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# Effects of constant low-temperature storage on the performance of a commercial strain of *Aphidius colemani*

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## Abstract

This study determined the effects of cold storage of mummies on the biological characteristics of *Aphidius colemani* Viereck (Hymenoptera Braconidae), a solitary, koinobiont endoparasitoid of more than 40 species of aphids. The effects of three constant temperatures (5, 7 and 9 °C) at five time periods (5, 10, 15, 20 and 30 days) were evaluated on eight quality control parameters of the parasitoid (adult emergence, sex ratio, time elapse before emergence, adult longevity, length of the hind tibia, flight capacity, percent parasitism and sex ratio of F1 wasps). The results showed that all parameters except the sex ratio of F1 were negatively affected by cold storage duration in comparison to control treatment (25 °C). Based on the adult emergence and sex ratio, *A. colemani* could be stored for 15 days at 5 and 7 °C and for 10 days at 9 °C but it is important to consider other quality control parameters (time elapse before emergence, adult longevity, adult size, flight capacity and percent parasitism). However, when the mummies of *A. colemani* were stored for longer than 10 days, their quality criteria were more negatively affected at all three temperatures. The results are discussed in order to facilitate the planning of *A. colemani* mass rearing and aphid biocontrol programs.

**Key words:** cold storage, quality control, aphid parasitoid, Braconidae, biological control, mass rearing.

## Introduction

Aphids are a major threat to agriculture worldwide, as they can adversely affect crop yield and quality. Aphid infestations not only weaken plants, but, depending on species, they may also result in the transmission of virus or phytoplasma diseases ultimately causing the plant death, if efficient control methods are not applied (Blackman and Eastop, 2006; van Emden and Harrington, 2007; Boivin *et al.*, 2012; Stokes *et al*., 2019). The indiscriminate use of chemical insecticides to control aphid infestations not only causes aphid resistance to pesticides but also negatively impacts on the aphid natural enemies (van Emden *et al.*, 1969). Biological control, in particular the augmentative release of aphid parasitoids or predators, is considered as an appropriate alternative measure to control the infestation of these insect pests both in greenhouse and open field (Boulanger *et al.*, 2019). Hence, it is necessary to optimize mass rearing and mass release procedures of aphid biocontrol agents in order to use them in augmentation biological control programs, including seasonal inoculative releases in glasshouse (Singh, 1982; Morales Ramos *et al.*, 2014; Rezaei *et al.*, 2018; 2019a).

*Aphidius colemani* Viereck (Hymenoptera Braconidae) is a solitary, koinobiont endoparasitoid of more than 40 species of aphids, in particular the cotton aphid, *Aphis gossypii* Glover (Hemiptera Aphididae), and the green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera Aphididae) (Rakhshani *et al.*, 2005; Zamani *et al.*, 2007; Boivin *et al.*, 2012; Tomanovic *et al.*, 2014). This polyphagous parasitoid species probably originated in northern India or Pakistan, but it is currently spread in various parts of the world, i.e. North and South America, Australia and Europe (Starý, 1975; Starý *et al.*, 2000). *A. colemani* is considered as one of the most suitable biological control agents to be used in augmentative release against aphids (Zamani *et al.*, 2007). Many commercial insectaries currently produce and commercialize this parasitoid to be mass released in protected crops or open field (Heimpel and Lundgren, 2000).

A successful biological control augmentation program with different *Aphidius* species depend on the optimization of their mass production (Wei *et al.*, 2003; Rezaei *et al.*, 2019a). One of the crucial steps of this process is the development of effective methods to store the parasitoid without lowering its quality parameters (Colinet and Boivin, 2011; Rathee and Ram, 2018). Storage of beneficial insects is frequently needed to ensure the availability of a huge number of biocontrol agents for release at the right time (Archer *et al.*, 1973; Scopes *et al.,* 1973; Benelli *et al.*, 2017; 2018).

Storage at suboptimal temperatures (often called "cold storage") can be a useful tool in the mass rearing of entomophagous biocontrol agents (Benelli *et al.*, 2017). Stockpile at low temperature allows greater flexibility in the beneficial insect production and shipment and facilitates the synchronization between the availability of the reared parasitoids in the commercial insectary and their release time in the target site (Lins *et al.*, 2013; Rathee and Ram, 2018). In appropriate cold storage situations, insects may enter a dormant state which is an immediate response to adverse environmental conditions (e.g., low temperature) and results in slowed or halted development. When removed from storage, insects should develop and behave as the control specimens (not subjected to low temperature) (Bayram *et al.*, 2005; Colinet *et al.*, 2006a; 2006b; Lins *et al.*, 2013). To date there has been a great number

of studies dealing with the effects of cold storage on various quantitative and qualitative control parameters of biological control agents (Hofsvang and Hagvar, 1977; Bayram *et al.*, 2005; Chen *et al.*, 2008; Colinet and Hance, 2010; Mahi *et al.*, 2014; Kidane *et al.*, 2015; Benelli *et al.*, 2017; 2018). All these studies suggest that optimal conditions to store parasitoids may vary and should be investigated in detail for each species separately (Bayram *et al.*, 2005). Most research, however, showed that the optimum temperature for storage of beneficial insects ranges from 5 °C to 15 °C (van Lenteren and Tommasini, 2003; Kidane *et al.*, 2015) although some species of Aphidiidae could be stored at 0-5 °C for several days (Archer *et al.*, 1973; Hofsvang and Hagvar, 1977; Ismail *et al.*, 2014). Depending on species, cold storage may negatively affect important biological parameters of insects (including immature survival, sex ratio, adult longevity, flight capacity and fertility) due to cold, starvation and desiccation conditions (Colinet and Boivin, 2011).

