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Exploring the Viability of Parasitoid Bee Pupae and Whole Insects Storage Under Cold Conditions

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ABSTRACT

One of the important stages of mass breeding of parasitoid bees is storing these bees at low temperatures in insectariums. The purpose of this study was to investigate the effect of cold on the possibility of keeping whole insects and pupae of parasitoid bees. In this research, 5-day-old pupae and one-day-old bee insects were stored for different periods in refrigerator conditions (absolute darkness and temperature of five degrees Celsius) and the effect of cold on various parameters of reproduction and Bee biology was evaluated. In storage for 30 days or more, one hundred percent of the pupae died, while after one week of storage, nearly 93% of the pupae turned into full-fledged insects, which is different from the control. Nevertheless, storing the pupae in the cold even for one week caused a significant reduction in the fertility and lifespan of the bees removed compared to the control. Storing whole bee insects in the refrigerator hurt their survival according to the duration of storage. The highest losses of female bees were observed after 60 days of storage, while female bees suffered losses in one and two weeks of storage. Unlike pupae, the storage of female bees in the refrigerator did not hurt the lifespan and egg-laying rate of the survivors. According to the results of this research, it is not recommended to store bee pupae in the refrigerator for one week, but female bees can be stored in the refrigerator for one week. The results of this study can be used in the mass breeding and storage of parasitoid bees in insectariums.

Keywords: Parasitoid bees, Insects, Cold, Biology, Reproduction.

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INTRODUCTION

The wasp *Bracon hebetor* Say is one of the most well-known and effective parasitoids that is used in most parts of the world as a biological agent to control scaly pests, especially Noctuidae Pyrallidae and Gelchiidae families [1-4]. This bee first paralyzes the last instar larvae of the host by injecting poison and then lays eggs in groups on their body surface [5, 6]. This parasitoid bee multiplies every year in a large number of insectariums, and to control various pests, especially the cotton bollworm, it is released on the surface of various fields, including cotton, tomato, and soybean [7, 8].

Low-temperature (cold) storage, the history of which dates back to more than 85 years ago [9], is an essential step in the process of mass and commercial breeding of natural enemies in insectariums [10]. In this stage, the natural enemy is stored at a low temperature (usually between zero and 15 degrees Celsius) before being released in the field and until its population reaches a sufficient number so that its metabolism and feeding are temporarily stopped [10, 11]. This technique increases the shelf life of the cultured natural enemy, and as a result, a sufficient and stable stock of it is provided for use in biological control programs [10, 12]. In addition, mass storage provides this possibility, during the outbreak of the pest in the field, the release of the natural enemy is done quickly and in sufficient numbers, and thus, the chance of success of the biological control program increases significantly [13, 14].

Although cold storage is an important part of the mass breeding process of natural enemies in insectariums and has many benefits for breeders [10], severe and long-term cold may affect various aspects of life. Natural enemies, especially parasitoids, also have negative effects [11]. The negative effect of exposure to long-term cold on the sex ratio, lifespan, fertility, length of development period, and the profile of parasitoids has been reported in different studies [10, 15]. In general, the resistance of parasitoids to cold is a complex process that is affected by a variety of external (non-living) factors such as temperature and storage duration, and internal (biological) factors such as nutritional status, sex, age, and developmental stage [11, 16]. The constant or fluctuating storage temperature has also been reported to be effective on the severity of injuries caused by cold so that if a parasitoid regularly and periodically experiences a temperature higher than the storage temperature during storage, the negative effects of cold on him will be reduced. Therefore, to maintain the quality of parasitoids stored in the cold, it is necessary to choose the right temperature for storage, the duration of cooling, and the appropriate growth stage for storage. However, the efficiency of parasitoid storage at low temperatures is often determined by the result of two factors, temperature and duration of storage [13, 17], which in some sources is called the term “cold exposure dose” to show the result of these two factors [18].

Several reports have been published on the storage of *B. hebetor* bees in the cold. The results of a study showed that after a month of storing the female insects of this bee in the refrigerator, their parasitism power decreased, but no change was observed in the parasitism rate of the offspring of the next generation. Also, the storage of female bees in the cold hurt the development period of the immature stages, but it did not have a significant effect on the sex ratio of the offspring of the first and second generations [17]. The results of another study showed that the females in diapause of this bee were more resistant to cold than the females without diapause, and it was possible to store them in the refrigerator for up to 8 weeks [13]. In another study, after three months of storage of whole insects, more than 50% of this parasitoid died at a temperature of 12 degrees Celsius. In contrast, the longevity of female bees, the survival of immature stages, and the sex ratio of the offspring of the next generation were not affected by cold [19]. In addition to the temperature and duration of storage, the effect of the development stage of this bee has also been studied on its resistance to cold and its eggs, larvae, and pupae have been reported to be more sensitive to cold compared to whole insects [20, 21].

