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# Eggs exposure to low temperature reasons dwindled viability and impeded biology in green lacewing (*Chrysoperla carnea* Stephen)

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## Abstract

Green lacewing, *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) is a well-known generalist predator. Integrated pest management (IPM) strategies for various crops belongs to its predatory performance. The possibility of storing *Chrysoperla* eggs at low temperature and the effects of storage on viability, emergence, quality of predator and its progeny were tested. Eggs were stored in full darkness up to a month at 10 ± 1°C, RH = 40 ± 5%. Percent hatching, incubation period, larval duration, pupal duration, pupal weight and percent adult emergence were evaluated after every 24 hours. Hatching were endured only 51.25% just after seven days of cold storage. However, 100 % eggs viability were lost after 18$^{th}$ day. In incubation period, 20.7 and 41.4% increase was observed at 7$^{th}$ and 18$^{th}$ day of cold storage respectively. Larval duration was increased six hours at 7$^{th}$ day. However, this increase extended up to 24 hours. Big upsurge was found in pupal period during last 7 days, which raised up to 42 hours as compared to control. Considerable reduction was found in pupal weight as cold storage increased and on average it recorded up to two milligrams/pupae. Percent emergence was significantly reduced as cold storage days increased. However, 2-18% reduction was recorded during the studies. No significant effects were found on hatching of F1 and F2 progenies. The results of this study reveal that for more efficient biological control, there is an urgent need to improve the method of storing eggs of *C. carnea*.

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**Introduction**

Green lacewing, *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) is one of the most prolific insect found on various crops in many parts of the world (Pasini *et al.*, 2017). Inundative release of their individuals on various crops showed potential for biological control of various insect pests (Resende *et al.*, 2017). With the dawn of the organic farming concept, there has been a considerable change in adaptation protocols for insect pest management (IPM). Natural enemies play a vital role in ecosystem, posing a valid substitute to, or integration with, other control strategies (McEwen *et al.*, 2007). Therefore, the development of IPM seeks to upsurge natural control by conservation and preservation of entomophagous fauna (Rumpf *et al.*, 1997; Rogers *et al.*, 2007). Thus, it is necessary to improve knowledge on the impact that different management practices exert over such biocontrol agents. The success of these biological control agents relies not only on the amount of individual released but also on their quality and time of release (Tezze and Botto, 2004). The development of cost effective and efficient storage methods constitutes one of the supreme challenging problem in rearing of targeted insects. It is particularly important for inundative or augmentative release of high quality biological control agents (Tauber *et al.*, 1993).

Storage of biological agents assure their availability in adequate number at the time of release in targeted surrounding. Thus, developing protocols of storage for biocontrol agents is considered of ultimate significance to provide efficiency and flexibility in mass rearing. This could be helpful to synchronize the preferred stage of development for peak release that also make available standardized stock for use in different studies. Progress in this field would help commercial and public insectaries and farmers as well because efficient storage protocols would transform into effective use of limited and costly resources (personnel, space and equipment), increase capacity to meet ultimate demands for production and distribution and reduced investment in rearing amenities. Ultimate availability of natural enemies would overwhelmed one of the key barrier to incorporation of biocontrol agents in to integrated pest management (Chen *et al.*, 2017). Therefore, the protocol development most preferably for short term storage, without significant loss of quality, is really critical for the commercialization of chrysopids and other predators that would be hastened if convenient and effective storage techniques were presented.

To date, no sufficient studies have been undertaken to investigate the effect of cold storage on the eggs of *C. carnea*. Only Osman and Selman, (1993) described *C. carnae* egg storage only possible at 8°C for approximately 29 days. López-Arroyo *et al.*, (2000) reported temperature range for successfully storing of eggs of *C. externa* is 14 to 21 days at 10 to 15.6°C. There is no general guideline to follow because of difference present between species in their tolerance of cold temperatures.

When considering storage of trash-resonant lacewings mainly in the genus *Chrysoperla*, we were challenged four main stumbling blocks that hamper even greater use of these predators in pest management. First is lack of mechanized and low cost methods for mass culturing. Second is lack of convenient, standardized procedure of storage of these predators. Third, techniques for short term storage are needed to promote augmentative release of *C. carnea*. Forth, adults show significant mortality when they are held at low temperature (López-Arroyo *et al.*, 1999).