Several studies about cold storage have involved Aphidiinae parasitoids (Colinet *et al.*, 2006a; 2006b; Colinet and Hance, 2010; Frere *et al.*, 2011; Silva *et al.*, 2013; Mahi *et al.*, 2014). In former studies, Archer *et al.* (1973) and Scopes *et al.* (1973) respectively stored the mummies of *Lysiphlebus testaceipes* (Cresson) and *Aphidius matricariae* Haliday at various constant low temperatures for 30 days with adult emergence up to 92%. After that, Hofsvang and Hagvar (1977) reported that *A. colemani* was clearly less tolerant to cold storage than *Ephedrus cerasicola* Stary. Moreover, Ismail *et al.* (2010) showed that storage of 1-day-old mummies of *Aphidius ervi* (Haliday) at 7 °C for two weeks negatively affected the fitness, female longevity and sex ratio of the parasitoid progeny. Colinet and Boivin (2011) reviewed the different factors that should be taken into account when designing cold storage experiments. Also, it is reported that the optimal cold storage conditions for the mummies of *A. ervi* largely depend on the host species (Frere *et al.*, 2011). Taking into consideration mortality, mummy weight, longevity, fertility and flight capacity as quality parameters, mummies of *Praon volucre* (Haliday), a parasitoid of 24 aphid species, could be stored at 5 °C for 5 days without parasitoid quality loss and for 10 days with only a little loss (lower percentage emergence of progeny, lower flight activity and a sex ratio slightly male-biased) (Lins *et al.*, 2013). Then, Silva *et al.* (2013) and Al Antary and Abdel-Wali (2015) investigated the optimized cold storage procedures of *Diaeretiella rapae* (McIntosh) and *A. matricariae*, respectively. In contrast to constant low temperatures, several studies have shown that exposing insects to fluctuating thermal regimes has some advantages on biological characteristics of parasitoids (Colinet *et al.*, 2006b; 2007; Ismail *et al.*, 2014, Mahi *et al.*, 2014; Rathee and Ram, 2018). For example, when mummies of *A. colemani* were exposed to 4 °C with periodic sudden transfers to 20 °C for 2 hours, survival of immature parasitoids was significantly improved (Colinet *et al.*, 2006b). However, the lower temperature thresholds for the development of *A. colemani* in *A. gossypii* and *M. persicae* were evaluated from linear regression equations and were 2.97 and 2.65 °C, respectively (Zamani *et al.*, 2007).

In order to improve mass rearing and mass release programs of *A. colemani*, knowledge of the relationship between storage period, temperature and performance of the parasitoid is of paramount importance (Colinet and Boivin, 2011; Lins *et al.*, 2013). It is crucial to find a right cold temperature/period of storage which can induce a temporary dormancy that may be interrupted easily, without taking into account the light and dark alternance. Thus, the aim of the current study was to optimize the method of cold storage of *A. colemani* mummies. Three temperatures, 5, 7 and 9 °C, for five time periods (5, 10, 15, 20 and 30 days) were tested.

## Materials and methods

### Aphid and parasitoid rearing protocols

The green peach aphids (*M. persicae*) were collected from the colony kept at the Department of Agricultural and Food Sciences (DISTAL; University of Bologna, Italy). The colony was established at DISTAL in 2003. The aphids were reared on seedlings of *Pisum sativum* L. in a growth chamber under controlled environmental conditions (20 ± 1 °C, 70 ± 5% RH and 16L:8D photoperiod) (Lanzoni *et al.*, 2004). Agroperlite medium (AGRILIT®3), in a 13 × 9 × 24 cm plastic container, was used to germinate and grow pea seeds. Regularly, aphids were transferred to new containers with fresh pea seedlings, in order to maintain the colony.

The parasitoid *A. colemani*, originating from commercial stocks provided by Bioplanet srl (Cesena, Italy) was reared on *M. persicae* under controlled environmental conditions (25 ± 1 °C, 70 ± 5% RH and 16L:8D photoperiod). The parasitoid adults were maintained in Plexiglas cages (20 × 20 × 20 cm) and fed on honey solution-soaked cotton balls (30% honey w/w). To obtain *A. colemani* mummies for the experiments, a small container (9 × 7 × 12 cm) of pea seedlings with 500-600 3-day-old aphid nymphs (Talebi *et al.*, 2006), were offered to 10 mated-female parasitoids (<48 hours old) in well-ventilated Plexiglas cages (20 × 20 × 20 cm) for 24 hours (Zamani *et al.*, 2007). After exposure, the aphids were removed from the cage and transferred onto seedlings of *P. sativum* until mummies appeared. Newly formed mummies were used for cold storage.

### Cold storage of parasitoid mummies

The effects of three constant temperatures (5, 7 and 9 °C) at five storage periods (5, 10, 15, 20 and 30 days) were evaluated on 1-day-old mummies of *A. colemani*. The relative humidity was 70 ± 5%. Besides, the control treatment consisted of mummies maintained at 25 ± 1 °C, 70 ± 5% RH. All mummies were placed in 3.5 cm Petri dishes and were covered with aluminum foil to produce dark condition (Colinet *et al.*, 2006a; 2006b; Colinet and Hance, 2010). The experiment was conducted as a completely randomized design. Different quality control parameters, including adult emergence, sex ratio, time elapse before emergence, longevity of adults, size of adults (length of the hind tibia), flight capacity, percent parasitism and sex ratio of produced wasps (F1) were evaluated as follows:

### Adult emergence and sex ratio of *A. colemani*

To evaluate the effect of storage on adult emergence, mummies were maintained at each temperature (5, 7 or 9 °C) for 5, 10, 15, 20 and 30 days. After the storage period, the mummies, placed in 3.5 cm Petri dish, were transferred to the standard environmental conditions (25 ± 1 °C, 70 ± 5% RH and 16L:8D photoperiod) and were monitored daily, at a specific time (from 10 to 11 a.m.), until the adult parasitoids emerged. The sex of the newly emerged adults was observed and recorded. Eight replications were carried out and each replication consisted of 10 mummies. For each treatment, the adult parasitoids were counted, and the percentage of adult emergence was calculated. The sex ratio, based on females, was also calculated. Since we did not have any adult emergence at 30-day-storage treatment for all temperatures and 20-day-storage treatment at 9 °C, the relevant quality control parameters were not calculated for these treatments.

