşmıştır. Diyapoz süresi vücut yağ rezervlerine etkisi olmamıştır. Diyapoz süresi glikojen düzeyine etki etmiştir ($P < 0.05$). Glikojen düzeyi diyapoza girmeden önce (174.52 ± 2.81 mg/g) düşük seviyede iken daha sonra 30 güne kadar vücudundaki toplam şekeri glikojene çevirerek vücudunda saklamaya başlıyor. 30 günden sonra ise glikojeni kullanmaya başlıyor. Farklı diyapoz süreleri

toplam şeker seviyesine etki etmiştir ($P<0.05$). Diyapoza girmeden önce toplam şeker miktarı (143.87 ± 7.73 mg\g) iken 90 günde (107.9 ± 4.56 mg\g) kadar düşmüştür. Diyapoz süresinin hayvanın vücut kül seviyesine etkisi olmamıştır. Çözünabilir protein ile glikojen seviyesi arasında negatif korelasyon mevcuttur ($r=-0.805$, $P<0.001$). 30. ve 70. günler arasında çözünabilir proteinin seviyesinin artması önemlidir. Diyapozda oluşan bu değişimin sebebinin anlaşılması açısından daha fazla araştırmaya ihtiyaç vardır.

Anahtar Kelimeler: *Bombus terrestris*, Diyapoz, Vücut ağırlığı, Besin madde kaybı

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1. INTRODUCTION

Bumblebees (*Bombus terrestris*) have ecological importance because of the pollination services they provide in natural and agricultural ecosystems. If any failure happen to these pollinators may result great concern for ecosystem functioning (Gündüz and Gülel, 2010;Huth-Schwarz *et al.*, 2012). Bumblebees need suitable nesting sites which vary between species. In commercial production of bumblebees, they are produced in nest in controlled environments and diapause is one of the main phases in the life cycle of this important insect. After mating, the queen must enter the diapause after certain amount of time. When the dormancy phase finishes, they can start to lay eggs and produce a new bumblebee colony. The diapause is possible at 2-4 °C, 82-86% humidity. During the diapause period, the queen dose not eats anything and use body nutrition reserves to survive. Gürel *et al.* (2011) reported that body weight loss of bumblebees was higher and survival rate was lower when diapause time was extended. There are many studies for different animals which go to hibernation, for these animals, during the hibernation animal uses body fat to survive. There are not enough studies on bumblebees to measure nutrient loss during diapause time. Our purpose of this study was to determine bumblebee queen weight loss and the nutrition loss (lipid, protein, glycogen, sugar and ash content) during different diapause duration.

2. SUMMARY OF LITERATURE

2.1. History of the bumblebee rearing and production

Since Sladen's research (1912), a large number of interested naturalists as well as professional biologists, mainly in Europe and North America, have studied bumblebees and published their results. These researchers collect spring queens from the field and let them establish in nest boxes or observation hives. Five species of bumblebees are currently used for crop pollination, the major ones being *Bombus terrestris* from Eurasia and *Bombus impatiens* from North America. As a result of their frequent use in foreign territories, they have been reported that *Bombus terrestris* becoming established, as well as interactions causing competition with local bumblebee fauna. Commercially many vegetables, fruits and seed crops are pollinated by bumblebees. In 2004, 40000 hectares of tomato crops were pollinated, with a crop value of € 12000 million. The growers benefits from bumblebee pollination are lowered production costs and improved crop quality (Velthuis and Doorn, 2006).

2.2. Bumblebee

Bumblebee queens emerge from diapause in late winter or spring, and at this time of the year they can often be seen searching for suitable nest sites. The timing of emergence differs markedly between species; such as *Bombus terrestris*, emerge early in February or March while other species such as *Bombus sylvarum*, emerges lately in May or in June (Goulson, 2010^a), the queen supply her nest with pollen molded it into a lump so that she will be able to lay her eggs in it. At the beginning she will lay 4-16 eggs in this first batch Figure 1 (Lopez-Vaamonde *et al.*, 2007). Then the pollen lump is covered with a layer of wax, secreted from the ventral abdominal surface of the queen, mixed with pollen. The queen also forms a wax pot near the entrance of the nest to stores the collected nectar. Bumblebee queen incubate her brood by embracing the top of the grooved pollen lump, maintaining close contact between the lump and her ventral surface (Goulson, 2010^a). She will start laying diploid eggs to produce female workers. When the count of emerged workers exceeds ten it becomes a

colony. In pollination process, the most important stage is the larva stage. Since the colony needs to collect a lot of nectar and pollen to feed these larvae, so the efficiency of the pollination process is depend on the density of larvae in the colony.

While in the laboratory the queen had been brought from a reliable source and a good environment was prepared (temperatures $30\pm 0.5^{\circ}\text{C}$ under constant humidity of $60 \pm 5 \%$) (Gösterit and Gürel, 2008), for the nest (plastic box), prepared sugar syrup and pollen cake so that the queen starts to develop her nest for the colony (Lopez-Vaamonde *et al.*, 2007).



Bumblebee egg stage (5 day)

Bumblebee larvae stage (12 day)

Figure 1. Picture of Bumblebee egg and larvae stage

The time taken by an egg to be an adult bumble bee is about 4-5 weeks Figure 2 (Anonymous, 2015^b). Most Hymenoptera, including bumblebees, have trichromatic vision. The three color receptors are stimulated by ultraviolet, blue and green wavelengths, with the peak sensitivities at 345nm, 440nm, and 550nm respectively (Morandin, 2000). So to control the nest or colony in the laboratory red light should be used, since the insects cannot see in this wavelength so they cannot fly.



Bombus terrestris audax, queen (Pierre Rasmont, 2009)



Bombus terrestris sassaricus, worker (Pierre Rasmont, 1998).



Bombus terrestris male (Ryton Wood, 2010)

Figure 2. Picture of Bumblebee queen, worker and male

After the first worker emerges, there is about one week available to transfer the nest to the colony box, big box with enough volume to grow and develop. When the colony population reaches 50-60 workers it is the saleable stage which is ready to do pollination services in the greenhouse. The first worker emerges in the 4-5th week and a colony reaches saleable stage in the 12th week (Gösterit, 2011). As it mentioned before pollination efficiency depends on the population size of the larvae stage in the colony, pollination will last up to 30-40 days. After that the life cycle of the colony is about to end, new queens and males emerges and there are no more larvae growing in the colony. It's time for the new generation of gynes and drones. Drones leave their colony first then the gynes leave. After that the drones and all other insect from the old colony died, only the mated queens will go for diapause Figure 3.

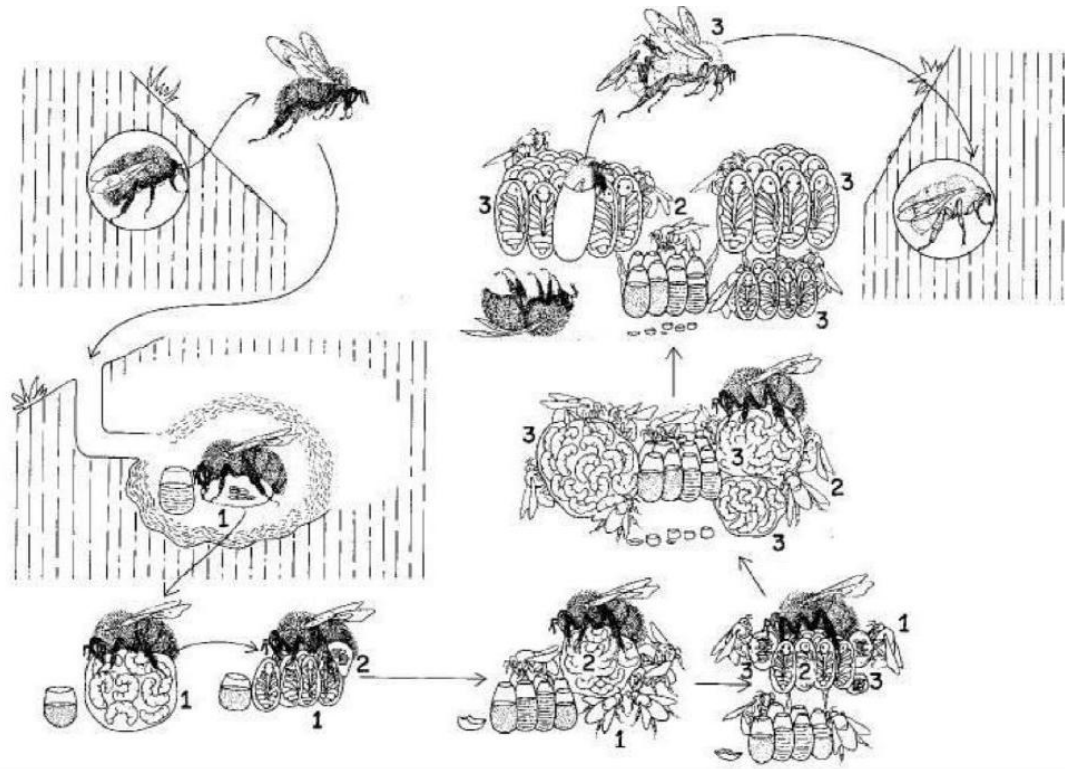


Figure 3 Life cycle of a bumblebee colony. The numbers (1, 2 and 3) represent the successive broods, (3) being the new generation constituted of virgin queens and males (Coppée, 2010).