Considering the necessity of storing breeding populations of parasitoid bees in insectariums and the need to investigate the response of populations to cold, this research was conducted to comprehensively investigate the lethal and sub-lethal effects of cold on whole insects and pupae of this bee. The results of this research can be used in the storage of complete insects and pupae of this bee in insectariums by supplementing the previous information.

MATERIALS AND METHODS

In this study, the larvae of *Anagasta kuniella* Zeller were used as an alternative host for the breeding of *B. hebetor* bees. Pans containing a mixture of flour and wheat bran after being infected with moth eggs of the host in room conditions (temperature 2±26 degrees, the relative humidity was 10%±60%, and the photoperiod was 16 hours of light to 8 hours of darkness). The larvae of the late stages of moths (fourth and fifth instars) were collected from the rearing pans and were given to parasitoid bees as hosts.

To breed parasitoid bees, plastic containers (height 25 cm and diameter 17 cm) were used. For this purpose, the initial population of parasitoids was confined inside these containers and their opening was blocked using white net cloth (40 mesh). Then, large host larvae (fourth and fifth instars) were provided to the bees in the container on a sheet of paper. Every 24 hours, the old host larvae were placed in closed containers with newly replaced larvae and old parasitized larvae and were kept in the rearing room until the full bees left. After five generations of rearing pupae and complete insects, the required number of bees were collected from the colony using an aspirator or manually and were gradually used in the desired experiments.

Five hundred pupae of the same age (5 days old) along with cocoons were randomly collected from the bee breeding colony and after dividing them into ten populations of 50, each population was placed separately in a transparent plastic cup with the diameter of the mouth. 10 cm and 12 cm height were transferred (each population of 50 pupae was considered as a replication). The opening of the glasses is blocked with a white lace cloth and the glasses are kept for a certain period (according to the experimental treatment, 7, 14, 21, 30, 45, and 60 days) in the refrigerator (temperature 5±1 degrees Celsius and total darkness) were kept. In the control treatment, the pupae were kept in the germinator instead of the refrigerator (temperature of 26±1 degrees Celsius, relative humidity of 60±5%, and photoperiod of 16 hours of light and 8 hours of darkness). After the period considered for each treatment, the pupae of that treatment are taken out of the refrigerator, and after being transferred into the germinator, their hatching percentage (percentage of complete insects) and the sex of the bees removed (based on the presence or absence of ovulation at the end of the abdomen) was measured and recorded separately.

To investigate the effect of cold on the lifespan and reproduction of bees that came out of pupae stored in the cold (sub-lethal effects of cold), 20 pairs of male and female bees were randomly selected from among the bees that came out of the pupae stored in the cold. Daily, ten-fourth or fifth instar larvae of the Mediterranean flour moth as a host and some 30% honey juice were provided to the bees in the glasses through a piece of wet cotton. At the end of every 24 hours, the larvae were removed from the glasses and the number of eggs laid on them was counted and recorded. This process continued until the death of the bees inside the glasses, and thus, the lifespan and daily egg-laying of the bees that came out of the cold-exposed pupae were measured and recorded.

Five hundred male bees and five hundred one-day-old female bees were collected from the bee breeding colony using an aspirator. Then, each of them was divided into ten populations of 50 and each population was transferred separately into a clear plastic cup as an experimental repetition (a total of 20 cups each containing 50 bees in each treatment). The mouths of the glasses were blocked with a white net cloth and the bees inside were fed with 30% honey water for 24 hours before entering the refrigerator. After feeding with honey, the glasses containing the bees were transferred to the refrigerator and kept there for a certain period. After the desired period in each treatment had passed, the glasses related to that treatment were taken out of the refrigerator and after being placed in room conditions, the number of dead people inside them was counted and recorded separately and by gender. Dead bees were those who were not able to move and fly normally until 3 hours after leaving the refrigerator. Accordingly, a small number of bees that were only able to move their arms and legs or their antennae after this period, but were not able to move normally, were counted among the dead. The glasses related to the control treatment were stored in the germinator instead of the refrigerator.