### Time elapse before emergence and longevity of *A. colemani* adults

Time elapse was evaluated from the end of treatment (when parasitoids where still inside the mummies) to adult emergence. Adult emergences were monitored once a day, at the same time. The lower temperature threshold for development of *A. colemani* were reported as 2.8 °C (Elliot *et al.*, 1995), 2.36 °C (Sampaio *et al.*, 2003) or 2.65 °C (Zamani *et al.*, 2007). We considered the evaluation of Zamani *et al.* (2007) since the origin of the parasitoid was in the same biogeographic realm (Palearctic). Although the strain used by Zamani *et al*. (2007) was not the same as this commercial strain of *A. colemani*, a starting threshold point was necessary to estimate temperature accumulation. Based on $T_0$ = 2.65 °C (Zamani *et al.*, 2007), the parasitoids inside the mummies would accumulate about 2.35, 4.35 and 6.35 degree-day (DD) per day during cold storage at 5, 7 and 9 °C, respectively, whilst at 25 °C, the immature stage of parasitoid would accumulate about 22.35 DD each day. To estimate the temperature accumulation for the parasitoids, we used the following equation (developed by Ismail *et al.*, 2010): temperature accumulation = A × B with A = differences between the temperature and the $T_0$, and B = days under cold storage or at room temperature (23-24 °C). Same as adult emergence, eight replications were considered for each treatment.

To determine the longevity of *A. colemani*, for each treatment 10 newly emerged adults (5 females and 5 males) were placed in a glass tube (10 × 1.5 cm) and maintained in an incubator at the standard environmental conditions (25 ± 1 °C, 70 ± 5% RH and 16L:8D photoperiod). The adult parasitoids were fed on honey solution-soaked cotton balls (30% honey w/w) until their death. Every individual was considered as a replication. In order to avoid mould infestation, the cotton ball was replaced every 48 hours until parasitoid death.

### Size of *A. colemani* adults

Hind tibia length is commonly used as a standard to assess the size of adult parasitoids (Godfray, 1994). The right hind tibiae of 10 *A. colemani* adults that emerged from each treatment were measured. For this purpose, each parasitoid was photographed using an Axiocam digital camera attached to a Carl Zeiss Axioskop light microscope. Then, the AxioVision 4.8 software was used to determine the length of the hind tibia of the parasitoid.

### Flight capacity of *A. colemani*

Flight capacity was determined using a method like that described by Lins *et al.* (2013). One-day-old *A. colemani* adults from each storage temperature and storage period (except 30-day-storage at all temperatures and 20-day-storage at 9 °C) were fed on 30% honey solution. They were then placed in an acrylic tube (3 cm diameter and 3 cm height), which was put in an open Petri dish (20 cm diameter). The open Petri dish formed the bottom of a cylinder. The cylinder had opaque walls, it was 20 cm high and had a diameter of 10 cm. The cylinder was placed inside the Petri dish. A transparent sticky lid was placed at the top of the cylinder. Insects were attracted to the top by a lamp (Lexman LED lamp, 60W, 860 Lumens) placed 10 cm above the cylinder. To prevent the parasitoids walking off to the top of the cylinder, the Petri dish was surrounded by water. The number of parasitoids stuck to the underside of the cover at the top and wall of the cylinder was recorded after 3 hours. The experiment was conducted in a climatic room (24 ± 3 °C, 70 ± 10% RH) with 3 replications per treatment and each replication consisted of 5 adult parasitoids. The percent of flight capacity was calculated as a ratio based on the number of parasitoids stuck to the top and wall of the cylinder to the sum of adults used for each treatment.

### Percent parasitism and sex ratio of F1 *A. colemani*

To investigate the parasitism potential of the cold stored *A. colemani* females, a female parasitoid (<24 hours old), mated with a control male from the stock colony, was exposed to 30 three-day-old *M. persicae* in a ventilated cylinder dish (5 cm diameter and 7 cm height). In order to obtain the cohort-colony of *M. persicae*, 8-10 adult aphids were placed on seedlings of *P. sativum* in each cylinder dish (5 cm diameter and 7 cm height) and removed after 24 hours. Then, the aphid offsprings were fed for three days on the seedlings and the number of aphids was counted at each container (30 *M. persicae* per dish). The aphids were exposed to *A. colemani* for 24 hours and then the female parasitoid was removed, and the aphids were fed on pea seedlings until mummification. In any treatment the mummies were counted and maintained in 3.5 cm Petri dishes until the adult parasitoids emerged. The newly emerged adults were counted, and their sex was checked and recorded. The experiment was conducted at a constant environmental condition (25 ± 1 °C, 70 ± 5% RH and 16L:8D photoperiod) with six replicates for each treatment. The percent parasitism was calculated as a ratio of the parasitoids emerged from

mummies to the sum of host aphids. Since we did not have any adult emergence at 30-day-storage treatment for all temperatures and 20-day-storage treatment at 9 °C, the percent parasitism was not calculated for these treatments.

### Statistical analysis

Differences in measured parameters were analysed using one-way ANOVA. An arcsine transformation was used to transform percent values for analysis. Furthermore, statistical differences among means were evaluated using Tukey's test ($P < 0.05$). Prior to analysis, the data were tested for normality using the Kolmogorov-Smirnov test. The results of Kolmogorov-Smirnov tests showed that there was no evidence that the error distributions of samples depart significantly from normality. All statistical analysis was completed using SPSS 22 software.

## Results

### Adult emergence and sex ratio

The percentage of *A. colemani* adult emergence was separately analysed for each temperature. For all temperatures, this parameter was significantly affected by storage period, 5 °C ($F = 58.41$; df = 5, 42; $P < 0.001$), 7 °C ($F = 15.56$; df = 4, 35; $P < 0.001$) and 9 °C ($F = 37.42$; df = 3, 28; $P < 0.001$) (figure 1). The percentage of adult emergence decreased according with the increasing storage period (figure 2). Compared with the control treatment, storage of mummies for 5 days at all temperatures had no negative effect on adult emergence. Since parasitoids completed their development and emerged during the storage time, there was no adult emergence from the mummies stored for 30 days at 7 °C and for 20 and 30 days at 9 °C. For the storage period of 30 days at 5 °C, a very few adults emerged; therefore, we could not evaluate further parameters for this treatment.

