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THE EFFECTS OF SHORT-TERM COLD STORAGE ON THE SURVIVAL OF THE ENDOPARASITOID *OOENCYRTUS PITYOCAMPAE* MERCET (HYMENOPTERA ENCYRTIDAE)

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Binazzi F., Sabbatini Peverieri G., Benassai D., Roversi P.F. – The effects of short-term cold storage on the survival of the endoparasitoid *Ooencyrtus pityocampae* Mercet (Hymenoptera Encyrtidae).

In the Mediterranean basin the Pine Processionary Moth (PPM), *Thaumetopoea pityocampa* is often responsible for damaging coniferous stands. The parthenogenetic thelytochous *Ooencyrtus pityocampae* because of its host location strategy and capacity to develop on alternative hosts plays a key role in the control of PPM. In the context of biological control, storage at low temperatures of parasitoids proved to be effective in improving their mass rearing for field release. Unfortunately, cold storage is often associated with high mortality even above the freezing temperature. Therefore, the present work focused on *O. pityocampae* juvenile stages submitted to 10-days storage at 15°C and then transferred to 26°C and 30°C until adult hatching. Two different hatching temperatures were used to test the hypothesis whether the speeding of *O. pityocampae* development could affect its survival and synchronization of emergencies. After the treatment, adult emergence percentage, adult emergence time and synchronization of emergencies were evaluated. Results showed that all life stages successfully survived the thermal regime.

Nonetheless, pupae because of their high emergence rates and synchronization of adult hatching appeared to be the best stage for short time storage.

KEY WORDS: *Thaumetopoea pityocampa*, biological control, cold storage, endoparasitoid, *Ooencyrtus pityocampae*.

INTRODUCTION

In several areas of the Mediterranean basin the Pine Processionary Moth (PPM), *Thaumetopoea pityocampa* (Denis & Schiffermüller) (Lepidoptera Thaumetopoeidae), is often responsible for damaging coniferous stands (TSANKOV *et al.*, 1998). Its main targets are *Pinus* ssp. but *Cedrus* spp. and *Pseudotsuga menziesii* can be attacked as well (ROQUES *et al.*, 2002). Moreover the urticating setae of larvae may represent a public health concern causing contact dermatitis (LAMY, 1990) and even more serious reactions such as anaphylaxis and conjunctivitis (VEGA *et al.*, 1999). In the last decades, hymenopteran endoparasitoids have been evaluated as pest control agents against this lepidopter. Among these natural enemies, the parthenogenetic thelytochous *Ooencyrtus pityocampae* (Mercet) (Hymenoptera Encyrtidae) because of its host location strategy and capacity to develop on alternative hosts plays a key role in the biological control of PPM (BATTISTI *et al.*, 1988; HALPERIN, 1990).

In Israel, the role of *Ooencyrtus pityocampae* as a control agent of *Thaumetopoea wilkinsoni* Tams, a major pest of coniferous stands in the eastern Mediterranean basin, has been investigated since 1961 (HALPERIN, 1990). Promising results were obtained in terms of increased parasitization of pest batches but further studies showed that such a level of control could be only achieved in years with mild and dry autumns (HALPERIN, 1990). More recently, several interventions were carried out in Tuscany and Lazio (Italy) in order to control the PPM.

Populations of *O. pityocampae* were released in specific sites synchronizing their activity with the egg stage of PPM. The analysis of PPM batches collected in the same stands in the following years showed an increase of parasitization rates compared with those recorded in the pre-treatment surveys (TIBERI, 2009).

Mass rearing of beneficial insects is an essential step of biological control programs, particularly of those depending on augmentative field releases (VAN LENTEREN & TOMMASINI, 2002). However a strong limiting factor in mass production of natural enemies is represented by the difficulty of storing them before the release. The organisms used in pest control programs often have a relatively short shelf life. For this reason, they have to be produced just before their use. Therefore, the development of adequate storage procedures is of key importance in limiting the cost of biological control by extending the production period (COLINET & BOIVIN 2011).