The use of cold storage techniques could be helpful for industrial rearing of natural enemies as well as for research under laboratory conditions. Shortage of natural enemies assure their availability in sufficient number at the time of release in the field for particular insect. Consequently, we investigated for how long *C. carnea* eggs remain viable and amenable to storage at low temperature for short period. Whether there are significant post-storage effects on performance of larvae and adults that could yield less mortality and less loss of fecundity and fertility after storage. In particular, the hypothesis that the quality of *C. carnea* decrease with increase in the period for which it is kept in cold storage.

**Materials and methods**

The experiment was carried out under constant laboratory conditions (25 ± 1°C and 60 ± 5% RH) at biological control laboratory, Nuclear Institute of Agriculture (NIA), Tando Jam, during the year of 2017.

*Sitotroga cereallela (Olivier) culture*

Eggs of Angoumois grain moth, *S. cereallela* were produced for rearing of *C. carnea*. For that matter, wheat grains were stored and disinfected by Phostoxin® tablets (tablets contain aluminum phosphide, which produce phosphine gas) and then poured into jute sack and get sterilized with boiling water for 2 minutes. It is then expose to sun light to eliminate stored grain mites and other potential pests. These wheat grains were then transferred to 4 liters (20.5cm L x 55.8cm D) glass jars (0.5 kg/glass jar). Freshly laid eggs of *S. cereallela* were add to each glass jar (5ml eggs/jar). After mixing them properly, jars were kept in laboratory at 25 ± 1°C and 60 ± 5% RH and 15:7 (L:D). After hatching, young larvae of *S. cereallela* were fed on wheat grains and on emergence adults were collected after 30 days by motorized aspirator and shifted to eggs collecting jars (1 liter plastic jar having a fine mesh (mesh size = 50) at the bottom). Eggs collecting jars were placed over starch and fresh eggs were collected and these eggs were used in experiments.

*Rearing of C. carnea*

Our experiment used 175$^{th}$ generation of laboratory reared *C. carnea* larvae which have been collected from biocontrol lab of Plant Protection Division, Nuclear Institute of Agriculture (NIA), Tando Jam. To avoid the cannibalism, individual of 1$^{st}$ instar larvae were sealed in 2 inches polypropylene transparent straw with three pin holes for aeration. To enhance the genetic base of stock culture, these larvae were provided with food (*S. cereallela* eggs) and placed in controlled biocontrol laboratory at 25 ± 1°C and 60 ± 5% RH and 15:7 (L:D) till pupation. These tubes were cut out and small open pieces of the tube along with pupae were then placed on glass Petri plates (9cm diameter and 1.5cm height) for emergence. On emergence, adults were transferred to plastic insect rearing cage (24.5 x 24.5 x 24.5cm).

Parental pairs were maintained in individual cages and provided with an artificial diet consisting honey, sugar, yeast and distilled water (1:2:1:2) throughout reproductive phase *C. carnea* during pre-oviposition, oviposition and post-oviposition period. Cages were provided with black cloth fitted at the top for egg laying. Eggs were harvested with steel razor after 24 hours interval.

*Storage of C. carnea eggs*

Hatching of *C. carnea* eggs were evaluated at common used refrigerator temperature (10°C,) with RH 40 ± 5%. Unstored eggs were kept at 25 ± 1°C served as control. After 24 hours of oviposition, eggs were collected, and placed (30 eggs) in individual hatching cloth (HC) (black, 26 x 20cm).

Total 90 HCs were placed for storage. These were kept at 25 ± 1°C before being shifted to lower temperature. These were kept and maintained under experimental condition for a month. After every 24 hours (a day) interval, three HCs were placed again at 25 ± 1°C for data recording. This process was carried out for a month. Timing and incidence of hatching were recorded of eggs of each hatching cloth after low temperature exposure at specific time.