To investigate the effect of cold on longevity and reproduction (daily average of egg laying), queen bees stored in the refrigerator from among the surviving bees at the end of each period, 30 pairs of male and female bees were randomly selected and each pair (as a replication) were placed separately in a beaker. The mouths of the glasses were blocked using a net cloth and placed upside down on the bottom of a white plastic tray. The glasses containing parasitoid bees were kept in the germinator under the conditions mentioned above. Daily and until the death of the female bee in each glass, ten larvae of Mediterranean flour moth (fourth or fifth instars) were provided to the bees in the glass. Also, the bees in the glasses were regularly fed with honey water using a dropper and the method mentioned above. Daily and regularly, paper slices containing host larvae were replaced with slices containing fresh host larvae, and the number of eggs laid on old larvae was counted and recorded. Daily and regularly, paper slices containing host larvae were replaced with slices containing fresh host larvae, and the number of eggs laid on old larvae was counted and recorded. Thus, in addition to the lifespan of male and female bees, the average daily laying of female bees in each treatment was also measured and recorded.

All experiments were conducted in a completely randomized design with seven treatments separately for pupae and whole insects. First, the normality of the data was evaluated using Minitab 13 software, and then the data were analyzed using one-way variance analysis using SAS statistical software. For statistical comparison between groups, the LSD supplemental test was used at the five percent probability level, and Excel 2007 software was used to draw graphs.

RESULTS AND DISCUSSION

Storage of pupae

The results showed that the storage of *B. hebetor* bee pupae in the refrigerator had a significant effect on the percentage of emergence of whole insects ($P<0.0001$, $F_{6, 35} = 253.2$). With the increase in storage time, the percentage of full insects emerging from the pupae decreased, so that in 30 days and more, all the pupae were destroyed and no complete insects came out of them. The percentage of losses (no emergence of complete insects) of pupae that were stored in the refrigerator for 7 days (7.33%) was not significantly different from the control (pupae kept at 26 degrees Celsius), but in periods of more than one During the week, the amount of pupae losses was significantly higher than the control. The results showed that storing the pupae in the cold causes a significant decrease in the lifespan of male and female bees that come out of them ($P<0.0001$, $F_{3, 76} = 39.2$; $P<0.0001$, $F_{3, 76} = 49.8$, respectively). In addition, the storage of pupae in the refrigerator significantly reduced the sex ratio (percentage of female individuals) and reproduction of released bees ($P<0.001$, $F_{3, 20} = 12.02$; $P<0.0001$, $F_{3, 76} = 55.7$, respectively). The average lifespan of male bees in the control treatment was 12.45 days, but with the increase in the storage period, their lifespan decreased and reached 2.4 days in the 21-day treatment. The average lifespan of female bees in the control treatment was 26.8 days, but with the increase in the duration of pupae storage in the refrigerator, the lifespan of the released female bees decreased and reached 4.7 days in the 21-day treatment. In the 14-day treatment, the average lifespan of female bees was 7.55 days, which was not significantly different from the 21-day treatment, but it was significantly less than the control and 7-day treatments **(Table 1)**.

Table 1. Average fertility, sex ratio, and lifespan of full-fledged bee insects after emerging from pupae stored at five degrees Celsius.

Storage time (days)	Fecundity	Percentage of female bees	Longevity of Male (day)	Longevity of Female (day)
0	16.21±0.59[a]	72.12±2.52[a]	26.8±2.19[a]	12.45±1.17[a]
7	7.55±1.09[b]	58.33±2.63[b]	11.85±1.50[b]	5.15±0.67[b]
14	4.26±1.11[c]	56.67±1.11[b]	7.55±0.61[c]	4.30±0.33[bc]
21	1.61±0.37[d]	69.50±1.78[a]	4.70±0.58[c]	2.40±0.21[c]

$P < 0.05$, LSD-test

Storing pupae in the refrigerator also significantly reduced the proportion of female individuals and the average daily laying (birth) among the released bees. So the percentage of female bees increased from 72.12% in the control treatment to 56.67% in the 14-day treatment with a significant decrease. The percentage of female bees in the 21-day treatment slightly increased compared to the 7- and 14-day treatments and reached 69.5%. The average egg-laying of the female bees that came out of the pupae in the control treatment was 16.21 eggs per day, but with the increase in the duration of pupae storage in the refrigerator, the average egg-laying of the bees decreased and reached 1.61 eggs per day in the 21-day treatment. In treatments of 7 and 14 days, the average egg laying of female bees was 7.55 and 4.26 eggs per day, respectively, both of which were significantly reduced compared to the control **(Table 1)**. In other words, storing the pupae of this bee in the refrigerator for one, two, and three weeks, respectively, caused a 53, 74, and 90% decrease in the average daily egg laying of the released female bees compared to the control.