2.2.1. Commercial production of bumblebee in controlled environment

The best way to start a nest is by joining a mated queen with a new emerged one day old bumblebee worker, to stimulate the queen to lay eggs faster (Gürel and Gösterit, 2008). The other important factor to be sure that the queen did not mate with a relative male, by buying the queens from a reliable source, to prevent any diploid male or other genetic failure (Cook and Crozier, 1995).

After the mated queen had been purchased from commercial producing company, the mated queen needs to be anesthetized with carbon-di-oxide once for 30 min, then it was putted in the nest box with a new emerged one day old bumblebee worker, to stimulate the queen to lay eggs faster. The queen starts her colony under laboratory conditions ($30 \pm 0.5^\circ\text{C}$, 60 % humidity, red light, food, pollen and sugar syrup ad libitum) (Gösterit and Gürel, 2008). After all workers of the first brood emerge, colony is transferred to a larger rearing box.

Newly emerging young queens and drones are collected from first generation colony and placed inside mating cage in Figure 4 and 5. To avoid inbreeding effects and genetics failure, young queens should be mated with males from different colonies. Mated queens are put into controlled diapause condition at 4°C for 45 days and then they are transferred back to the controlled environment and they are allowed to start the second generation colonies following the same procedure as described above (Gürel and Gösterit, 2009).



Figure 4. Picture of mating cage

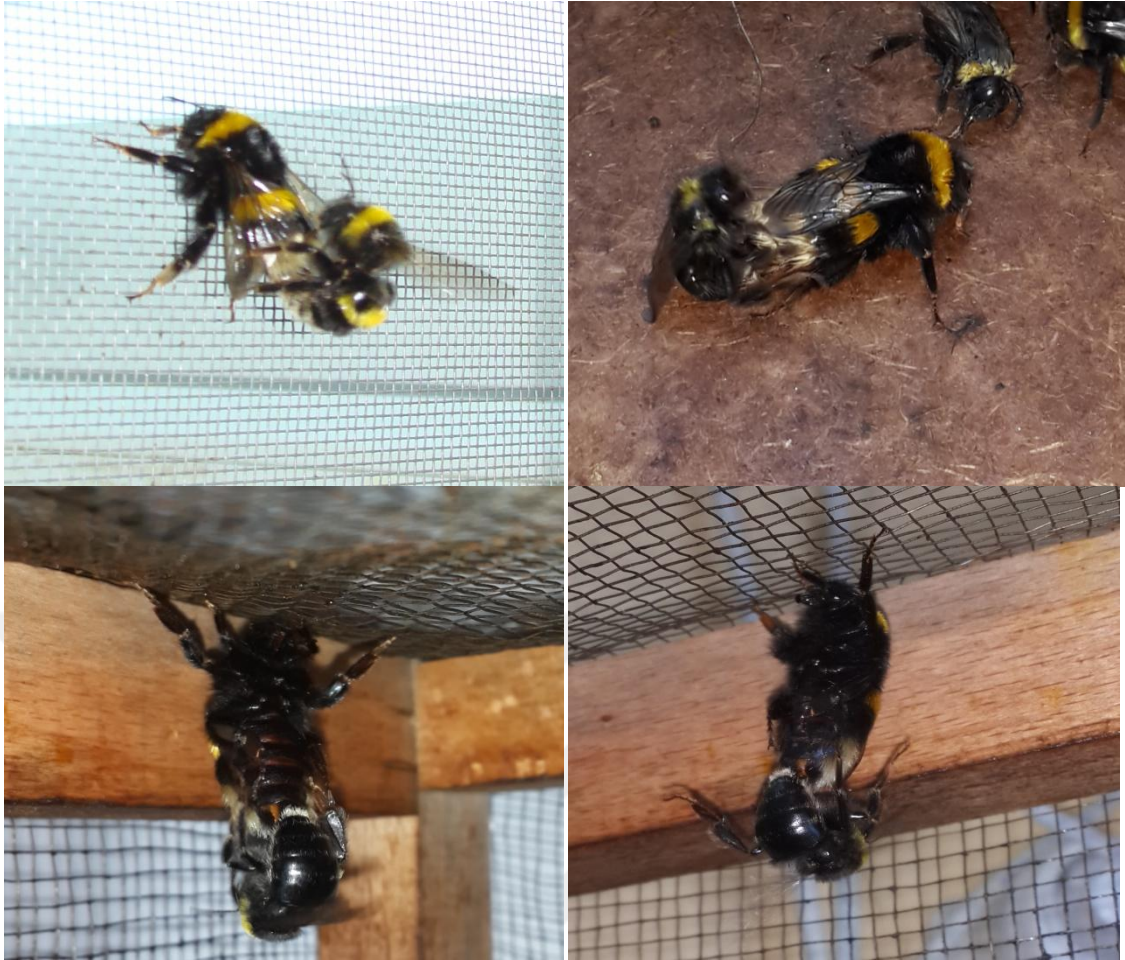


Figure 5. Picture of mating *Bombus terrestris* queens and males

2.2.2. Diapause

In insects, the life cycle of each species encompasses four main phases: reproduction, dormancy (diapause), growth and development and, movement and migration. The seasonal timing of each of these phases is adapted, in part, to avoid unfavorable physical factors and in part to synchronize activity with favorable physical conditions and seasonally available biotic requisites, such as food, mates, oviposition sites and periods free from natural enemies (Tauber and Tauber, 1978).

Diapause has two broad roles in insect life. It permits escape in time, but it also interacts with development rates to synchronize life cycles and determine patterns of voltinism. Diapause is a form of development in insects like hibernation in higher animals. It enables the insects and related arthropods to escape from harmful seasons. Winter is most commonly avoided in temperate

zones, but diapause is also used to avoid hot, dry summers and periods of food shortage. Diapause is a developmental response that is expressed only during a specific developmental stage, which depends on the species of insect. For example, the commercial silkworm (*Bombyx mori*) always diapauses as an early embryo, the European corn borer (*Ostrinia nubilalis*) as a fifth instar, the cecropia moth (*Hyalophora cecropia*) as a pupa, and the Colorado potato beetle (*Leptinotarsa decemlineata*) Diapause as an adult insect. Few species are capable of entering diapause several times, but this usually occurs only in species living at high latitudes, which several years may be required for the completion of development (Denlinger, 2009). If the diapause occurs in response to environmental cues, it is referred as facultative diapause, but if it occurs during each generation regardless of the environmental cues it receives, it is considered an obligatory diapause, facultative diapause is the more common one but several important species such as the gypsy moth (*Lymantria dispar*) have an obligatory diapause (Denlinger, 2009).