When the sex ratio of emerged adult parasitoids was analysed, there was a significant difference ($F = 4.27$; df = 4, 35; $P < 0.01$) among the different storage periods at 5 °C. On the contrary, the female proportion was not significantly different from the control temperature at 7 °C ($F = 1.64$; df = 4, 34; $P = 0.19$) and 9 °C ($F = 0.40$; df = 3, 26; $P = 0.75$). Tables 1, 2 and 3 present the results for the sex ratio of *A. colemani*, exposed to different temperatures for various time periods. It is remarkable that, at 5 °C, the proportion of females was higher for the longest storage periods.

### Time elapse before emergence and adult longevity

The time required to develop into adult stage once brought back to 25 °C after cold storage varied significantly among the storage periods at 5 °C ($F = 38.50$; df = 4, 35; $P < 0.001$), 7 °C ($F = 57.87$; df = 4, 35; $P < 0.001$) and 9 °C ($F = 45.16$; df = 3, 28; $P < 0.001$). The elapsed time to adult emergence decreased gradually as the length of cold storage increased (for example, at 5 °C, the elapsed times were 5.17 days and 3.09 days for the storage periods of 5 days and 20 days, respectively) (tables 1, 2, 3). As shown in tables 1, 2 and 3, at 5 °C, the immature stages of the parasitoids accumulated about 11.75, 23.5, 35.25 and 47 DD in 5, 10, 15 and 20 days, respectively. Otherwise, at 7 °C, the immature stages of the parasitoids accumulated about 21.75, 43.5, 65.25 and 87 DD in 5, 10, 15 and 20 days, respectively. Finally, at 9 °C, the immature stages of the parasitoids accumulated about 31.75, 63.5 and 95.25 in 5, 10 and 15 days, respectively.

Male ($F = 19.16$; df = 4, 20; $P < 0.001$) and female ($F = 13.13$; df = 4, 20; $P < 0.001$) longevity of *A. colemani* was notably affected by the storage period at all three temperatures. The results obtained from the analysis of adult longevity of *A. colemani* are presented in tables 1, 2 and 3. As expected, the low temperatures had negative impact on the longevity of the males and females of *A. colemani*.

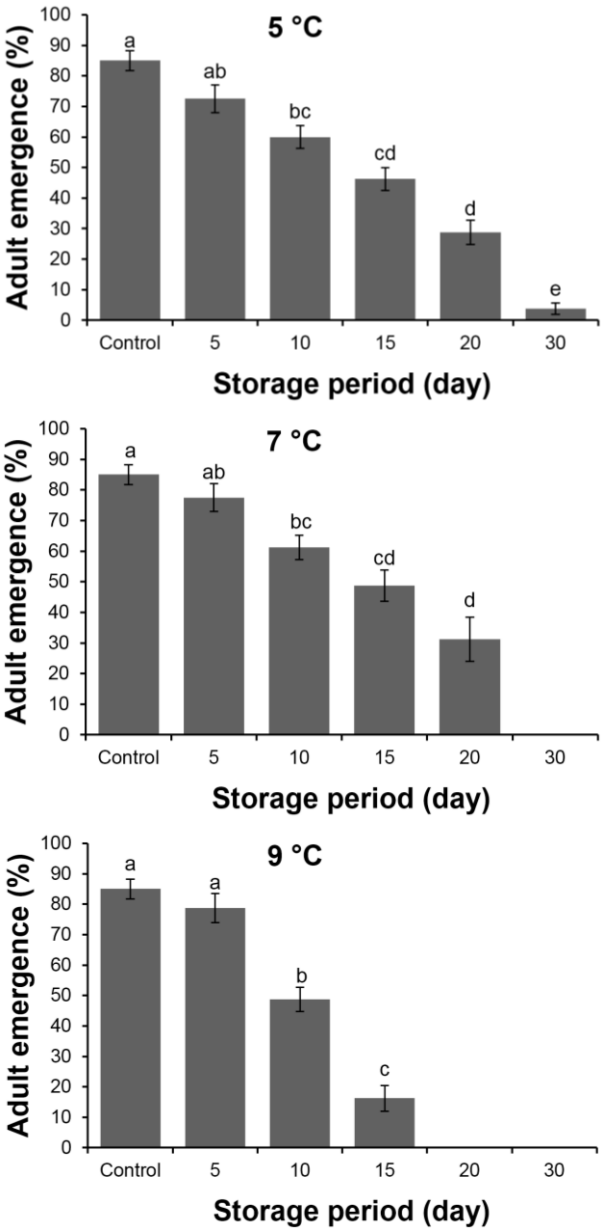


**Figure 1.** Adult emergence percentage (mean ± SE) of *A. colemani* according to the different storage periods (5, 10, 15, 20 and 30 days) at three temperatures (5, 7 and 9 °C). Means followed by same letter did not differ significantly (one way ANOVA followed by Tukey's test) ($P < 0.05$).

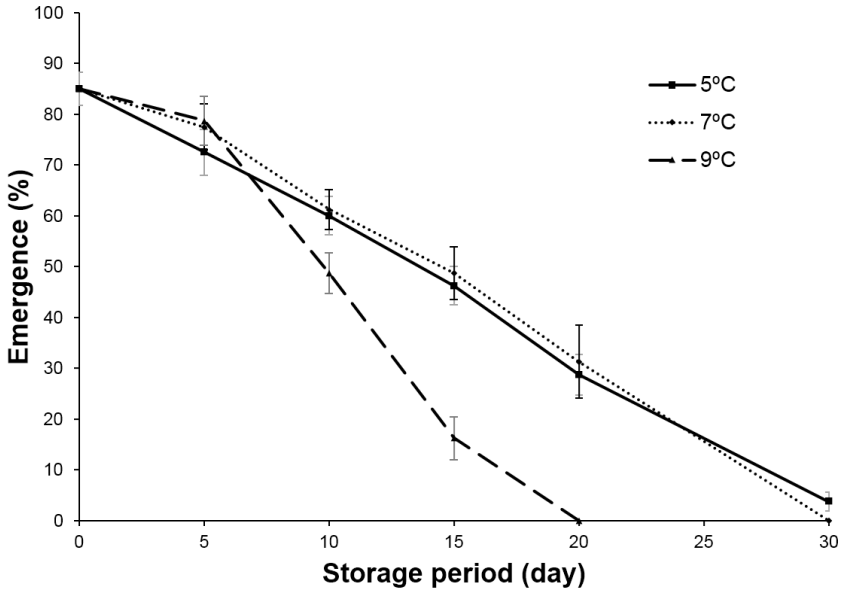


**Figure 2.** Percentage emergence (± SE) of *A. colemani* adults stored from 5 to 30 days at three low temperatures, 5, 7 and 9 °C, including the control mummies (day 0).

