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The Impact of Cold Storage on the Survival and Viability of Parasitoid Bee Pupae and Whole Insects

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ABSTRACT

An important aspect of large-scale parasitoid bee breeding involves maintaining the bees at cold temperatures within insectariums. This study aimed to evaluate the effect of cold storage on the viability of both pupae of parasitoid bees and adult insects. Five-day-old pupae and newly emerged one-day-old bees were subjected to different storage durations under refrigeration (complete darkness at a constant temperature of 5 °C). The effects of cold exposure on various biological and reproductive parameters were analyzed. Pupae maintained for 30 days or more experienced complete mortality, while approximately 93% of those refrigerated for one week successfully emerged as adult insects, which was different from the control group. However, even short-term cold storage of pupae significantly reduced the lifespan and fertility of emerging bees compared to the control group. Storing adult bees under refrigeration negatively affected their survival, with losses increasing as storage duration prolonged. The highest mortality rates were recorded among female bees after 60 days of storage, although mortality was also observed after 1 and 2 weeks. Unlike pupae, cold storage of adult female bees did not negatively affect their lifespan or egg-laying capacity. Based on these findings, refrigeration of bee pupae for one week is not recommended, whereas female bees could be stored under cold conditions for up to one week without severe consequences. These insights contribute to improving parasitoid bee mass breeding and storage strategies in insectariums.

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

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Introduction

Bracon hebetor Say, a widely recognized and highly effective parasitoid wasp, is extensively utilized worldwide as a biological control agent against scaly pests, particularly those belonging to the Noctuidae, Pyralidae, and Gelechiidae families [1-4]. This parasitoid bee incapacitates the final instar larvae of its host by injecting venom, leading to paralysis, and subsequently deposits groups of eggs on the larval body surface [5, 6]. Due to its efficacy, *B. hebetor* is mass-reared annually in numerous insectariums and deployed to manage pest populations, particularly cotton bollworm infestations, across various agricultural fields, including tomato, cotton, and soybean crops [7, 8].

The practice of cold storage, which dates back over 85 years [9], plays a critical role in the large-scale and commercial rearing of natural enemies in insectariums [10]. During this process, natural enemies are kept at low temperatures, typically ranging between 0 and 15 °C, to temporarily suppress their metabolism and feeding

activity before they are released into the field [10, 11]. This approach extends the shelf life of parasitoids, ensuring a stable and sufficient stock for integration into biological control strategies [10, 12]. Furthermore, large-scale storage enables the rapid release of parasitoids in significant numbers when pest outbreaks occur in the field, substantially enhancing the success rate of biological control programs [13, 14].

While cold storage is a crucial component of mass-rearing enemies in insectariums and offers numerous advantages for breeders [10], prolonged or extreme exposure to low temperatures can negatively impact various biological aspects of parasitoids [11]. Several studies have documented the detrimental effects of extended cold exposure on factors such as lifespan, sex ratio, fertility, development duration, and overall physiological traits of parasitoids [10, 15].

The ability of parasitoids to withstand cold conditions is a different process influenced by both external (abiotic) factors, such as levels of temperature and duration of storage, and biological factors, including age, sex, nutritional condition, and developmental stage [11, 16]. Additionally, whether the storage temperature remains constant or fluctuates has been shown to affect the extent of cold-induced damage. Research suggests that periodically exposing parasitoids to temperatures higher than the primary storage temperature during storage may mitigate some of the adverse effects [11, 16].

To preserve the quality and viability of parasitoids during cold storage, selecting the appropriate temperature, storage duration, and developmental stage for preservation is essential. However, the effectiveness of parasitoid storage at cold temperatures largely depends on two key factors: temperature and the length of storage [13, 17]. Some sources refer to the combined impact of these two variables as the "cold exposure dose," which helps determine the overall effects of cold storage on parasitoids [18].

There have been numerous studies on the cold storage of *B. hebetor* parasitoid bees. One study indicated that after one month of refrigeration, the parasitism ability of female bees diminished, though there was no noticeable change in the parasitism rate of their subsequent generation. Furthermore, cold storage negatively impacted the development duration of the immature stages, yet the ratio of sex in both the 1st and 2nd generations remained largely unaffected [17]. Another study found that diapause females of this bee species exhibited greater cold tolerance than non-diapause females, allowing them to be stored for up to eight weeks in the refrigerator [13]. In a different investigation, more than half of the parasitoids died after 3 months of storage at 12 °C. However, cold storage did not significantly alter the longevity of the females, the survival rate of the immature stages, or the ratio of sex in the next generation's offspring [19].

The impact of storage conditions, such as temperature and duration, as well as the developmental stage of *B. hebetor*, has also been explored. Larvae, eggs, and pupae of the species have shown higher sensitivity to cold when compared to whole insects [20, 21].