Complete insect storage

The results of this research showed that the storage of whole male and female insects in the refrigerator significantly reduced their survival ($P<0.0001$, $F_{6, 63} = 377.5$; $P<0.0001$, $F_{6, 63} = 316.1$, respectively). By increasing the storage time in the refrigerator, the percentage of male and female bees lost increased, so that in the 60 days of treatment, one hundred percent of male bees and 97.14% of female bees died. On the other hand, in one-week storage, the percentage of female bees lost was only 4.42%, which was not significantly different from the control. However, the percentage of deaths of male bees in the same period (25.08%) was significantly higher than the control.

Storage of male and female bees in the refrigerator significantly affected their lifespan ($P<0.0001$, $F_{4, 145} = 14.33$; $P<0.001$, $F_{4, 145} = 5.59$, respectively). In general, the bees stored in the refrigerator had a longer life span than the control bees if they tolerated the cold and survived. So that the average lifespan of male bees in the control treatment was 4.7 days, while in the 14-day treatment, this amount reached 20.37 days. Female bees also had similar conditions in terms of lifespan, so their lifespan increased from 16.7 days in the control treatment to 29.87 days in the one-month treatment, although no significant difference was observed between different storage periods. In proportion to the increase in the lifespan of the female bees stored in the refrigerator, the length of their egg-laying period also increased significantly ($P<0.001$, $F_{4, 145} = 39.2$) and from 11 days in the control treatment to 18.5 days in the 30-day treatment reached, but no significant difference was observed between the treatments of 14, 21 and 30 days in this regard.

Although the bees stored in the refrigerator had a longer lifespan compared to the control, their average daily laying and clutch size (the number of eggs laid on one host larva) did not have a statistically significant difference in different treatments ($P>0.05$, $F_{4, 145} = 1.83$; $P>0.05$, $F_{4, 145} = 0.66$, respectively). The average fertility of female bees in the control and 30-day treatments were calculated as 10.42 and 11.16 eggs per day per female bee, respectively, which were not significantly different from each other. At the same time, the size of the bee clutch had very limited changes and reached 3.54 eggs per host larva in the control treatment to 3.71 eggs in the 30-day treatment.

The results of the present study showed that the effects of storing bee insects in the refrigerator were only limited to the parent bees and the offspring in the next generation were not affected by the storage of the parents in the refrigerator. So there was no statistically significant difference between the development period of immature stages in different storage periods ($P>0.05$, $F_{4, 145} = 1.89$; $P>0.05$, $F_{4, 145} = 2.15$, in male and female bees, respectively). In male and female bees, the average length of the development period of immature stages in different treatments fluctuated from 11.9 to 12.77 days in male bees and 12.07 to 12.8 days in female bees. Like the length of the development period, the proportion of female bees in the next generation offspring was not affected by the storage of the parents in the refrigerator ($P>0.05$, $F_{4, 70} = 2.02$) and the average percentage of female bees in the next generation offspring was in the range of 68.87% in the control treatment until 61.44% fluctuated in 30 days treatment.

Considering the importance of the parasitoid bee *B. hebetor* in the biological control of scaly pests, especially the cotton bollworm, and the increasing expansion of its mass breeding in insectariums, it is necessary to provide solutions for the efficient and economical storage of this parasitoid at low temperatures. Cold resistance of parasitoids is a complex process that is affected by a variety of external and internal factors such as storage temperature, storage duration, developmental stage, and nutritional status [16]. Although temperatures between zero and 15 degrees Celsius have been recommended for parasitoid storage [16], technically it is easier for insectarium owners and farmers to provide a temperature of five degrees Celsius (refrigerator temperature) than other temperatures and bee storage is often done at this temperature. On the other hand, one of the important and influencing factors on the tolerance of parasitoids to cold is their developmental stage [16]. Most efforts to store this bee at low temperatures have focused on the complete insect [13, 17] and pupa [20, 22, 23], and there are very few reports on the storage of other developmental stages [21, 24].

The results of the present research regarding the storage of bee pupae at refrigerator temperature showed that according to the duration of storage, the qualitative characteristics of the pupae, including hatching percentage, as well as life span, egg-laying power and the proportion of female bees that came out of them decreased significantly. The negative effects of storing pupae in the cold on the quality characteristics of this bee have been reported in several studies [20, 25].