2.2.3. Preparation of diapause

In preparation for diapause, the insect usually store additional energy reserves and moves to a site that is slightly protected from the severe environmental conditions, These sites may be underground, under scrap in the soil, within plant tissues or inside cocoons or other structures constructed by the insect(Resh and Cardé, 2009). A migration flight may be a preparatory step for diapause, there could be a short flight to a local wooded area or it can be a long-distance flight from Canada or northern regions of the United States to Mexico or California by monarch butterfly (*Danaus plexippus*) to spend the winter in an adult diapause (Denlinger, 2009). Usually feeding stops during diapause, so the insect is forced to survive on the energy stored before entering diapause. It is not unusual for an insect designed for diapause to store twice as much lipid reserves compare to same size insect that is not programmed to enter diapause (Anonymous. 2015^e). Another challenge faced by diapausing insects is that the lack of access to water, although some insects may drink during diapause, but certain stages such as embryos and pupae do not have this option (Denlinger, 2009). This lack of water poses special constraints for an organism as small as

an insect. Their large surface to volume ratios make insects particularly vulnerable to water loss across the surface of their skeleton shell. Two features appear to be common adaptations for maintaining water balance during the long months of diapause, the cuticles of many diapausing insects are coated with extra thick layers of wax that are effective in retarding water loss and some of diapausing insects are capable of absorbing atmospheric water vapor through their cuticle using a mechanism that is not yet clearly understood. (Resh and Cardé 2009)

2.3. Thermoregulation of the nest

Depending on the latitude at which they live, bumblebee queens have about 2-7 months to find a nest and breed colony that contains several hundreds of workers, and then produce the next generation of reproductive gyens and drones (Goulson, 2010^c). That means the queen needs to compress the cycle into short period of time. The immature stages must be incubated to develop without delay so all bumblebee queens incubate their brood by using of their abdomen heat, after the first batch of eggs has been laid the queen spends most of her time incubating the laid eggs. The queen builds the brood clump with a groove on the dorsal surface at which she sits, this will make a close contact between the brood and the ventral surface of her abdomen and thorax. (Heinrich, 1974) found that *Bumbus vosnesenskii* queens can maintain a brooding temperature of 25°C above ambient temperature even in the absence of insulation (Heinrich, 1974). Silvola (1984) estimated that a *Bumbus terrestris* queen uses about 600 mg of sugar per day at the typical temperature range of central Europe, and to do that she may visit up to 6,000 flowers. In absence of the queen the brood will rapidly cool, so availability of plentiful surrounding flowers near to her nest is vital (Silvola, 1984). The thermoregulatory capacity of the established bumblebee nests is impressive, Heinrich, (1974) attempted to kill a commercial colony of *bumbus terrestris* by placing its entirety in a domestic freezer at -30°C for 24 hours, he found that the colony is still alive and buzzing loudly. The workers gathered into a tight clump at the top of the brood and were presumably shivering at their maximum capacity while the queen was in the center of the crowd (Heinrich, 1974). If colony temperature is low some

bumblebee workers begin to incubate brood, if the temperature drops further more bumblebees switch to incubation mod. At the opposite if the nest temperature increases, bees start to do fanning behavior, and if the nest continues to get warmer, more bumblebees switch to fanning as well (Heinrich, 1974; Goulson, 2010^c).

2.4. Pollination

Pollination is defined as the transfer of pollen from the anthers of one flower to the stigma of the same or a different flower (Anonymous, 2015^d). In the majority of plants, pollination is necessary for established seed. A variety of vectors can do the pollination by transport pollen including wind, water, birds and bats. On the other hand significant majority are pollinated by insects. Unlike other insect groups, adult bees feed their offspring on pollen. To gather sufficient resources for its offspring, a bee has to maintain a high work rate compared to a butterfly, which stops at flowers only to feed itself. Because of this work rate they make excellent pollination, and many plants are adapted primarily for bee pollination (Goulson, 2010^a). There is now growing appreciation for bumblebee as they are alternatives to the honeybee and in some situations bumblebees may be better (Westerkamp, 1991).

Willmer *et al.* (1994) reported that honeybees do have a number of advantages as pollinators: they form vast colonies that can pollinate large areas of crops and they provide honey. However, they also have disadvantages. First, honeybees are fair weather foragers, in cold conditions and when it is raining, they will not forage. Second, honeybees are not able to adequately pollinate some crops. They have short tongues so they are not keen to visit crops with deep flowers such as red clover (*Trifolium pratense*). In some plants, such the Solanaceae (which includes tomatoes and potatoes) the pollen is presented in poricidal anthers, these are essentially similar to an inverted salt cellar, to obtain the pollen the insect has to shake the anthers (known as buzz pollination). Honeybees are not able to do buzz pollination thus they cannot efficiently pollinate these crops. Finally, reliance on a single species for pollination of crops is an inherently risky strategy. Most recently in North America mysterious phenomenon probably

attributable to viral diseases called as Colony Collapse Disorder has devastated commercial hives and threatens yields of bee-pollinated crops (Goulson, 2010^b). In contrast, bumblebees are hardy and will forage in very cold conditions and even when it is raining. In North America, bumblebee queens have been seen foraging when the air temperature was below freezing, while in the Scandinavian summer they will forage for 24 h each day. Because of their greater cold tolerance, bumblebees begin foraging earlier in the day than honeybees. For example, *Bombus impatiens* began foraging 15 to 40 min before honeybees on cucurbit crops (Goulson, 2010^b).

Bumblebees are capable of buzz pollination and make excellent pollinators of Solanaceae such as tomatoes (King, 1993). The anthers of these flowers only release pollen when vibrated, which bumblebees achieve by placing their thorax close to the anthers and contracting their flight muscles at a frequency of about 400 Hz (King, 1993).

There are two alternative approaches to use bumblebees as pollinators; they can be bred for this purpose and the colonies can be placed near the crop field or in the greenhouse. The grower can exploit natural populations of bumblebees. The former approach is perhaps best suited to high value crops grown intensively in greenhouses (Goulson, 2010^b).

2.5. Bumblebee in greenhouse

The amount of colonies needed in the greenhouse depends on the crop type and the size of the greenhouse. Two hives of bumblebees can pollinate 3000 m² of tomato greenhouse, one hive of bumblebee can pollinate 3000 m² to 5000 m² of sweet pepper greenhouse and one hive of bumblebee can pollinate 1000 m² of strawberry greenhouse (Rubio, 2015). It is usual for bumblebees to start pollinating within 1 or 2 days, sometimes full pollination activity may not be evident after 5 days. Bumblebees will perform best at temperatures less than 30°C (Rubio, 2015).

2.6. Bumblebee diseases

Gösterit. and Erkan (2012) reported that some important parasites and diseases are *Locustacarus buchneri*, *Nosema bombi*, *Crithidia bombi* and *Apicystis bombi* in mass rearing production of bumblebees, and these parasites and diseases prevent the colony foundation of queens, decrease their longevity, cause to poor colony growth and premature colony death in commercial rearing.

2.7. Studies on bumblebee body nutrition reserves

In bumblebee life cycle, after mating, the queen must enter dormancy phase (over-winter) to grow and develop. To survive during this tough phase the queen needs to prepare a suitable nest for protection from bad environmental condition and store enough nutrients for metabolic needs. During diapause the insect is not able to collect any nectar or pollen to stay alive. It is completely dependent on her stored supply. It is important to investigate the losses of nutrient and to find the compounds controlling the survival; because, if the queen dose not collects enough resources, it cannot fall in diapause. Fliszkiewicz and Wilkaniec (2007) reported that the contents of amino acids (serine, cystine, glycine and alanine) in the fat body of inseminated bumblebee queens may control entering the diapause. Higher contents of serine, cysteine and alanine were reported in the diapausing queens, while the non-diapausing queens were characterized by a significantly higher content of glycine in their fat body (Fliszkiewicz and Wilkaniec 2007) and also they looked fat content of the diapause and non-diapause queens. They could not find any differences between their fatty acid profiles. Gündüz and Gülel, (2010) compared the protein in recently emerged unfed female and male of *Bracon hebetor* Say (Hymenoptera: Braconidae) to those in 5 days and 10 days old wasps fed with honey. The protein level in both sexes increased significantly from emergence to day 5 and then returned to emergence levels on day 10 in females, but remained constant in males during the same period. Akman Gündüz *et al.*, (2010) compared glycogen and total sugar levels in completely starved (no water or sugar provided), honey fed, sucrose fed and sugar starved (only water provided) females of the parasitoid *Bracon hebetor* Say (Hymenoptera: Braconidae), and quantified the glycogen and total sugar levels in newly

emerged unfed (day 0) female wasps. They found that sucrose and honey fed females had significantly higher glycogen levels than emergent, completely starved and sugar starved females. Also there was no significant difference between completely starved and sugar starved females for glycogen levels. Emerging females had significantly lower total sugar levels than those receiving other diet treatments. Total sugar levels did not significantly differ between completely starved and sugar starved females. Similarly, honey fed and sucrose fed females had similar levels of total sugars (Akman Gündüz *et al.*, 2010).

2.8. Fat body: the nutrition storage site

The insect fat body is a mesodermal tissue which has similar function of the mammalian liver. The major function of the fat body, especially of its wall layer, is the accumulation of storage nutrients (protein, lipid, and glycogen) used at various stages of life (Arrese and Soulages, 2010). Young bumblebee queens accumulate, mainly in the abdomen, considerable feed reserves in the form of a fat body as early as in the first days of their life (Fliszkiewicz and Wilkaniec, 2007). In the period of diapause, those reserves are utilized almost completely. Nutrition reserves accumulated in the fat body must be large enough to survive the entire period of diapause. Fat body cells, having homeostatic functions related to metabolism, respond to nutritional and hormonal cues that regulate and modulate blood sugar, lipid, and protein at larval and mature stages (Resh and Cardé 2009; Anonymous, 2016).