Considering the significance of preserving breeding populations of parasitoid bees, this study aims to find out the lethal and sub-lethal impacts of cold exposure on both *B. hebetor* pupae and whole insects. The findings from this research will contribute to improving the storage practices for both the complete insects and pupae of this species in insectariums, adding to the body of knowledge on the subject.

Materials and Methods

For this study, *Anagasta kuniella* Zeller larvae were chosen as an alternative host for breeding *B. hebetor* bees. A mixture of flour and wheat bran was placed in pans and inoculated with moth eggs under room conditions (temperature of 26 ± 2 °C, relative humidity of 60 ± 10%, and a 16-hour light/8-hour dark photoperiod). Late-stage larvae (4th and 5th instars) were harvested from the pans and provided to the parasitoid bees as hosts.

Parasitoid bees were bred in plastic containers (25 cm in height and 17 cm in diameter), with their openings sealed with a fine white mesh cloth (40 mesh). Host larvae (4th and 5th instars) were offered to the bees on sheets of paper inside the containers. After 24 hours, the parasitized larvae were replaced with fresh ones, and the parasitized larvae were put in separate containers to allow the bees to emerge. After 5 generations of breeding, both pupae and fully developed bees were collected manually or using an aspirator and used in the experiments as needed.

In this study, 500 five-day-old pupae along with their cocoons were selected randomly from the bee colony. These pupae were grouped into ten separate populations of fifty each. Each population was put in a plastic cup. The openings of the cups were sealed with white lace cloth. The pupae were stored in the refrigerator at a temperature of 5 ± 1 °C and total darkness for varying periods (7, 14, 21, 30, 45, and 60 days), depending on the experimental

treatment. In the control group, the pupae were stored in a germinator at 26 ± 1 °C, 60 ± 5% relative humidity, and a 16-hour light/8-hour dark photoperiod. After the designated storage period, the pupae were transferred from the refrigerator to the germinator, where their hatching success (percentage of pupae that hatched into insects) and the gender of the emerging bees were recorded.

To evaluate the sub-lethal effects of cold on bee lifespan and reproduction, 20 male and 20 female bees that emerged from cold-stored pupae were randomly selected. These pairs were placed in separate containers, and every day, they were provided with 10 fourth or 5th instar larvae of the Mediterranean flour moth and 30% honey water via a piece of moist cotton. The larvae were replaced daily, and the number of eggs laid on them was counted and documented. This procedure was repeated until the bees died, allowing for the measurement of their lifespan and daily egg-laying capacity.

Additionally, 500 male and 500 one-day-old female bees were collected using an aspirator from the bee colony. These bees were also divided into 10 populations of fifty each, and placed in separate clear plastic cups (a total of twenty cups, with fifty bees in each treatment). The openings of the cups were sealed with white net cloth, and the bees were fed 30% honey water for a day before refrigeration.

Following the honey feeding, the cups of bees were placed in the refrigerator and stored for the designated time according to each treatment. Once the storage period for each treatment was complete, the cups were removed from the refrigerator and allowed to return to room temperature. The number of dead bees was then counted and recorded separately by gender. A bee was considered dead if it was unable to move or fly normally within 3 hours of being removed from the refrigerator. Bees that could only move their legs, arms, or antennae during this period but could not resume normal movement were also counted as dead. The control treatment cups were kept in the germinator rather than the refrigerator.

To examine how cold storage influences the lifespan and egg-laying behavior of queen bees, thirty pairs of bees from the surviving population at the end of each storage period were randomly selected. Each pair was placed in an individual beaker, which was considered a replication, with the opening covered by a net cloth. The beakers were inverted and set inside a plastic tray. These containers were then placed in the germinator under the controlled conditions mentioned earlier. During the experiment, ten larvae (4th or 5th instars) of the Mediterranean flour moth were given to the bees daily, along with regular honey water feedings via a dropper. The larvae were replaced with fresh ones each day, and the number of laid eggs on the larvae was recorded. The study monitored both the longevity of the bees and the average number of laid eggs by the female bees per day.

The experiments followed a random design, with 7 different treatments for both pupae and whole insects. The data were checked for normality using Minitab 13 software and analyzed using one-way ANOVA in SAS software. Statistical comparisons between the groups were made with the LSD test at the 5% significance level, and graphs were generated using Excel 2007 software.

Results and Discussion

Pupae storage results

The findings indicated that refrigerating *B. hebetor* pupae had a marked impact on the emergence rate of adult bees ($P < 0.0001$, $F_{6, 35} = 253.2$). As storage time increased, the emergence rate of fully developed insects decreased. After 30 days or more of cold storage, all pupae failed to produce any complete insects. The loss rate of pupae after 7 days in the refrigerator (7.33%) was not different from the control group (pupae stored at 26 °C), but longer storage periods resulted in significantly higher losses compared to the control. The analysis also revealed that cold storage significantly reduced the lifespan of both female and male bees that emerged from the pupae ($P < 0.0001$, $F_{3, 76} = 39.2$; $P < 0.0001$, $F_{3, 76} = 49.8$, respectively). Furthermore, cold storage negatively affected both the sex ratio (the percentage of females) and the reproductive success of the emerging bees ($P < 0.001$, $F_{3, 20} = 12.02$; $P < 0.0001$, $F_{3, 76} = 55.7$).