In the present study, the mortality percentage of pupae that were stored in the refrigerator for one week was reported to be much less than 70%. Also, in the period of two and three weeks, the mortality percentage of the pupae in the present study was much lower compared to the reported values. The results of the present study regarding the possibility of storing bee insects in the refrigerator showed that it is proportional to the storage period. Complete insect populations also suffered mortality like pupae, and these losses were significantly higher in male bees than in female bees. In long-term storage [26] and 60 days like pupae, almost 100% of male bees and more than 80% of female bees were lost. While in one-month storage, unlike pupae, approximately 50% of full-fledged female insects survived.

On the other hand, unlike pupae, whose storage in the refrigerator significantly reduced the life span and reproduction of whole insects, in the storage of whole insects, the cold did not have any negative effect on the life span and reproduction of the surviving individuals, and even the lifespan of cold-stored bees was significantly increased compared to control bees. The negative consequences of storing whole insects of bees and other parasitoids in the cold have been reported in several studies [27, 28]. In almost all the studies conducted regarding the storage of *B. hebator* whole insects in the cold, the negative effect of cold on the survival of the whole insects has been reported. While the reports regarding the negative effects of cold on other biological and reproductive characteristics of parasitoid bees including *B. hebator* (sub-lethal effects) are contradictory, in several reports, contrary to the results of the present study, the sub-lethal effects of cold such as A decrease in lifespan and egg laying on whole insects of some parasitoids have been reported [29, 30] showed that the average *oviposition* of *B. hebetor* was significantly reduced after long-term cold storage of its whole insects. Depletion of food and water reserves and the accumulation of toxic metabolites have been reported as possible reasons for this decrease [17].

The temperature and photoperiod conditions of bee breeding before cold storage play an important role in the percentage of losses during storage. In the present study, the bee rearing conditions before storage was a non-diapause condition, while Chen *et al*. [13] showed that rearing this bee at a temperature between 17-20 degrees Celsius and a photoperiod of 10 hours of light to 14 hours of darkness (diapause conditions) causes diapause in the bee and as a result increases its tolerance to cold and reduces losses during storage at refrigerator temperature. In general, considering that the *B. hebator* bee spends the winter in the form of a complete insect [31], therefore, complete insects are more resistant to cold compared to pupae [32]. On the other hand, the results of some studies related to the storage of whole insects of parasitoids in the cold [14, 33] like the present study, have reported no negative effect of cold on the reproduction of parasitoids.

The results of the present study showed that the parasitoids stored in the refrigerator had a longer lifespan if they survived. In some studies related to the investigation of the effect of stresses on parasitoids [34, 35], this phenomenon is referred to as type B reaction, during which high intensities of environmental stress (such as cold) after the occurrence of severe losses in A population causes a better selection of individuals more resistant to cold and these surviving individuals have better performance in terms of longevity and egg laying. According to the results of the present research, the duration of development and the appearance of immature stages (egg to complete insect) and the sex ratio in the offspring of the next generation were not affected by the storage of parent bees in the cold, which indicated that in the storage of complete insects *B. hebetor* bees were stored in the refrigerator, the negative effects of cold were limited to the parent bees, and these effects were not transferred to the offspring of the next generation.

Similar to the present study, the results of Chen *et al*. [17] also showed that after storing *B. hebetor* whole insects at low temperatures, the sex ratio of the released bees in the next generation was not affected by cold. Chen *et al*. [17] showed that the length of germination and appearance of the immature stages of *B. hebator* was affected by cold, but there was no linear relationship between the duration of cold storage and the duration of germination and appearance. In addition, in the results of their research, the maximum difference in the length of emergence and appearance in different storage periods was two days, which can be ignored in the mass breeding programs of this bee.

CONCLUSION

The results of this research showed that the storage of *B. hebetor* bee pupae at a temperature of five degrees Celsius for more than a week caused a significant decrease in the percentage of the emergence of full-fledged insects, as well as the lifespan and average laying of female bees. In the one-week storage of the pupae, although their hatching percentage did not change significantly compared to the control, the life span and average egg laying of the released bees decreased significantly. Therefore, it is not recommended to store the pupae of this bee in the refrigerator even for a short period. Of course, in insectariums and herbal medicine clinics, this bee is delivered to farmers in the form of a complete insect, and pupae are stored in some special cases and often to preserve the colony, in which case the pupae can last up to a week. On the other hand, comparing the findings of this study with the results of other studies showed that probably older pupae (5 days old) are more resistant to cold compared to young pupae (one day old). Also, due to fewer losses of whole insects in the cold and no negative effect of low temperature on the lifespan and egg-laying of female bees, it seems that the whole insects of this bee are more suitable for storage at low temperatures compared to pupae. The whole insects of this bee can be stored in the refrigerator for up to a week.

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