**Table 1.** Different measured parameters of *A. colemani* after various time periods of cold storage under 5 °C temperature (mean ± SE). Numbers of replicates are given in parentheses under the means.

| Temp. | Time period (days) | Elapsed time to emergence (days) | Female longevity (days) | Male longevity (days) | Hind tibial length (mm) | Sex ratio (female) | Sex ratio of progeny (female) | Flight capacity (%)[2] | Parasitism (%)[1] |
|---|---|---|---|---|---|---|---|---|---|
| 25 °C | Control | 5.38 ± 0.10 a (8) | 7.2 ± 0.73 a (5) | 6.0 ± 0.45 a (5) | 0.542 ± 0.008 a (10) | 0.593 ± 0.042 b (8) | 0.533 ± 0.031 a (6) | 86.67 ± 13.33 a (3) | 55.00 ± 2.55 a[3] (6) |
| 5 °C | 5 | 5.17 ± 0.10 ab (8) | 6.0 ± 0.45 a (5) | 5.2 ± 0.37 a (5) | 0.528 ± 0.012 a (10) | 0.586 ± 0.025 b (8) | 0.496 ± 0.020 a (6) | 66.67 ± 13.33 ab (3) | 51.11 ± 3.72 a (6) |
| | 10 | 4.65 ± 0.13 b (8) | 5.2 ± 0.58 ab (5) | 4.4 ± 0.40 ab (5) | 0.506 ± 0.012 ab (10) | 0.563 ± 0.030 b (8) | 0.515 ± 0.010 a (6) | 53.33 ± 13.33 ab (3) | 39.44 ± 2.00 b (6) |
| | 15 | 3.54 ± 0.18 c (8) | 3.6 ± 0.51 bc (5) | 2.8 ± 0.58 bc (5) | 0.497 ± 0.010 ab (10) | 0.696 ± 0.039 ab (8) | 0.567 ± 0.066 a (6) | 33.33 ± 6.67 b (3) | 29.44 ± 2.34 b (6) |
| | 20 | 3.09 ± 0.21 c (8) | 2.2 ± 0.37 c (5) | 1.4 ± 0.24 c (5) | 0.481 ± 0.014 b (10) | 0.760 ± 0.060 a (8) | 0.500 ± 0.050 a (6) | 26.67 ± 6.67 b (3) | 18.89 ± 2.22 c (6) |
| | 30 | | | | | | | | |

[1] Percent parasitism calculated as a ratio of the emerged mummies to the number of host aphids for the adult parasitoids after cold storage periods; [2] Percent of flight capacity calculated as a ratio based on the number of parasitoids stuck to the top and wall of the cylinder to the number of adults used in each treatment; [3] Values followed by the same letter within each column are not significantly different at $P < 0.05$ (Tukey's HSD multiple range test).

**Table 2.** Different measured parameters of *A. colemani* after various time periods of cold storage under 7 °C temperature (mean ± SE). Numbers of replicates are given in parentheses under the means.

| Temp. | Time period (days) | Elapsed time to emergence (days) | Female longevity (days) | Male longevity (days) | Hind tibial length (mm) | Sex ratio (female) | Sex ratio of progeny (female) | Flight Capacity (%)[2] | Parasitism (%)[1] |
|---|---|---|---|---|---|---|---|---|---|
| 25 °C | Control | 5.38 ± 0.10 a (8) | 7.2 ± 0.73 a (5) | 6.0 ± 0.45 a (5) | 0.542 ± 0.008 a (10) | 0.593 ± 0.042 b (8) | 0.533 ± 0.031 a (6) | 86.67 ± 13.33 a (3) | 55.00 ± 2.55 a[3] (6) |
| 7 °C | 5 | 4.77 ± 0.13 b (8) | 6.2 ± 0.73 ab (5) | 5.4 ± 0.40 a (5) | 0.527 ± 0.017 a (10) | 0.610 ± 0.022 a (8) | 0.490 ± 0.025 a (6) | 60.00 ± 11.55 ab (3) | 53.33 ± 2.43 a (6) |
| | 10 | 4.49 ± 0.15 b (8) | 3.6 ± 0.75 bc (5) | 2.6 ± 0.51 b (5) | 0.516 ± 0.008 a (10) | 0.582 ± 0.034 a (8) | 0.520 ± 0.037 a (6) | 46.67 ± 6.67 ab (3) | 42.22 ± 2.53 b (6) |
| | 15 | 3.05 ± 0.15 c (8) | 2.4 ± 0.51 c (5) | 2.0 ± 0.32 bc (5) | 0.510 ± 0.009 a (10) | 0.666 ± 0.036 a (8) | 0.471 ± 0.026 a (6) | 33.33 ± 6.67 b (3) | 30.00 ± 1.92 c (6) |
| | 20 | 2.92 ± 0.18 c (8) | 1.4 ± 0.24 c (5) | 0.8 ± 0.20 c (5) | 0.505 ± 0.011 a (10) | 0.710 ± 0.066 a (7)[4] | 0.488 ± 0.045 a (6) | 26.67 ± 6.67 b (3) | 20.56 ± 2.34 d (6) |
| | 30 | | | | | | | | |

[1] Percent parasitism calculated as a ratio of the emerged mummies to the number of host aphids for the adult parasitoids after cold storage periods; [2] Percent of flight capacity calculated as a ratio based on the number of parasitoids stuck to the top and wall of the cylinder to the number of adults used in each treatment; [3] Values followed by the same letter within each column are not significantly different at $P < 0.05$ (Tukey's HSD multiple range test); [4] Only 7 replications were considered, because in one replication no adults emerged after the storage period.