*Post-storage effects*

It was examined whether eggs stored at relatively low temperature affects development and reproduction of post-storage *C. carnea*. To do so, we separated the first instar larvae from each hatching cloth and were sealed in 2 inches polypropylene transparent straw provided with eggs of *S. cereallela*. We extended our evaluation regarding effect of storage till post-embryonic life stages. All larvae from different treatment (storage time) were kept under at 25 ± 1°C and recorded the data of incubation period, hatching percentage, larval duration, pupal duration, pupal weight and adult emergence. Added effect of loss of quality by cold storage was also evaluated. Subsequently, adult were paired and released in the round glass jars (14.5 height x 19.5 diameter), provided with black cloth at top for egg laying. Eggs were collected and determined the hatching percentage for two generations (F1 and F2).

*Statistical analysis*

Data of experiments were statically analyzed to check the significant difference between treatments using SAS JMP® Pro 12.2.0 software. ANOVA was used to determine the effect of cold storage days on life parameters like of incubation period, hatching percentage, larval duration, pupal duration, pupal weight and adult emergence. Tukey's honestly significant difference (HSD) test was used to compare the means of different response parameters. Linear regression analysis was also done to check the relation of each parameter with the cold storage time.

**Results**

Hatching percentage (*F = 61.8, n = 84, df = 20, p = 0.000)* was 90 ± 2.1 percent for most of the samples in the control group. The proportion of hatched larvae decreased significantly with increase in cold storage time (Fig. 1.). Percentage remained 51.3 ± 4.3 at 7$^{th}$ day of cold storage and continued to decreased till 0% just after 18 days of cold storage. With the increase of cold storage time the incubation period *(F = 11.3, n = 76, df = 18, p = 0.000)* increased sigificantly (Fig. 1.). Although the proportion of incubation period were significantly altered by cold storage, they were not different from the control up to 7 days treatment. Significant effect was found on the larval duration (*F = 3.76, n = 76, df = 18, p = 0.001*) of *C. carnea* when eggs were subjected to cold storage (Fig. 2.). Maximum larval period (207 ± 5.7 hours) was recorded at 15$^{th}$ day of cold storage to onward. Likewise, pupal period (*F = 8.25, n = 76, df = 18, p = 0.000)* was also significantly affected by cold storage duration. Increase in pupal duration was found directly proportion to increase in cold storage time (Fig. 2.). Maximum pupal period (183 ± 7.6 hours) was found at 16$^{th}$ day of cold storage and found constant till 20$^{th}$ day. Even though, larval and pupal duration were found significantly affected by cold storage time but they were not statistically different from control group up to 13$^{th}$ day of cold storage treatment. Total 13.11 % and 30 % increase was observed in larval and pupal duration respectively with respect to control group.

Pupal weight (*F = 1.14, n = 76, df = 18, p = 0.343*) did not show significant differences as *C. carnea* eggs were subjected to different cold storage timing (Fig. 3.). Percent emergence was found really disturbed. The proportion of emerged adults (*F = 5.28, n = 76, df = 18, p = 0.000*) decreased significantly with increase in cold storage time (Fig. 3.). In control group, 90 ± 2.04 % adult emergence was recorded as maximum, while no significant difference was found till 14$^{th}$ day of treatment. At the end, 19.6 % decrease was recorded as compare with control group.