2.9. Used techniques to examine protein, lipid, glycogen and sugar level of insects

There are many techniques to detect protein level in unknowns sample like Kjeldal methods (ExpotechUSA, 1998), Lowry method (Peterson, 1983) and Bradford Protein Assay (Pierce Biotechnology, 2005). The Kjeldahl method was developed in 1883 by Johann Kjeldahl to investigate total protein in the food product (ExpotechUSA, 1998). The sample contains protein digested by heating it in the existence of sulfuric acid so that it releases nitrogen which can be determined by a suitable titration technique. The amount of protein present is then calculated from the nitrogen concentration of the sample (ExpotechUSA, 1998). But the Kjeldahl method had disadvantages; it does not give a measure of the true protein because all nitrogen in the foods is not in the form of protein. Different proteins need different correction factors because they have different amino acid sequences. The method is slow and uses strong oxidizing acids requires special safety precautions in the laboratory. The chemical waste from the test is not environmentally acceptable for normal sewer systems (Hughes and Gray, 1998). In the other side the techniques that used spectrophotometer like Lowry method and Bradford Protein Assay are more reliable and sensitive, The disadvantages of Bradford Protein Assay is the procedure produces a nonlinear standard curve and it needs more protein standard concentrations to cover working range(Pierce Biotechnology, 2005). The modified Lowry method described by Peterson (1983) which includes acceptable disadvantages such as it takes long time to finish the assay because of the using of folin reagent which increases complexity of the procedure and practical limits of using only 20 samples per run(Peterson, 1983).

Emile Van Handel (1985) published an article about determinations of total lipids in mosquitoes. Female mosquitoes depended on stored lipid to survive in unfavorable conditions. It had been used hot sulfuric acid to convert unsaturated lipids to water-soluble sulfonic acid derivatives (after extracted by methanol-chloroform), combined with vanillin-phosphoric acid will develop a reddish color measured by spectrophotometer at 525nm (Van Handel, 1985^a).

It's the same technique that had been used to investigate lipid levels in *Bracon hebetor* by Akman Gündüz et al., (2010).

In the case of carbohydrate contents of the mosquitoes Emile Van Handel (1985^b) mentioned a method for rapid determination of glycogen and sugar, the method reduce the time needed to analyze group of 12 mosquitoes from one day to less than one hour. He reported used anthrone reagent after extracting the sugar and glycogen by sodium sulfate (to precipitate glycogen) and methanol (to separate sugar form precipitated glycogen), and read the absorbent by spectrophotometer at 652 nm.

3. MATERIALS AND METHODS

3.1. Bumblebee rearing

One thousand fifty mated bumblebee queens were used for this research. All these queens were supplied from Agricultural Research and Application Center (Tarımsal Araştırma ve Uygulama Merkezi)/ bumblebee laboratory in Süleyman Demirel University. In late winter 2015, the production of bumblebee colonies had been started (about 300 nests) with one mated queen and a young bumblebee worker in small box, with continues darkness and temperature $26\pm 2^{\circ}\text{C}$ with humidity $68\pm 5\%$. The nests were controlled 2 times per week for dead queens, checked the brood, and added the sugar syrup and pollen cake and nest box cleaned. Meanwhile when the first brood appeared it was necessary to remove the helper worker and leave the queen take to care of her nest. If the first pupa emerged as a male, this means that the helper worker had laid the eggs, so the new male had removed and left the queen to lay her first brood. When the population of the nest increased to 10-15 workers, it was relocated to a colony box (larger box) to give it the space which needs to develop and grow. In late May most of the colonies had new emerged queens and males, the mating proses started in the middle of June to give time to the gyens and drones to fully mature. In the mating room, the drones were put in the mating cage and then combined with the queens with high density florescent light and temperature $21\pm 2^{\circ}\text{C}$ with humidity $68\pm 5\%$. These ambiances represent the natural environments for the bumblebees' couples in nature. In the mating cage the

mature drones tried to ride on the back of the queen in an attempt to mate with her, when this happened the couples were attached for about 15 minutes. Drones were removed after the mating. Mated queens were collected in diapause box, each box had 50 mated queens.

All queens were grouped depending on diapause period of seven different duration (0, 15, 30, 45, 60, 75 and 90 days) with three replicates.

3.2. Bumblebee samples preparation

Each diapause box with 50 mated queens were labeled and recorded accordingly as the amount of queens, wet weight, date of wet weight, date of enter diapause and the group number. After measuring the wet weight, all groups were immediately freeze at -20 °C for euthanasia, and then put all the groups in hot air oven at 55°C for 3 days to dry. After drying, we had been measured the dry weight and noted it for each replicate. Then the dried queens stored in glass jar filled with carbon dioxide to prevent the oxidation and the activity of microorganisms.

This procedure was done for all samples, all replicates were grinded to fine pieces with coffee grinder and stored in refrigerator in glass jar filled with carbon-dioxide.

3.3. Chemical analysis

3.3.1. Protein determination with Lowry method

Reagents, required chemicals and equipment

- Trichloroacetic acid (TCA), 10% (w/v)
15 g of trichloroacetic acid to 150 mL in volumetric flask with purified distill water
- Ethanol (95%)
- 1% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 0.2% (w/v) sodium and potassium tartrate solution:
0.5g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 1g of sodium and potassium tartrate to 50 mL in volumetric flask with purified distill water.
- 10% (w/v) Na_2CO_3 :
5g of Na_2CO_3 to 50 mL in volumetric flask with purified distill water.
 - CTC reagent: slowly add 0.1% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.2% (w/v) sodium and potassium tartrate solution to 10% (w/v) Na_2CO_3 while stirring, this solution need to be kept under refrigeration(about 10°C)
- 0.8M NaOH:
1.6 g of sodium hydroxide pellet in volumetric flask add carefully 50 mL purified distill water while cooling
- 5% (w/v) Sodium dodecyl sulfate:
5 g of Sodium dodecyl sulfate to 100 mL in volumetric flask with purified distill water
 - **Reagent A:** mix one part of CTC Reagent, tow part of 5% (w/v) Sodium dodecyl sulfate (SDS) and one part 0.8M NaOH. This reagent is stable for 2-3 weeks in room temperature (20°C), if white precipitate appear because low temperature it can be re-dissolve easily by heating, but if there are a lot of dark precipitate then it must be discard but small amount of dark precipitate will not affect color development.
- 2N Folin-Ciocalteu phenol reagent

- **Reagent B:** mix one part of 2N Folin-Ciocalteu phenol reagent with five parts distilled water in darkness. This reagent is stable for months at room temperature (20°) in amber container but it will be better to prepare it in the same day of experiment.
- Bovine serum albumin standard(BSA) 0.5 mg/mL:
BSA is water soluble protein but it take time to be dissolve, so the stock solution had been prepared 30 min before starting the experiment
- Centrifuge : protek lab model MP-1000R
- Plane tube 10 mL
- Automatic Pipet
- Test tube rack
- Spectrophotometer (GENESYS 10S UV-VIS)

Procedure

1. 0.05 gram of ground samples was weighted and put in plan test tube
2. 1 mL of distilled water was added
3. 1.5 mL 10% TCA was added and mixed by vortex
4. Centrifuged for 20 minutes at 4100rpm (3080g)
5. The supernatant was decanted, 1.5 mL 10% TCA added and mixed by vortex
6. Centrifuged for 20 minutes at 4100rpm 3080g
7. The supernatant were decanted, 3mL 95% ethanol alcohol added to wash the precipitate
8. Centrifuged for 20 minutes at 4100rpm (3080g)
9. The supernatant were decanted, 3mL 95% ethanol alcohol added to wash. This procedure repeated to give a lipid-depleted residue for soluble protein (Plummer, 1987).
10. At this point, the protein had been extracted and the pellet ready to re-dissolved. 2 mL distilled water had added
11. 2 mL reagents A were added mix with vortex and allowed to stand at room temperature for 10 minutes.

12. During this 10 minutes reagent B was prepared as mentioned before, 1mL reagent B was added and mixed immediately and allowed to stand at room temperature in darkness for 30 minutes.
13. After 30 minutes, the 750 nm was used to read the samples absorbance. Standards and unknowns had been run in duplicate. The standard curve was used to calculate the sample amount. (Peterson, 1983).