**Table 3.** Different measured parameters of *A. colemani* after various time periods of cold storage under 9 °C temperature (mean ± SE). Numbers of replicates are given in parentheses under the means.

| Tem. | Time period (days) | Elapsed time to emergence (days) | Female Longevity (days) | Male Longevity (days) | Hind Tibial Length (mm) | Sex ratio (female) | Sex ratio of progeny (female) | Flight capacity (%)[2] | Parasitism (%)[1] |
|---|---|---|---|---|---|---|---|---|---|
| 25 °C | Control | 5.38 ± 0.10 a (8) | 7.2 ± 0.73 a (5) | 6.0 ± 0.45 a (5) | 0.542 ± 0.008 a (10) | 0.593 ± 0.042 b (8) | 0.533 ± 0.031 a (6) | 86.67 ± 13.33 a (3) | 55.00 ± 2.55 a[3] (6) |
| 9 °C | 5 | 4.33 ± 0.11 b (8) | 6.8 ± 0.37 ab (5) | 5.4 ± 0.40 a (5) | 0.539 ± 0.009 a (10) | 0.586 ± 0.023 a (8) | 0.528 ± 0.019 a (6) | 53.33 ± 6.67 ab (3) | 52.22 ± 4.01 a (6) |
| | 10 | 3.97 ± 0.16 b (8) | 4.8 ± 0.37 bc (5) | 3.6 ± 0.51 b (5) | 0.522 ± 0.018 a (10) | 0.619 ± 0.031 a (8) | 0.552 ± 0.014 a (6) | 40.00 ± 11.55 b (3) | 43.33 ± 2.72 ab (6) |
| | 15 | 2.73 ± 0.30 c (8) | 2.8 ± 0.58 c (5) | 2.4 ± 0.40 b (5) | 0.514 ± 0.011 a (10) | 0.667 ± 0.114 a (6)[4] | 0.587 ± 0.019 a (6) | 26.67 ± 6.67 b (3) | 32.78 ± 2.34 b (6) |
| | 20 | | | | | | | | |
| | 30 | | | | | | | | |

[1] Percent parasitism calculated as a ratio of the emerged mummies to the number of host aphids for the adult parasitoids after cold storage periods; [2] Percent of flight capacity calculated as a ratio based on the number of parasitoids stuck to the top and wall of the cylinder to the number of adults used in each treatment; [3] Values with the same letter within each column are not significantly different at $P < 0.05$ (Tukey test); [4] Only 6 replications were considered, because in two replications no adults emerged after the storage period.

### Hind tibial length

In the case of 5 °C only, there was a significant difference ($F = 4.52$; df = 4, 45, 20; $P < 0.01$) among different storage periods for hind tibial length of *A. colemani*. Tibia size of *A. colemani* was large when stored for 5 days at 5 °C and showed the descending gradient in size as the storage period was increased. The mean hind tibia length of *A. colemani* varied from 0.481 mm (20 days storage at 5 °C) to 0.542 mm (control treatment) (table 1, 2 and 3).

### Flight capacity

The percentage of flight capacity of *A. colemani* in the control treatment was 86.67% and decreased significantly with increasing length of the storage period to 26.67% at 5 °C ($F = 5.01$; df = 4, 10; $P < 0.05$), 7 °C ($F = 6.17$; df = 4, 10; $P < 0.01$) and 9 °C ($F = 6.43$; df = 3, 8; $P < 0.05$) (figure 3; tables 1, 2, 3).

### Percent parasitism and F1 sex ratio

Percent parasitism by *A. colemani* emerged from cold storage treatments was influenced by storage period at 5 °C ($F = 32.81$; df = 4, 25; $P < 0.001$), 7 °C ($F = 38.92$; df = 4, 25; $P < 0.001$) and 9 °C ($F = 11.35$; df = 3, 20; $P < 0.001$) (tables 1, 2 and 3). The duration of storage at all three low temperatures had a negative effect on the percent parasitism of *A. colemani* as there was a significant decrease in the percentage parasitism with increase in storage period.

Sex ratio of F1 generation was not affected by cold storage of the parents in the mummies. It can be seen from the data in tables 1, 2 and 3 that the mean female proportion of F1 progeny varied from 0.471 (15 days at 7 °C) to 0.587 (15 days at 9 °C).

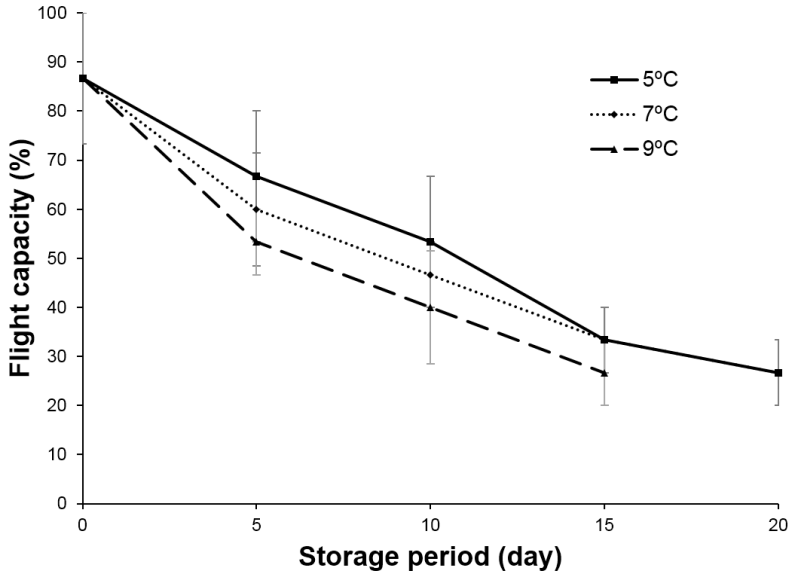


**Figure 3.** Linear decreasing of flight capacity percentage (± SE) of *A. colemani* after cold storage at three low temperatures, 5, 7 and 9 °C, for different time periods, 5, 10, 15 and 20 days, including the control treatment (day 0).

## Discussion

Insect storage at low temperature is a valuable means for increasing the shelf life of biological control agents, including parasitoid insects (Rathee and Ram, 2018). *A. colemani* is frequently mass reared and released in augmentation biological control program as one of the effective biocontrol agents against several economically important aphid pests (Heimpel and Lundgren, 2000; Vásquez *et al*., 2006; Karacaoğlu *et al*., 2018). Our results showed that all measured parameters including adult emergence, sex ratio, time elapse before emergence, longevity, hind tibial length, flight capacity and parasitism potential, except sex ratio of F1 generation, were affected by cold storage duration.