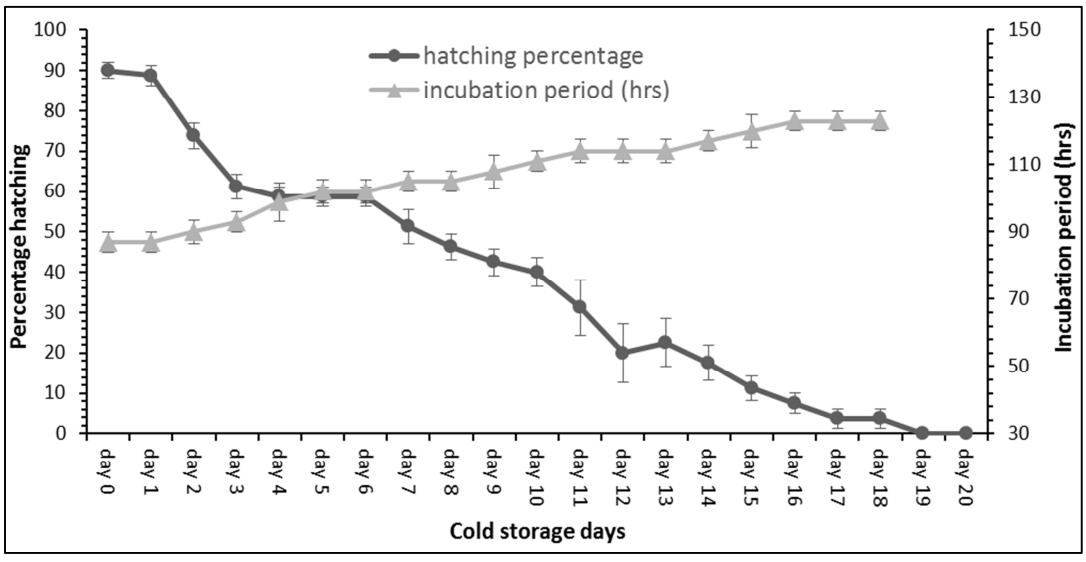


**Fig. 1.** Average hatching percetage and incubation period in specific cold storage days. Day 1 is presenting as control treatment. Gery and black broken lines presenting mean of parameters. All verticle bars indicate ± SEM and overlapping bars are not stasitically different with each others.

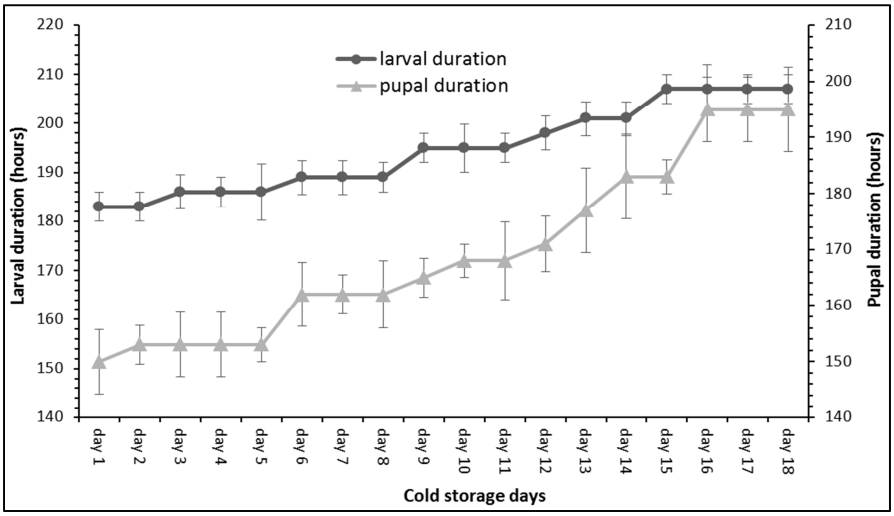


**Fig. 2.** Average larval and pupal duration in specific cold storage days. Day 1 is presenting as control treatment. Gery and black broken lines presenting mean of parameters. All verticle bars indicate ± SEM amoung them overlapping bars are not stasitically different with each others.