3.3.2 Lipid determination

Reagents, required chemicals and equipment

- Vanillin
- 85% Phosphoric acid
 - Vanillin-phosphoric acid reagent: dissolve 150 mg vanillin in 25 ml hot water and then add it slowly to 100 ml of 85% Phosphoric acid.
- Soya been oil used as standard
- Sulfuric acid (95-98%)
- Chloroform-Methanol (1:1)
- Plan tube (10 ml)
- Water bath set on 100°C approximately
- Plan tube rack
- Vortex
- Centrifuge : protek lab model MP-1000R
- Spectrophotometer (GENESYS 10S UV-VIS)

Procedure

1. 0.05 gram of ground samples was weighted and put in plan test tube
2. 1 mL chloroform-methanol (1:1) was added and gently mixed
3. Centrifuge for 5 minutes at 4100rpm (3080g)
4. The supernatant was transferred to another plan tube without any queens tissue
5. It was putted in hot water bath on 100°C approximately to evaporate the solvent for about 2-3 minutes
6. After the solvent completely evaporated, 0.2 mL concentrated sulfuric acid was added and heated for 10 minutes in hot water bath on 100°C approximately
7. After cool, 4.8 mL vanillin reagent was added, mixed with vortex and allowed to stand at room temperature for 10 minutes (the reddish color is stable from 5 to 30 min)
8. The 525nm was used to read the samples absorbance. Optical densities for unknown sample higher than 1.00 was diluted (200µL from original to 4800µL vanillin reagent) (Van Handel, 1985^a)
9. The standard curve was used to calculate the sample amount.

3.3.3 Glycogen and total sugar determination

Reagents, required chemicals and equipment

- Sulfuric acid (95-98%)
- Anthrone
 - In 2 liter Erlenmeyer flask 300 mL distilled water was poured then while cooling carefully 760 mL concentrated sulfuric acid was added. Then, it was dissolved 1.5 gram of Anthrone in this diluted sulfuric acid. The yellow reagent can be stable for many weeks in the refrigerator.
- Pure glycogen (sigma G875-5G glycogen from oyster) and pure glucose was used for standard solutions
- Sodium sulfate 2%
- Methanol
- Test tube 10 mL
- Plan tube rack
- Centrifuge : protek lab model MP-1000R
- Water bath set on 92°C approximately
- Vortex
- Spectrophotometer (GENESYS 10S UV-VIS)

3.3.3.1 Procedure to determination glycogen

1. 0.05 gram of ground sample was weighted and put in plan test tube
2. 0.2 mL 2% sodium sulfate was added and mixed well by vortex
3. 1 mL methanol was added and mix well by vortex
4. Centrifuge for 10 minutes at 4100rpm (3080g)
5. The supernatant was taken to other clean tube (for sugar determinations) and the precipitate putted in hot water bath to evaporate the solvent completely
6. 5 mL of Anthrone Reagent was added, mixed by vortex and heated in hot water bath at 92-90°C for 17 minutes

7. The absorbance was read at 625 nm. Optical densities for unknown sample higher than 1.00 had been diluted (100 μ L from original to 4900 μ L Anthrone Reagent) (Van Handel, 1985^b).
8. The calculation of the unknown sample was calculated according to glycogen standard (1 mg/ml) curve.

3.3.3.2 Procedure to determination total sugar

1. 0.05 gram of ground sample was weighted and putted in plan test tube
2. 0.2 mL 2% sodium sulfate was added and mixed well by vortex
3. 1 mL methanol was added and mix well by vortex
4. Centrifuge for 10 minutes at 4100rpm (3080g)
5. The supernatant was taken to other clean tube and putted in hot water bath to evaporate the solvent to about 0.2 mL
6. 4.8 mL of Anthrone Reagent was added, mixed by vortex and heated in hot water bath at 92-90°C for 17 minutes
7. The absorbance was read at 625 nm. Optical densities for unknown sample higher than 1.00 had been diluted (50 μ L from original to 4950 μ L Anthrone Reagent) (Van Handel, 1985^b)
8. The calculation of the unknown sample was calculated according to glucose standard (1 mg/ml) curve.

3.4 Dry matter and ash determination

1. Hot air oven
2. High temperature furnace
3. Ceramic crucible
4. Sensitive scale

The samples were dried at 103°C in hot air oven for four hours for dry matter calculations and then burned at 550 °C for ash (Bulgurlu and Ergül, 1978).

3.5 Statistics

By using MINITAB software the statistics calculation was done. The data were analyzed with one way ANOVA and Tukey test was used to determine the differences between the averages as a result of analysis of variance.

4. RESULT AND DISCUSSION

4.1. Bumblebee body weight losses and survival rate during different diapause duration

Diapause duration had a significant effect on the body weight losses ($P < 0.05$). Table 1. shows the body weight losses \pm standard errors and survival rate of the diapause queens during the different diapause duration. Body weight losses increased when the diapause time increased Figure 6. Mean body weight losses (15.79 %) were highest at the 90 day of diapause. Survival rate was 100% until 60 days. There were some dead queens after 60 days of diapauses, but it was lower than reported by Gösterit and Gürel, (2009). In our research queens average weight were higher than the reported values, because queens were selected as healthy, big and similar size.

Table 1. Bumblebee body weight losses \pm standard errors and survival rate during different diapause duration

Diapause days	Wet weight mean (g)	After diapause weight mean (g)	Body weight losses (%)	Survival rate (%)
0	0.83 \pm 0.019	Non-diapause	Non-diapause	-
15	0.90 \pm 0.029	0.874 \pm 0.029	3.16 ^F	100
30	0.92 \pm 0.14	0.86 \pm 0.011	6.7 ^E	100
45	0.92 \pm 0.008	0.84 \pm 0.004	7.82 ^{CE}	100
60	0.97 \pm 0.014	0.89 \pm 0.011	9.84 ^{BC}	98.89
75	0.95 \pm 0.004	0.84 \pm 0.003	11.99 ^B	97.33
90	0.99 \pm 0.005	0.83 \pm 0.007	15.8 ^A	97.33

^{A-F}: the values in the same column with different letters are statically different ($P < 0.05$).

4.2. Bumblebee protein levels during different diapause duration

Proteins can be informally divided into three main classes, which correlate with typical tertiary structures: globular proteins (soluble protein which mainly enzymes), fibrous proteins (collagen or exoskeletons protein (cuticle)), and membrane proteins. These three different protein structures can be determined based on the extraction procedure. The soluble protein extraction method was used to see the protein metabolisms of the bumblebee queens during diapause. The total protein level was determined with Lowry method which is commonly used and most cited method. Diapause duration had a significant effect on the mean of soluble protein levels ($P < 0.05$). During different diapauses duration, soluble protein levels were 493.13 ± 8.01 mg/g before enter diapause and then went down to 307.9 ± 6.19 mg/g at day 30, after that it went up to 461.36 ± 4.6 mg/g at day 75. At 90 day of diapause, it started to go down again Figure 6 shows the changes for mean of soluble protein values during different diapause duration. According to our result, there is something happening with protein metabolism. First 30 days, bumblebee soluble proteins decreased but after 30 days they started to mobilize the protein metabolism. Rinehart *et al.*, (2001) reported that an interesting biochemical changes were recorded in pupal diapause of the flesh fly, *sarcophaga crassipalis*, The protein ultra-spiracle (the dimerization partner of the ecdysone-receptor) was not detectable between 20 and 40 days of diapause while it reappears in late diapause in day 50 (Rinehart *et al.*, 2001). Another evidence for grow and develop during diapause, growth was recorded during the larval diapause of a drosophilid fly *Chymomyza costata* both the central nervous system and prothoracic wing discs increased in size during diapause (Kostal, *et al*, 2000). Low levels of juvenile hormone allow the previtellogenic growth of ovaries in diapause females of the carabid beetle *Pterostichus angustatus*, vitellogenesis (also known as yolk deposition) is blocked until diapause complete (Hodek, 2002). The other research support our finding, Fliszkiewicz, 2002 the measurements of ovary length, showed that laboratory raised bumblebee queens experiencing diapause had ovaries that were significantly longer than those of non-diapause bumblebee queens, this characteristic can be taken as an indicator of ovary development as it does not change during the queen's lifetime (Fliszkiewicz, 2002). In *Bumbus ignites* the

optimal chilling duration for break diapause must be at least two months (Yoon and Lee, 2014). Since the diapause time is the preparation time for the production after the diapause (laying eggs and producing workers), it is normal to see increased protein metabolisms during this time.