Adult emergence after cold storage period is a paramount parameter for the successful application of the biocontrol agents. In agreement with former studies (Rathee and Ram, 2018), the percentage of adult emergence decreased with the increase of the storage duration at all three temperatures (5, 7 and 9 °C). The low emergence percentage at 7 and 9 °C was because some parasitoids completed their development and emerged during the storage time. We, however, discarded these parasitoids from the calculation of adult emergence. As mentioned by Hofsvang and Hagvar (1977), average developmental times of *A. colemani* from mummification to adult emergence at 7 and 10 °C were 23.5 and 16.6 days, respectively. Based on our results, long term (> 20 days) storage at 7 and 9 °C seems, however, unsuitable for *A. colemani.* As shown in tables 1, 2 and 3, the percentages of adult emergence for 15-day storage at 5, 7 and 9 °C were 46.25, 48.75 and 16.25%, respectively. Earlier findings on other Aphidiinae parasitoids, however, showed different results (Archer *et al.*, 1973; Frere *et al.*, 2011; Mahi *et al.*, 2013; Ismail *et al.*, 2014). For instance, the percentage emergence of *P. volucre* mummies accumulated at 5 °C for 15 days was estimated as 64.6% (Lins *et al.*, 2013). In comparison to other Aphidiinae parasitoids, our *A. colemani* strain proved to be less tolerant to cold storage, as also shown by Colinet and Hance (2010).

Sex ratio can be distorted by insect storage at low temperature. Distortion may result from a differential mortality among sexes when immature stages are exposed to low temperatures (Colinet and Boivin, 2011). This quality control parameter is important in cold storage experiments since the high number of females is considered as one of the key factors for successful parasitoid release (Rathee and Ram, 2018). In the current experiment, the sex ratio of parasitoids emerged from mummies stored at 5 °C was significantly affected by duration of exposure. At this temperature, the increase of the storage period resulted in a significantly higher female-biased sex ratio compared to the control treatment. The results of the current study suggested that males may be more susceptible than females to cold injuries. A better withstanding to low temperatures of females compared to males has already been reported for *A. colemani* (Colinet *et al.*, 2006b) and *E. cerasicola* (Hofsvang and Hagvar, 1977). Also, Archer *et al.* (1973) showed that the storage of *L. testaceipes* mummies at 4.4 °C for 30 days led to more females than males. Instead, male-biased sex ratio after two weeks of storage at 7 °C was reported for *A. ervi* (Ismail *et al.*, 2010). However, there was no significant difference in the sex ratio of some Aphidiinae parasitoids exposed to low temperature, e.g. *A. matricariae* (Colinet and Hance, 2010), *D. rapae* (Silva *et al.*, 2013) and *L. fabarum* (Mahi *et al.*, 2014). Females and males, therefore, showed similar tolerance to low temperatures.

In most parasitoid wasp species, including *A. colemani*, there is a considerable body size and weight difference between sexes as females are generally larger than males (Godfray, 1994). Females may thus contain higher amount of energy. In low temperature conditions, energy reserves can be critically affected, as insects do not feed and maintain a low level of metabolism (Renault *et al.*, 2003). Consequently, Colinet *et al.* (2006b) suggested that male individuals of *A. colemani* exhaust their energy stock more rapidly than females, because of reduced initial stores and/or because of differential utilization. As mentioned by Hofsvang and Hagvar (1977) and Colinet *et al.* (2006b), the sex ratio is pretty dependent on external factors, so that general conclusions are difficult to make, but it is important to consider the effects of the current issue on augmentation biological control programs with *A. colemani*.

Cold storage can affect different aspects of development, especially the time lag before emergence. According to our results, the elapsed time for the parasitoids to develop into adult stage after removal from cold storage followed the expected patterns: it decreased with the duration of cold exposure and with increasing storage temperature (Colinet *et al.*, 2006b; Chen *et al.*, 2008). Since we used three temperatures above the development threshold, this indicates that, even at low temperatures, immature stage of parasitoids still developed slowly. In agreement with the current results, previous studies were reported that the time lag before emergence was markedly affected by the duration of cold exposure (Colinet *et al.*, 2006b; Colinet and Hance, 2010). For example, Ismail *et al.* (2010) showed that elapsed time to emergence for *A. ervi* after storage at 7 °C decreased as the length of cold exposure increased from 7 to 14 days. Based on estimated $T_0$ (2.65 ºC) reported for *A. colemani* by Zamani *et al.* (2007) and evaluated temperature accumulations, development occurred at more than 5 °C. Our observations thus support the data found in the former studies (Zamani *et al.*, 2007; Colinet and Hance, 2010), but the parasitoid strain used in the current study was different from Zamani *et al.* (2007). Conversely, the time elapse to adult emergence notably increased with duration of cold storage indicating a developmental delay as in *E. cerasicola* (Colinet and Hance, 2010). In addition to delaying emergence time, cold storage may also affect the distribution pattern of emergence. So, developmental responses to cold storage may vary from complete arrestment of development to slower development (Colinet and Boivin, 2011).

In this study, the longevity of *A. colemani* male and female adults markedly decreased as the storage duration at all three temperatures increased, which is in agreement with previous finding of Rathee and Ram (2018). The negative impact of low temperature on adult longevity has also been observed for other parasitoids either hymenopterans (Rundle *et al.*, 2004; Lins *et al.*, 2013; Silva *et*

*al.*, 2013; Al Antary and Abdel-Wali, 2015; Kidane *et al.*, 2015) or dipterans (Dindo *et al*., 2003; Dindo and Grenier, 2014; Benelli *et al.*, 2017). Longevity may be very short, since adults may die within the first few hours after emergence (Levie *et al.*, 2005). The parasitoid longevity is strongly linked to the amount of fat reserve (Silva *et al.*, 2013; Kidane *et al.*, 2015). Colinet *et al.* (2006a) stated that the amount of fat reserves available for emerging adults of *A. colemani* declined linearly with duration of cold exposure and a corresponding decrease of adult longevity occurred. Similar results were observed by Ismail *et al.* (2010) for *A. ervi*, Silva *et al.* (2013) for *D. rapae* and Kidane *et al.* (2015) for *Encarsia sophia* (Girault et Dodd). Adult longevity can be affected by the amount and type of food consumed in the adult stage (Uçkan and Gülel, 2001). The newly emerged adult parasitoids were fed on 30% honey solution, but this food did not compensate the injury caused by storage at low temperatures including a possible depletion of fat content, as reported by Colinet *et al.* (2006a).