In this context, storage at low temperatures of either egg parasitoids or hosts has been thoroughly investigated in order to develop effective programs for the production of biocontrol agents (SABBATINI PEVERIERI *et al.*, 2014.). Efforts to rear parasitoids on refrigerated, frozen or cryo-stored host eggs have been recently carried out in order to improve parasitoid production. Rearing on stored eggs was successful in several groups of endoparasitoids but detrimental effects were often reported as a function of

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time and temperature of storage (CHEN & LEOPOLD, 2007; KIVAN & KILIC, 2005; MAHMOUD & LIM, 2007; SABBATINI PEVERIERI *et al.*, 2014). On the other hand, cold storage of parasitoids under sub-ambient temperatures above 0°C, reduces their metabolic rate, slowing down their development and allowing an extension of their shelf life (COLINET & BOIVIN, 2011). Therefore, shipment of natural enemies from their original production sites to their final releasing areas is considerably simplified. Moreover cold storage allows a precise synchronization between field releases and the most critical phases of pest outbreaks (MCDONALD & KOK, 1990; VENKATESAN *et al.*, 2000).

In addition, cold storage proved to be essential not only for insect industrial production but also for maintaining colonies under laboratory conditions for research purposes (COLINET & BOIVIN, 2011).

Unfortunately, such techniques are often associated with major fitness costs even above the freezing temperature (HANCE *et al.*, 2007; VAN BAAREN *et al.*, 2005) and mortality often represents the extreme consequence of a progressive accumulation of sub-lethal perturbations occurred during chilling (COLINET & BOIVIN, 2011).

HALPERIN (1990) noted that *O. pityocampae* is able to develop generation after generation all the year round when it is reared at temperatures ranging from 24°C to 32°C. Conversely, diapause could be artificially induced when mature larvae were exposed to temperatures below 15°C (HALPERIN, 1990) and easily interrupted by simply warming them up to 28-30°C for about three weeks (MASUTTI, 1964). So far, low-temperature studies of *O. pityocampae* have involved the use of field-parasitized egg clusters of the natural host *Thaumetopoea* spp. and neither the employment of alternative hosts nor comparisons between the cold tolerance of different life stages have been evaluated. However, since rearing of *Thaumetopoea* spp. is often complex and unsuitable for mass production, treatments and controls in our trial were carried out using *Graphosoma lineatum* (L.) (Heteroptera Pentatomidae) as an alternative host for *O. pityocamape* (TIBERI *et al.*, 1991). This pentatomid exhibits facultative diapause and can be easily reared in laboratory conditions. Moreover *O. pityocampae,* though reared for several generations on pentatomids, is reported to readily accept its natural host *T. pityocampa* (TIBERI *et al.*, 1991). Most egg parasitoids overwinter as immature stages (BOIVIN, 1994), but exceptions often occur as in *Ooencyrtus kuwanae* and *Oencyrtus nezarae* that were reported to survive the cold season overwintering as diapausing adults (GRANEK & MCDONOUGH, 1973; NUMATA, 1993). On the contrary, there is evidence that *O. pityocampae* parasitoids overwinter as juvenile stages (HALPERIN, 1990). Consequently, this trial, which was part of a broader investigation on *O. pityocampae* cold tolerance, focused on immature larvae, mature larvae and pupae submitted to a short-time storage. After the thermal treatment, two different temperatures were selected for parasitoid hatching in order to test the hypothesis whether the speeding of *O. pityocamape* development could affect its survival and synchronization of emergencies.

MATERIALS AND METHODS

ORIGIN AND REARING OF THE INSECTS

Adults of the pentatomid *Graphosoma lineatum* (L.) were collected from fennel plants (*Foeniculum vulgare* Mill.) in Tuscany-Italy (43°51 N, 11°15 E). Thereafter, insects were reared for several generations in cages and provided with seeds of *Foeniculum vulgare, Anethum graveolens* L. and *Pimpinella anisum* L. as a food source. Moreover young potted fennel plants were also added to improve *G. lineatum* diet. An adequate water supply for plants and insects was ensured by an automatic irrigation system. Cages were maintained in a rearing room at 27±2°C, 45±5% RH and 16:8 L:D photoperiod. Soft laboratory paper was attached inside each cage as a substrate for insect oviposition. Alternatively cardboard cylinders paved by cotton disks were used for the same purpose. Cotton disks and paper were daily inspected and egg batches collected for the trial (BINAZZI *et al.*, 2015).