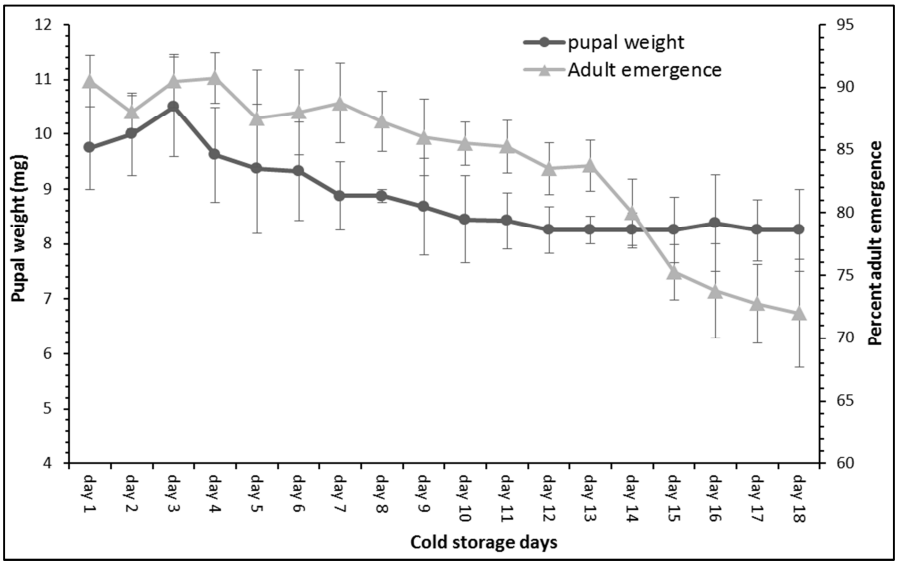


**Fig. 3.** Average pupal weight and adult emergence in specific cold storage days. Day 1 is presenting as control treatment. Gery and black broken lines presenting mean of parameters. All verticle bars indicate ± SEM amoung them overlapping bars are not stasitically different with each others.

Linear regression model was fitted to check the relationship between biological parameter of *C. carnea* with cold storage time (Fig. 4.). Hatching and adult emergence percentage have strong negative correlation with cold storage duration. Incubation period, larval and pupal duration were found positively correlated with cold storage time. The results of these dependent variables affected by cold storage (hatching (H), pupal weight (P) and adult emergence (E) were combined to show the additive loss of them. The mean values of these variables were used to calculate the following equation. The resulting AL values for different treatments are presented in Table 1.

$AL = [100 - (H + P + E)/3]$.

With respect to F1 and F2 progeny, cold storage treatment were not affected significantly (Table 2). Due to the no difference in hatching percentage of F1 and F2 progeny during cold storage treatments, further observation of other variables were not evaluated.

**Table 1.** Added effect of loss of quality by cold storage.

| Cold storage (days) | Added effect* |
|---|---|
| day 0 | 63.4 |
| day 1 | 63.0 |
| day 2 | 57.3 |
| day 3 | 54.1 |
| day 4 | 53.0 |
| day 5 | 51.9 |
| day 6 | 52.0 |
| day 7 | 49.6 |
| day 8 | 47.5 |
| day 9 | 45.7 |
| day 10 | 44.7 |
| day 11 | 41.6 |
| day 12 | 37.3 |
| day 13 | 38.2 |
| day 14 | 35.3 |
| day 15 | 31.6 |
| day 16 | 29.9 |
| day 17 | 28.3 |
| day 18 | 28.0 |

*As value of potentially useful predators.