The hind tibial length of parasitoid wasps is considered as the most common indicator of the total adult size (Godfray, 1994). According to this parameter, the size of adult parasitoids decreased significantly with the increase of the length of storage period at 5 °C. Similarly, Ismail *et al.* (2014) reported that *A. ervi* size was significantly affected by storage at 0 °C. The size of hind tibia of *A. ervi* stored at 7 °C for maximum 2 weeks, however, presented equal size to that of the controls (Ismail *et al.*, 2010). Moreover, when two host species, *S. avenae* and *A. pisum*, both parasitized by *A. ervi*, were stored as mummies at low temperatures (2 and 7 °C), the length of the hind tibia of parasitoids that emerged from mummies of *A. pisum* was notably longer than that of parasitoids that emerged from mummies of *S. avenae* (Frere *et al.*, 2011). However, the hind tibial size of *P. volucre* was not affected by the length of period of storage at 5 °C (Lins *et al.*, 2013). These studies suggested that the response of adult size of Aphidiinae parasitoids to storage at low temperature may be different, according to the species. In *A. colemani*, the lower temperature tested in this study (5 °C) had more impact on the adult size of the parasitoids than 7 and 9 °C.

Flight capacity, an important quality control criterion for the mass-reared natural enemies (van Lenteren *et al.*, 2003), may be negatively affected by the storage of parasitoids at low temperatures (Lins *et al.*, 2013). In comparison to the control treatment, our results showed that the flight capacity of *A. colemani* was decreased by increasing the storage period at all three temperatures. These findings seem to be consistent with other research which showed that the flight capability of *Encarsia formosa* Gahan (Hymenoptera Aphelinidae) and *E. eremicus* (Rose et Zolnerowich) declined with increased cold storage duration (Luczynski *et al.*, 2007). Energy consumption together with muscle alteration may explain why flight capacity is reduced after cold storage (Colinet and Boivin, 2011). Until now, there was not any report on the flight capacity of *A. colemani* after storage at low temperature, but it is important to consider this parameter in commercial production programs, when storage of the parasitoid is provided.

Successful parasitism (i.e., the number of mummies per parasitoid during a certain period) is a crucial quality control parameter for mass production of entomophagous insects (van Lenteren *et al.*, 2003). Considering the results, the percent parasitism decreased with the increase in the length of the period in storage at all three temperatures in comparison with control treatment. Likewise, some studies have reported reduction of percent parasitism with the increase of the length of cold storage of parasitoid wasps, for instance, *Telenomus busseolae* Gahan (Bayram *et al.*, 2005), *E. formosa*, *E. eremicus* (Luczynski *et al.*, 2007), *P. volucre* (Lins *et al.*, 2013), *D. rapae* (Silva *et al.*, 2013) and *E. sophia* (Kidane *et al.*, 2015). This result may be related with an adverse effect of storage at low temperatures on the number of eggs laid and the egg fertility of *A. colemani*. Also, the rate of parasitism takes into account the steps of the foraging behaviour such as host recognition, acceptance and discrimination (Colinet and Boivin, 2011; Rezaei *et al.*, 2019b). No study was found in the literature dealing with the effects of cold storage on foraging behaviour of *A. colemani*. It may be, however, hypothesized that the reduction in parasitism success after cold storage was related to a decrease in foraging capability of the parasitoid. These hypotheses, however, need to be tested.

In haplodiploid organism such as *A. colemani*, males develop from unfertilized eggs and females from fertilized eggs. Therefore, the sex of the egg is under the direct behavioural control of the mother according to the existing conditions (Jarosík *et al.*, 2003). The results of this study did not show any significant difference in progeny sex ratio of *A. colemani* that were stored at three temperatures for various time periods. This quality control parameter indicated that the effects of cold storage on the parental generation did not affect the sex ratio of F1 generation. In accordance with the present results, Chen *et al.* (2008) reported that the progeny sex ratio of the F1 and F2 generations of *Gonatocerus ashmeadi* Girault did not vary with storage duration of the parental generation. On the contrary, expression of damage in progeny sex ratio after cold storage has been reported by Bayram *et al.* (2005) who found that F1 progeny sex ratio of *T. busseolae* was more male biased with increasing length of storage treatment (Colinet and Hance, 2009).

In conclusion, the quality control criteria for *A. colemani* were mentioned by van Lenteren *et al.* (2003). An acceptable emergence rate from host mummies was reported as higher or equal to 45% and the sex ratio had to be female biased. Based on these values and on the results achieved in this study, we suggest that *A. colemani* can be stored for 15 days at 5 °C and 7 °C and for 10 days at 9 °C. However, it is important to consider other parameters (adult longevity, flight capacity, hind tibial length and the number of produced mummies per parasitoid) that we evaluated for these temperatures and time periods. For instance, when mummies of *A. colemani* were stored for 15 days at 5 and 7 °C the flight capacity, percent parasitism and adult longevity were adversely affected although the emergence ratio and sex ratio were acceptable (> 45%). Therefore, *A. colemani* mummies can be stored for no longer than 10 days without much loss of performance (e.g., adult longevity, adult size and

flight capacity) at all three temperatures. Moreover, the sex ratio of F1 progeny at all tested temperatures and time periods was not negatively affected. In order to facilitate the planning of the mass rearing and mass release programs of *A. colemani*, the delayed emergence stored at the three temperatures (5, 7 and 9 °C) can be useful. In particular, it is necessary to be cautious in concluding that the evaluation performed in this study can be generalized for all strains of *A. colemani*, but the data collected may be useful in the perspective of storing the parasitoid. Moreover, further studies on the efficiency of stored parasitoids against aphids under field and greenhouse conditions are needed.

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