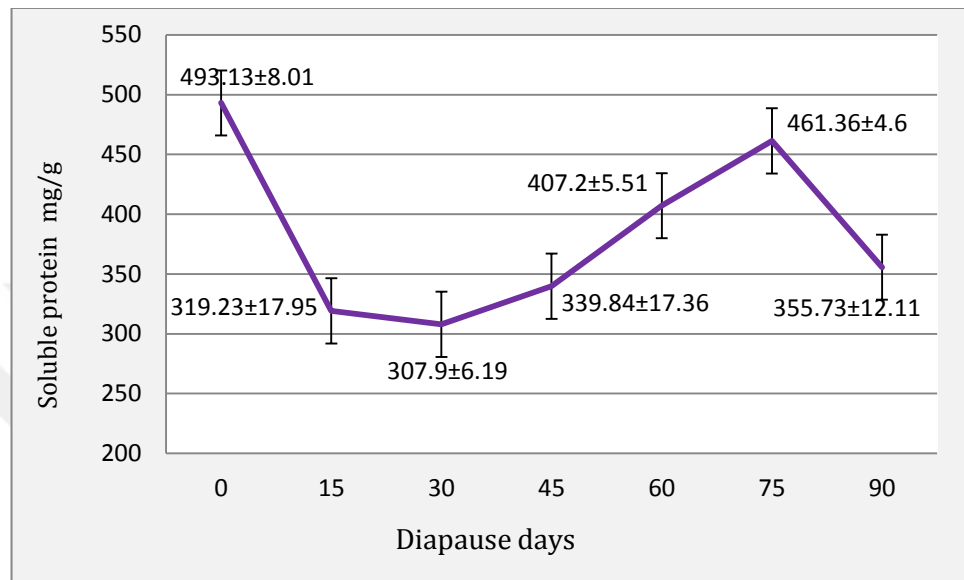


Figure 6. Bumblebee queens' soluble protein (mg/g) ± standard errors during different diapause duration

4.3. Bumblebee lipid levels during different diapause duration

Diapause duration had no significant effect on the lipids levels during different diapause duration Figure 7. Lipid level was 123.78±17.73 before diapause and 127.68±17.26 in 90 day of diapause, minimum level was 94.87±14.06 in 30 day of diapause and maximum level was 134.99±18.48 in 45 day of diapause. Fliszkiewicz and Wilkaniec (2007) reported similar result that diapause and non-diapause queens fatty acids levels were not different, but they reported that lipid and the body fat content is important to control entering diapause for bumblebee queen.

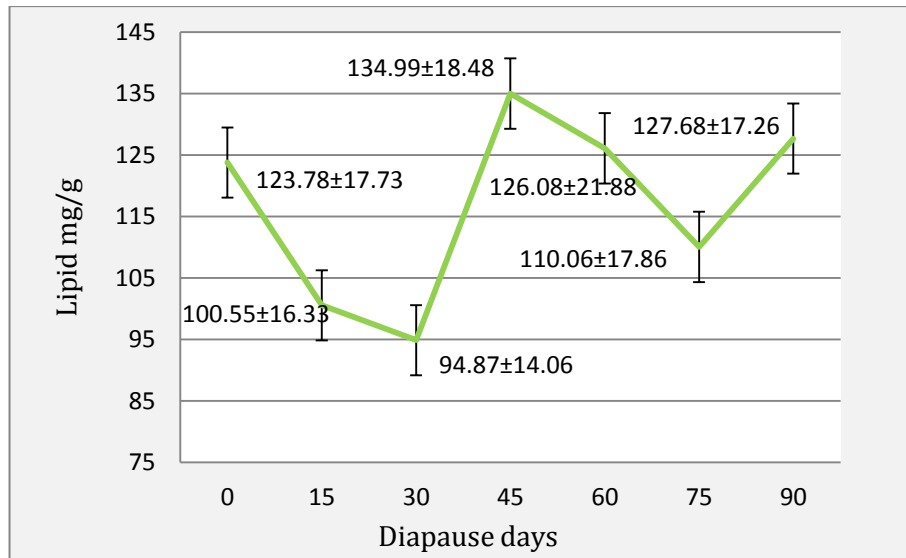


Figure 7. Bumblebee queens' lipid values (mg/g) ± standard errors for different diapause duration

4.4. Bumblebee glycogen levels during different diapause duration

Glycogen is a multi-branched polysaccharide of glucose that serves as a form of energy storage in animals and fungi which is similar to plant starch. In mammalian, glycogen is made and stored primarily in the cells of the liver and the muscles. Bumblebees store sugar as glycogen in the fat body cells. The fat body contains enzymes mediating the gluconeogenesis process as well as enzymatic systems. Glycogen is synthesized by glycogen synthase and hydrolyzed by glycogen phosphorylase in the fat body cells (Anonymous, 2016). Diapause duration had a significant effect on the glycogen levels during different diapause duration ($P < 0.05$). Glycogen levels were very low 174.52 ± 2.81 mg/g before diapause and suddenly go up 266.45 ± 8.26 mg/g when it started diapausing at day 15. Minimum level during diapause was 203.87 ± 6.55 mg/g in day 75. Figure8.

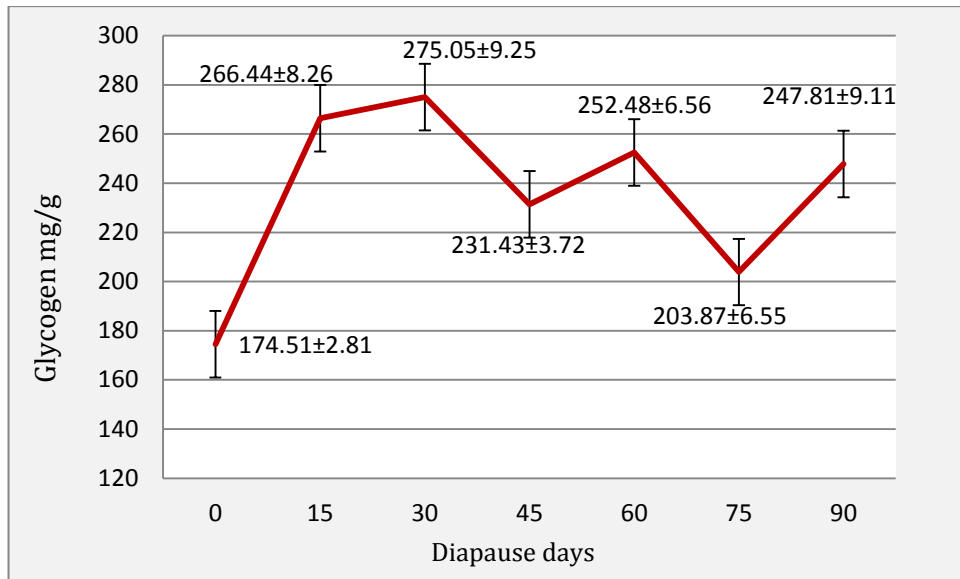


Figure 8. Bumblebee queens' glycogen values (mg/g) ± standard errors for different diapause duration

4.5. Bumblebee sugar levels during different diapause duration

Bumblebees have nectar as main carbohydrate (sugar) sources for energy requirements in nature. Bumblebees are fed with regular beetroot sugar. They store excessive sugar as glycogen and they can use glycogen as a sugar reserves in their body. Soluble sugar level is regulated in the animal body because it is toxic to the cells and the organs. Sugar level may increase after feeding, but it will be stored shortly as glycogen in fat body. When soluble sugar level goes down, glycogen reserves supplies the sugar requirement from fat body. Diapause duration had a significant effect on the sugar levels ($P < 0.05$) during different diapause duration. Total sugar levels were 143.87 ± 7.73 mg/g before diapause and goes up 158.81 ± 2.33 mg/g when it started diapausing at day 15. Minimum level during diapause was 107.9 ± 4.56 mg/g in day 90 Figure9.

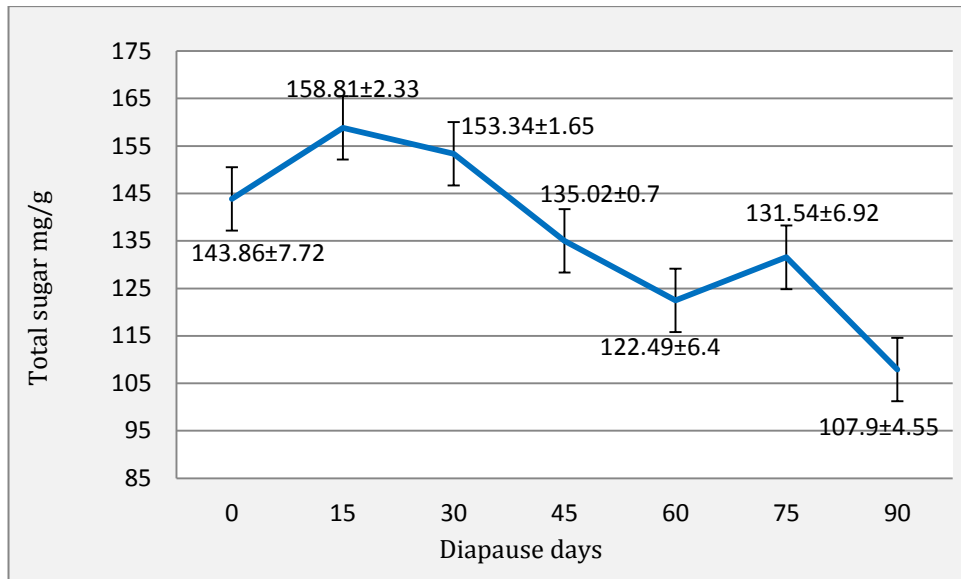


Figure 9. Bumblebee queens' total sugar values (mg/g) ± standard errors during different diapause duration

4.6. Bumblebee ash levels during different diapause duration

Diapause duration had no significant effect on the ash percentage levels during different diapause duration Figure10. Before diapause ash percentage was 1.5 ± 0.2 and in day 90 ash percentage was 1.26 ± 0.16 , maximum level of ash was before diapause 1.5 ± 0.2 and minimum level of ash was in 30 day of diapause.