For example, male bees in the control group lived for an average of 12.45 days, but this lifespan decreased significantly as storage time increased, dropping to just 2.4 days after 21 days of cold storage. Similarly, female bees in the control group had an average lifespan of 26.8 days, but their lifespan also decreased with longer storage durations, reaching only 4.7 days after 21 days. In the 14-day cold storage treatment, female bees had an average lifespan of 7.55 days, which, while not significantly different from the 21-day group, was still notably shorter than both 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 5 °C.

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}

Cold storage of *B. hebetor* pupae notably affected the emergence of female bees and their reproductive output. The proportion of female bees was significantly lower after refrigeration. In the control group, 72.12% of the emerged bees were female, but this dropped to 56.67% after 14 days of cold storage. A slight recovery to 69.5% occurred in the 20 first day of treatment, though it remained lower than the control group.

The reproductive capacity of female bees was also adversely affected by prolonged cold storage. Bees from the control group laid an average of 16.21 eggs daily. However, as the pupae were stored for longer periods, egg-laying diminished. After 21 days of refrigeration, egg-laying fell dramatically to 1.61 eggs per day. In the 7 and 14-day treatments, the mean daily egg count was 7.55 and 4.26, respectively—both significantly lower than the control group **(Table 1)**. Essentially, refrigeration of pupae for 7, 14, and 21 days resulted in a 53%, 74%, and 90% reduction in the mean daily egg-laying of the females, respectively, compared to the control group.

Storage of the whole *B. hebetor* male and female bees in cold conditions resulted in a significant reduction in their survival rate ($P < 0.0001$, $F6, 63 = 377.5$; $P < 0.0001$, $F6, 63 = 316.1$). As storage time increased, mortality rates rose, with all male bees and 97.14% of female bees dying after 60 days in the refrigerator. However, after one week of storage, the mortality of female bees was only 4.42%, which was not significantly different from the control group. In contrast, male bee mortality at the same time was 25.08%, a significant increase compared to the control group.

Cold storage also had a noticeable impact on the lifespan of both male and female bees ($P < 0.0001$, $F4, 145 = 14.33$; $P < 0.001$, $F4, 145 = 5.59$). For bees that survived the cold storage, their lifespan was extended compared to the control group. Male bees in the control group had an average lifespan of 4.7 days, while those in the 14-day treatment lived for 20.37 days. Similarly, female bees from the control group lived for an average of 16.7 days, and those stored for 30 days had a lifespan of 29.87 days, though no significant differences were observed across various storage durations.

As the lifespan of female bees increased with cold storage, the duration of their egg-laying period also significantly increased ($P < 0.001$, $F4, 145 = 39.2$), from eleven days in the control group to 18.5 days in the 30-day treatment. However, no significant differences were noted in egg-laying duration between the 14, 21, and 30-day treatments. Despite the increased lifespan of the refrigerated bees, their average daily egg-laying and clutch size did not show statistically significant variations across different storage periods ($P > 0.05$, $F4, 145 = 1.83$; $P > 0.05$, $F4, 145 = 0.66$). The fertility rate of female bees was nearly identical in the control (10.42 eggs per day) and 30-day treatments (11.16 eggs per day), with only minimal differences in clutch size, which ranged from 3.54 eggs per host larva in the control group to 3.71 eggs per host larva in the 30-day treatment.

The findings from this study revealed that cold storage primarily impacted the parent bees, with no significant effects on the offspring's development in the following generation. There was no notable difference in the developmental duration of immature stages across the various storage periods for either male or female bees ($P > 0.05$, $F4, 145 = 1.89$; $P > 0.05$, $F4, 145 = 2.15$). For both male and female bees, the mean development time ranged from 11.9 to 12.77 days for males and from 12.07 to 12.8 days for females. Similarly, the proportion of female bees in the next generation was unaffected by the cold storage of the parents ($P > 0.05$, $F4, 70 = 2.02$), with the percentage of female offspring varying from 68.87% in the control group to 61.44% in the 30-day treatment.