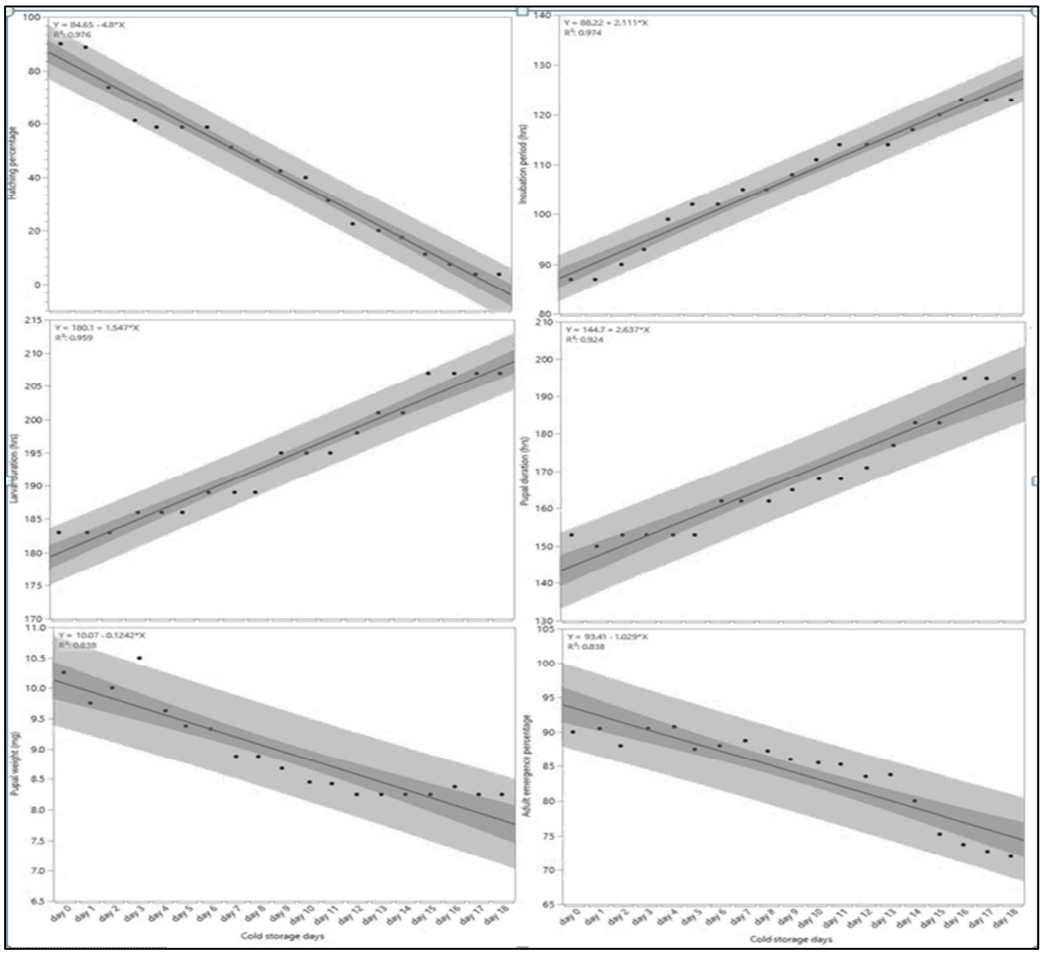


**Fig. 4.** displays the fit plot that shows the slope of the fitted line. Black spots are presenting the average of each parameter at particular days of storage. The 95% confidence limits in the fit plot are pointwise limits that cover the mean of each parameter for a particular cold storage days with probability 0.95. The prediction limits, which are wider than the confidence limits, show the pointwise limits that cover a new observation for a given cold storage days with probability 0.95.

**Table 2.** The effect of cold storage on the F1 and F2 progeny.

| Cold storage (days) | F1 progeny hatching percentage | F2 progeny hatching percentage |
|---|---|---|
| day 0 | 91.3 ± 0.7 | 91.5 ± 0.4 |
| day 1 | 91.2 ± 2.1 | 89.3 ± 0.9 |
| day 2 | 90.8 ± 0.9 | 89.9 ± 0.5 |
| day 3 | 90.9 ± 1.3 | 88.8 ± 1.3 |
| day 4 | 91.4 ± 0.7 | 90.1 ± 0.6 |
| day 5 | 90.8 ± 1.8 | 85.3 ± 2.8 |
| day 6 | 91.1 ± 1.6 | 89.4 ± 0.8 |
| day 7 | 90.4 ± 1.9 | 85.7 ± 2.3 |
| day 8 | 90.1 ± 3.3 | 86.4 ± 1.8 |
| day 9 | 89.5 ± 4.8 | 86.7 ± 1.4 |
| day 10 | 90.7 ± 4.3 | 85.4 ± 0.5 |
| day 11 | 88.5 ± 1.2 | 87.5 ± 1.7 |
| day 12 | 87.4 ± 4.2 | 85.9 ± 1.9 |
| day 13 | 89.1 ± 5.4 | 86.4 ± 1.3 |
| day 14 | 86.9 ± 4.9 | 84.6 ± 3.3 |
| day 15 | 88.9 ± 3.7 | 85.2 ± 4.8 |
| day 16 | 87.4 ± 5.3 | 86.9 ± 3.4 |
| day 17 | 86.3 ± 5.1 | 84.5 ± 2.6 |
| day 18 | 89.8 ± 3.9 | 83.9 ± 3.8 |

Values are presented as mean ± SE.

**Discussion**

For storage to be of value in the mass rearing of *C. carnea*, it is essential to fulfill several requirements: high hatching percentage, high incidence of survival and reproduction after storage, about to normal larval and pupal duration, sustained adult emergence along with high fecundity and fertility. Our experiment demonstrated that *C. carnea* eggs can be stored for as long as 7 to 15 days at normal refrigeration temperature (10°C ±1) and generally meet these requirements. Moreover, the outcomes provide vision into the additional experiments for improving the storage technique.