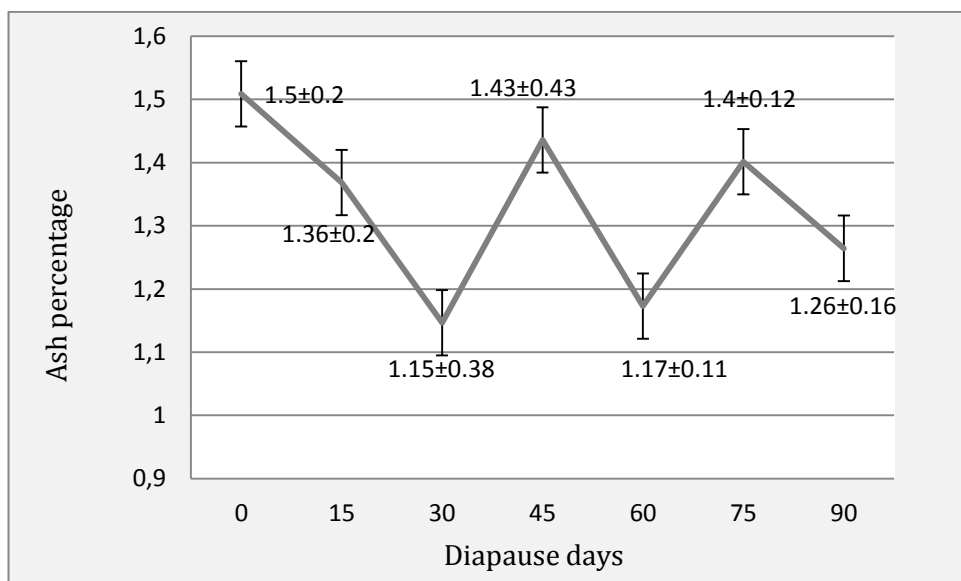


Figure10. Bumblebee queens' ash percentage values ± standard errors during different diapause duration

4.7. Correlations between bumblebee values of total sugar, glycogen, lipid and protein during different diapause duration

The analysis result showed a significant relationship between the nutrients during different diapause duration. There is a significant high negative relationship between levels of soluble protein and glycogen levels ($r=-0.805$; $P<0.001$) Figure 11. In general there is no significant relationship between other factors.

Lipid and protein had low positive relationship($r=0.266$) and sugar had low negative relationship with both lipid($r= -0.322$) and protein ($r= -0.193$) show in Table 2.

Table 2. Correlations between bumblebee queens' sugar, glycogen, lipid and protein levels during different diapause duration

Pearson Correlation	Sugar (r)	Glycogen (r)	Lipid (r)	Protein (r)
Sugar	-			
Glycogen	0.098	-		
Lipid	-0.322	-0.208	-	
Protein	-0.193	-0.805*	0.266	-

* $P<0.001$

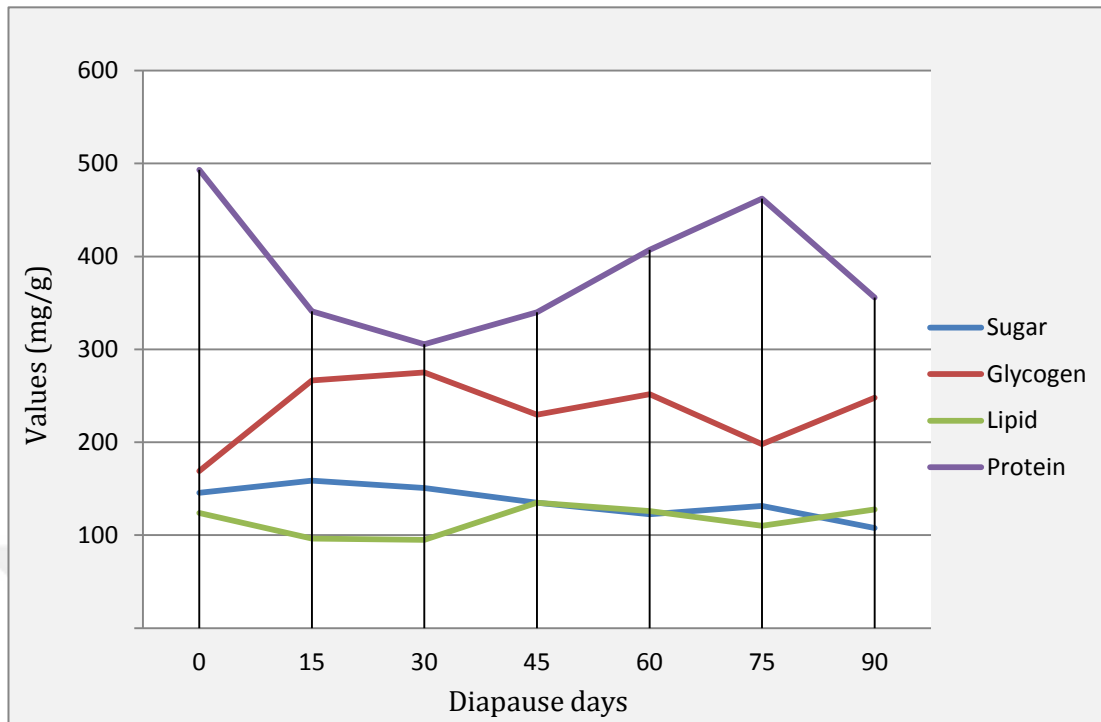


Figure11. Correlations between bumblebee queens' sugar, glycogen, lipid and protein values during different diapause duration

5. CONCLUSIONS AND RECOMMENDATIONS

According to our result of protein diapause period should not be less than 30 days because after 30 days the protein levels are increasing, there is seem to be growth and development in the body of the queens. Until day 75 the levels of soluble protein were high, but after 75 days the level of soluble protein went down and the survival rate decreased. Lipid levels were not different before and after different diapause durations. Lipit content of the bumblebee queens were important entering as reported by other researchers as mentioned before. Glycogen levels before diapause was low because the insect had all nutrients around and it took the energy from sugar directly however during diapause glycogen levels increased in the beginning and sugar levels decreased.

Bumblebee protein, glycogen and sugar metabolisms were active between 30 to 75 days. During this period queen prepare her body for producing the new colony. One of the important jobs is to laying eggs after diapause. There some researchers suggested that during diapause time bumblebees develops

ovaries and also they should be kept in diapause 45 days to get a good colony and surviving rate. Our finding partially explains their results. Feeding the animals before diapause is important issues for bumblebee production. Our findings produce more research questions to be answered such as, entering diapause time, temperature and nutrition content should be investigated thoroughly with a surviving rate and forming good colony after the diapause of the bumblebee queens.