Given the significance of *B. hebetor* as a biological control agent, particularly against pests like the cotton bollworm, and the growing demand for its mass production, it is crucial to develop effective and cost-efficient methods for storing this parasitoid at cold temperatures. The cold tolerance of parasitoids is influenced by several factors, including storage duration, temperature nutritional status, and developmental stage [16]. While temperatures ranging from 0 to 15 °C are often recommended for parasitoid storage [16], a refrigerator temperature of 5 °C is more practical for insectarium owners and farmers. Consequently, many use this temperature for bee storage. Additionally, a key factor in determining a parasitoid's cold tolerance is its

developmental stage [16]. Many studies on cold storage of *B. hebetor* have mentioned the whole insect [13, 17] and pupae [20, 22, 23], with relatively few reports addressing the storage of other developmental stages [21, 24]. The findings of this study regarding the cold storage of bee pupae indicated that as the storage duration increased, there was a significant decline in the quality of the pupae, including lifespan, egg-laying capacity, hatching rates, and the proportion of female bees that emerged. Similar detrimental effects of cold storage on the quality of *B. hebetor* pupae have been documented in previous research [20, 25].

In this study, the pupae stored in the refrigerator for a week showed mortality rates below 70%, with the two- and three-week storage periods also resulting in lower mortality rates compared to those reported in earlier studies. Additionally, the survival rate of complete insect populations, similar to the pupae, was negatively impacted by the cold, with male bees experiencing higher mortality than females. After long-term storage, particularly at 60 days, nearly 100% of male bees and over 80% of female bees died. In contrast, approximately 50% of female bees survived after one month of storage, unlike the pupae.

However, when considering whole bees, the cold storage did not significantly reduce the lifespan or reproductive capacity of the surviving insects, and their lifespan increased substantially when compared to the control group. This contrasts with the negative effects observed in previous studies, where cold storage of whole bees and other parasitoids led to mortality and reduced biological performance [26, 27]. Most studies on cold storage of *B. hebetor* have noted a decline in the survival rates of the entire insect. Despite this, the effects of cold storage on reproductive traits, such as egg-laying and lifespan, are inconsistent. Several studies have reported sub-lethal effects such as a decrease in both lifespan and egg-laying after prolonged cold exposure of parasitoid bees, which was not observed in this study [28, 29]. Some research suggests that a reduction in water and food reserves or the buildup of toxic metabolites might explain these sub-lethal effects [17].

The conditions in which bees are raised before cold storage, such as temperature and photoperiod, are also crucial in determining the extent of loss during storage. In this study, the bees were reared under non-diapause conditions, whereas other studies have shown that rearing *B. hebetor* under diapause conditions (17–20 °C and a photoperiod of ten hours of light and fourteen hours of darkness) enhances its cold tolerance and reduces mortality during storage at refrigerator temperatures [13]. Overall, as *B. hebetor* overwinters in its complete insect form [30], these stages are more resistant to cold than pupae [31]. Some studies on the cold storage of parasitoid insects also support the finding that cold does not negatively impact reproduction, in line with the present study's results [14, 32].

The findings of this study demonstrated that parasitoids stored in the refrigerator had an extended lifespan, provided they survived the cold storage period. This phenomenon, often referred to as a type B response in studies on parasitoid stress [33, 34], occurs when severe environmental stresses, such as cold, lead to significant population losses. However, the remaining individuals who endure these stresses tend to be more resilient and show improved performance in terms of both longevity and egg-laying. In the case of *B. hebetor* bees, the current research showed that cold storage did not affect the development time, emergence of immature stages (from egg to adult), or the ratio of sex of the next generation. This suggests that the detrimental effects of cold storage were confined to the parent bees and did not influence the offspring of subsequent generations.

These findings align with the results of Chen *et al*. [17], who also found that cold storage of *B. hebetor* whole insects did not impact the ratio of sex of their progeny. While Chen *et al*. observed that cold storage did influence the length of the immature stages' development, they found no direct correlation between the storage duration and the developmental period. Furthermore, in their study, the variation in emergence and developmental periods across different storage durations was minimal—only a two-day difference—which is considered negligible for mass breeding of this species.

Conclusion

In conclusion, the findings from this study indicated that storing *B. hebetor* bee pupae at 5°C for more than a week resulted in a significant decline in the emergence of fully developed insects, as well as in the egg-laying capacities and lifespan of female bees. Although there was no substantial change in the hatching percentage of pupae after one week of storage compared to the control group, there were notable reductions in the lifespan and egg production of the emerging bees. Therefore, short-term storage of these pupae in the refrigerator is not advisable. However, in insectariums and botanical medicine facilities, it is common for bees to be provided to farmers as fully developed insects, with pupae being stored only in specific cases, such as for colony preservation, where

they can last for a week. Furthermore, this study's results, when compared with previous research, suggest that older pupae (approximately five days old) exhibit greater cold resistance than younger ones (about one day old). Additionally, due to lower mortality rates and no detrimental effects on the egg-laying abilities and lifespan of adult bees, it appears that whole insects are better for cold storage than pupae. These insects can be stored in the refrigerator for a week without significant negative effects.

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