Hatching percentage was significantly affected by cold storage days, which remained only 51.3 ± 4.3 at 7$^{th}$ day of cold storage and continued to decreased till 0% just after 18 days of cold storage. López-Arroyo *et al.,* (2000) reported that generally *C. externa* eggs have very high hatching percentage at 12.8°C, and fell to 16 to 100% at 10°C. Literature reports three basic resaons for diminution of haching percentage: freezing of body water leads to cellular dehydrtion, mechanical damage to cells, and osmotic concentrtion of solutes (Muldrew *et al.*, 2004). Direct chilling may cause severe cold shock which cause denaturating of protein, dissociation of multimeric proteins and membrane lipid phase transitions (Dobrins *et al.*, 1993; Kunugi and Tanaka, 2002; Tsai *et al.*, 2002). The fact in deep denaturation of protein is depended on very specific temperature dependent interaction of peotein nonpolar group with water. In this phenomenon, haydration is advantageous thermody namically, i.e. the Gibbes energy of hydration is negative and increase in magnitude at a decreasing temperature. Due to which, tighlty packed polypeptide chain in compacted native structure, unfolds at significant cold temperature, exposing internal nonpolar groups to water that actually leads to the denatureation of protein (Babu *et al.*, 2004). The last reason is indirect chilling that depend on chronic esposures to relatively mild cold temperature, which leads various metabolic disorders include depeletion of energy substrates and/or ATP, oxidative stress and disturbance of ionic and osmotic homeostasis (Koštál *et al.*, 2016).

As incubation period increased significantly with increase in cold storage time. Comparative to the control group, 41.4% increase was observed. The duration spent by eggs at low temperature (i.e., less than 5°C) was negatively correlated to the time required by an egg to start hatching. Even though, cold temperature increase synchorny and percocity of hatching, chilling did not bring a significant diminution of hatching time (Chuche and Thiéry, 2009). Delay in hatching period may be due to a reason that storage at low temperature limits the

embryonic development without causing high damage rate. It has been reported that poor hatching success at low temperature is due to the lack of a sufficient energy supply for the hatching process (Evans, 2001).

This study showed that *C. carnea* larval (*F = 3.76, n = 76, df = 18, p = 0.001*) and pupal durtion (*F = 8.25, n = 76, df = 18, p = 0.000)* are amenable to cold storage time. Prolonged low temperature had a deterimental effect on juveline stages. Likewise, pupal weight was not found disturbed. Although, there was no significant difference found in the pupal weight of *C. carnea* stored at 10°C ± 1, viability of mature puape were found less than the control group. Some adult emergence were malfunctioned, we removed them because they were not able to mate and produce viable eggs. The level of accumulated injury is directly proportion to the duration of exposure and chilling injury gathers and ultimately become lethal (Koštál *et al.*, 2006). Cold storage affects young pupae relatively severely, probably due to have fewer energy stored as compared to other stages. Hence survival at low temperature is associated to the exhaustion of energy reserves (Foerster *et al.*, 2004; Colinet *et al.*, 2006).

Results of our study demonstrated that even hatching percentage of next two, F1 and F2 generations were not significantly affected by cold storage time. However, incubation, larval and pupal period were recorded significant positively correlated with increase in cold storage time. While hatching and emergence percentage are not. As the information in the literature on the effect of low temperature storage on *C. carnea* eggs are limited, the consequences of this research are more imperative in presenting how future studies might progress the method of storing *C. carnea* eggs and to improve efficiency of biocontrol agents. Further studies on low temperature storage of this predator using cryptopreservarion should be considered.

**Conclusion**

Storage of predator and parasitoids is a crucial factor in the execution of inundative or augmentative release (Collier and Van Steenwyk, 2004). it is nearly true for both predator and parasitoid, but perhaps it would be more problematic to implement for predator like *C. carnea*.

These investigations, consequently, specify that *C. carnea* eggs can be stored at 10°C ± 1 for upto 7 to 15 days with little loss of viability. One might expect that the eggs of *C. carnea* would not be the most practicle stage for long term storage as they are not well protected as much as the pupae are (Osman and Selman, 1993). However, still more work essentials to be carried out to determine the hatchability of stored eggs at different field temperature when mass rearing of this predator as eggs is struggled.

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**Conflict of author**

The authors declare that they have no conflict of interest throughout the manuscript.

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