REFERENCES

- Akman, G.E., Gülel, A., 2010. Protein , Lipid , and Glycogen Levels in the Parasitoid Bracon Hebetor Say (Hymenoptera : Braconidae). Journal of Zoology 34:243–48.
- Akman, G.E., Gülel, A., Varerı, I.Ö., Boz A., Cesur, Ö., 2010. Effects of Sugar Feeding on Lipid, Glycogen, and Total Sugar Levels of a Female Parasitoid, Bracon Hebetor (Say) (Hymenoptera: Braconidae). Turkish Journal of Agriculture and Forestry 34,(4):343–47.
- Anonymous., 2015^a. 6 most common bumblebees in the U. K. <http://www.bumblebee.org/key.htm>
- Anonymous., 2015^b. The yearly life cycle of the bumblebee colony <http://www.bumblebee.org/lifecycle.htm>
- Anonymous., 2015^c. The-Crankshaft Publishing’s what-when-how.com claims copyrights to documents only created by The-Crankshaft Publishing and its staff.<http://what-when-how.com/insects/diapause-insects/>
- Anonymous., 2015^d. Pollination. <https://en.wikipedia.org/wiki/Pollination>
- Anonymous., 2016. Fat Body (Insects).<http://what-when-how.com/insects/fat-body-insects/>
- Arrese, E.L., Soulagés, J.L., 2010. Insect Fat Body: Energy, Metabolism, and Regulation Insect Fat Body: an Overview. 207–228.
- Bulgurlu S., Ergul M., 1978. Physical, chemical and biological analyses methods of feeds. Izmir, Ege University Agriculture Faculty. Publish: 127
- Cook, J.M., Crozier, R.H., 1995. Sex Determination and Population Biology in the Hymenoptera. Trends in ecology & evolution (Personal edition) 10,(7):281–86.
- Coppée, A., 2010. *Bombus terrestris* (L . 1758) : A Complex Species or a Species Complex ? Impacts on the Speciation. Universite de Mons.
- Denlinger, D.L., 2009. Diapause. in encyclopedia of insects, edited by V. H. Resh and R. T. Cardé. Academic Press, 2009: 267–271
- ExpotechUSA., 1998. A Guide to Kjeldahl Nitrogen Determination Methods and Apparatus. Labconco 3–10.
- Fliszkiewicz, M., 2002. Causes Of The Lack Of Diapa Use In Bumble Bee Females(*Bombus latr.*, Apoidea). Journal of Apicultural Science 46,(1):31–40.
- Fliszkiewicz, M., Wilkaniec, Z., 2007. Fatty Acids and Amino Acids in the Fat Body of Bumblebee *Bombus terrestris* (L.) in Diapausing and Non-Diapausing Queens. Journal of Apicultural Science 51,(1):55–63.
- Goulson, D., 2010^a. Bumblebees: Behaviour, Ecology and Conservation. second edition. New York, NY: Oxford University Press: 5-12.

- Goulson, D., 2010^b. Bumblebees: Behaviour, Ecology and Conservation. second edition. New York, NY: Oxford University Press: 161-176.
- Goulson, D., 2010^c. Bumblebees: Behaviour, Ecology and Conservation. second edition. New York, NY: Oxford University Press: 12-20.
- Gösterit, A., 2011. Effect of Different Reproductive Strategies on Colony Development Characteristics. *Journal of Apicultural Science* 55,(2):45–51.
- Gösterit, A., Erkan, C., 2012. *Bombus Arısı Yetiştiriciliğinde Karşılaşılan Bazı Hastalık ve Zararlılar*. 3. Uluslararası Muğla Arıcılık ve Çam Balı Kongresi, 1-4 Kasım 2013, Muğla: 335-346
- Gösterit, A., Fehmi, G., 2008. Effects of Temperature Treatments on the Bumblebee (*Bombus Terrestris L.*) Colony Development. *Akdeniz Üniversitesi Ziraat Fakültesi Dergisi* 21,(1):75–78.
- Gürel, F., Gösterit, A., 2008. Effects of Different Stimulation Methods on Colony Initiation and Development of *Bombus terrestris L.* (Hymenoptera: Apidae) Queens. *Applied Entomology and Zoology* 43,(1):113–17
- Gürel, F., Gösterit, A., 2009. The Suitability of Native *Bombus terrestris Dalmatinus* (Hymenoptera: Apidae) Queen for Mass Rearing. *Journal of Apicultural Science*. 53, (1): 67-73,.
- Gürel, F., Gösterit, A., Karşlı, B.A., 2011. Effect of Diapause Duration on Queen Weight Loss and Survival in the *Bombus terrestris*. Glasgow: Society for Experimental Biology.
- Heinrich, B., 1974. Thermoregulation in Endothermic Insects. *Science* (New York, N.Y.) 185,(153):747–756.
- Hodek, I., 2002. Controversial Aspects of Diapause Development. *European Journal of Entomology* 99,(2):163–13.
- Hughes, C., Gray, I., 1998. Chemical Analysis in the New Zealand Dairy Industry. Chemical Processes in New Zealand.
- Huth-Schwarz, A., Settele, J., Moritz, R.F.A, Kraus, F.B., 2012. Factors Influencing *Nosema Bombi* Infections in Natural Populations of *Bombus terrestris* (Hymenoptera: Apidae). *Journal of invertebrate pathology* 110,(1):48–53.
- King, M.J., 1993. Buzz Foraging Mechanism of Bumble Bees. *Journal of Apicultural Research* 32,(1):41–49.
- Kostal, V., Shimada, K., Hayakawa, Y., 2000. Induction and Development of Winter Larval Diapause in a Drosophilid Fly, *Chymomyza costata*. *Journal of Insect Physiology* 46:417–428.
- Lopez-Vaamonde, C., Brown, R.M., Lucas, E.R., Pereboom, J.J.M., Jordan, W.C., Bourke, A.F.G., 2007. Effect of the queen on worker reproduction and new queen production in the bumble bee *Bombus terrestris*. *Apidologie*, 38(2), 171–180.
- Morandin, L.A., 2000. Bumble Bee (*Bombus impatiens*) Pollination Of Greenhouse Tomatoes. western Ontario.
<http://www.ncbi.nlm.nih.gov/pubmed/18605031>

- Peterson, G.L., 1983. Determination of Total Protein. in *Methods in enzymology*, Academic Press 91: 95–119
- Pierce Biotechnology, Inc. 2005. *Protein Assay Technical Handbook*. 1–40. (http://wolfson.huji.ac.il/purification/PDF/Protein_Quantification/PIERCE_ProteinAssayHandbook.pdf).
- Plummer, D.T., 1987. *An Introduction to Practical Biochemistry*. 3rd ed.:270-271
- Resh, V.H., Cardé, R.T., 2009. *Encyclopedia of Insects*. Academic Press is an imprint of Elsevier (Second Edi). Academic Press, 2009.
- Rinehart, J.P., Cikra-Ireland, R.A., Flannagan, R.D., Denlinger, D.L., 2001. Expression of Ecdysone Receptor Is Unaffected by Pupal Diapause in the Flesh Fly, *Sarcophaga crassipalpis*, While Its Dimerization Partner, USP, Is Downregulated. *Journal of Insect Physiology* 47,(8):915–21.
- Rubio, P.L., 2015. Guidelines for the Use of Biobest Bumblebee Hives (*B. terrestris*). (www.biobest.be).
- Silvola, J. 1984. Respiration and Energetics of the Bumblebee *Bombus terrestris* Queen. *Holarctic Ecology* 7,(2):177–81.
- Sladen, F. W. L. 1912. How Pollen Is Collected by the Honey-Bee. *Nature* 88,(2209):586–587.
- Tauber, M.J., Tauber, C.A., 1978. Evolution of Phenological Strategies in Insects: A Comparative Approach with Eco-Physiological and Genetic Considerations. in *Evolution of Insect Migration and Diapause*, edited by H. Dingle. Springer-Verlag New York. :. 53–71
- Van Handel, E. 1985^a. Rapid Determination of Total Lipids in Mosquitoes. *Journal of the American Mosquito Control Association* 1,(3):302–304.
- Van Handel, E. 1985^b. Rapid Determination of Glycogen and Sugars in Mosquitoes. *Journal of the American Mosquito Control Association* 1,(3):299–301.
- Velthuis, H.H.W., Van Doorn, A., 2006. A Century of Advances in Bumblebee Domestication and the Economic and Environmental Aspects of Its Commercialization for Pollination. *Apidologie* 37:421–51.
- Westerkamp, C., 1991. Honeybees Are Poor Pollinators - Why? *Plant Systematics and Evolution* 177,(1-2):71–75.
- Willmer, P.G., Bataw, A.A.M., Hughes, J.P., (1994). The superiority of bumblebees to honeybees as pollinators: insect visits to raspberry flowers. *Ecological Entomology*, 19,(3): 271–284
- Yoon, H.J., Lee K.Y., 2014. Optimal Timing and Duration of Cold Application for Breaking Diapause in Queens of the Bumblebee *Bombus ignitus*. *International Journal of Industrial Entomology* 28,(2):51–57.

APPENDICES

Appendix A Pictures



New nests in the laboratory



Queen with her brood in small nest



Queen with her brood and first worker emerged in small nest



Transfer the nest to bigger colony box



Final stages for the colony in the commercial box



Mixer (vortex)



Hot air oven model MLF 250



high temperature furnace



Spectrophotometer model GENESYS 10S UV-VIS



Centrifuge model MPS-1000

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