Immatures of the endoparasitoid *O. pityocampae* overwintering in egg clusters of *T. pityocampa* were originally gathered from coniferous stands (600 m asl) in Gargano, (Puglia-Italy) (41°50 N, 16°00 E). Egg batches were placed into glass tubes and transferred into a rearing room at 30±1°C, 40±10% RH and 16:8 L:D in order to interrupt parasitoid diapause. When adults hatched, a new colony was established. Parasitoids were then reared for more than 30 generations on *G. lineatum* host eggs. Afterwards the new colony was maintained in a climatic chamber (Binder KBWF 720) set at the standard rearing conditions of 26±1°C, 75±5% RH and 16:8 L:D. Adult females were fed with pure honey drops *ad libitum* (SHMIDT & TANZEN, 1998) that were refreshed three times per week.

STORAGE AT 15°C

Fresh egg batches (< 24h) of about 14 *G. lineatum* eggs were glued on paper stripes by a drop of distilled water (3.5x2.5cm). Stripes were then exposed to 5-days-old *O. pityocampae* females for 24h at a 1:5 parasitoid/host ratio and standard rearing conditions (BINAZZI *et al.*, 2013). Parasitized eggs were easily identified by the presence of an egg stalk protruding from the host chorion (MAPLE, 1937). After parasitization stripes were divided into smaller parts bearing each a batch of fully parasitized eggs. Egg clusters were subsequently placed into large glass tubes (15 cm long and 2 cm diameter, closed at both ends by cloth mesh). Thereafter, groups of batches were held at standard conditions for 1, 5 and the 11 day in order to obtain three different juvenile development stages within parasitized *G. lineatum* eggs: Immature larvae, mature larvae, and pupae (MAPLE, 1937; TAKSAU & HIROSE, 1989). The development stages of immatures were checked by dissections (MAPLE, 1937). Afterwards egg batches hosting parasitoid juveniles were directly transferred into a climatized chamber (TK 252 Nuve) set at 15°C (75±5% RH) and stored for 10 days in full darkness.

After the trial, batches from every development stage were separated in two equal groups and placed into two climatic chambers (TK 252 Nuve) set respectively at 26°C and 30°C (75±5% RH and 16:8 L:D) until parasitoid hatching. Controls involved a group of parasitized batches left at standard conditions until adult hatching and three groups maintained at standard conditions until respectively the 1th, 5th and the 11th day and then directly transferred into a new chamber set at 30°C, 75±5% RH and 16:8 L:D. Relative humidity and temperature within chambers were constantly recorded using data loggers (Escort 60D32).

For each control and treatment, 15 replicates (i.e. parasitized batches) were employed and the following parameters recorded: Adult emergence percentage

(Percentage of no. of emerged adults/no. of parasitized eggs), sex ratio (% females) and adult emergence time (developmental time from every treated stage to adult emergence, estimated as the time between the end of cold storage until adult emergence). All observations were carried out by a stereomicroscope Nikon SMZ - 1500 equipped with a cold light source.

Statistical analysis

The Shapiro-Wilk test was carried out in order to test the normality of data (Zar, 2010). Transformations were applied to adult emergence percentage and synchronization of parasitoid emergencies, but failed to normalize the data. Kruskal-Wallis nonparametric ANOVA was then used to analyse differences among treatments. When significant differences were detected, pairwise comparisons of groups were performed by the Mann-Whitney U test. For multiple comparisons, the significance level ($\alpha = 0.05$) was adjusted with Bonferroni correction, α/n, where n was the number of pairs in the multiple comparisons (Zar, 2010). Conversely, adult emergence time was square root transformed ($\sqrt{x+0.5}$) after the Shapiro-Wilk test and means separated by ANOVA and Tukey post-hoc test ($P< 0.05$). Synchronization of parasitoid emergencies was measured for every replicate (i.e. parasitized batch) in terms of

$$\text{Coefficient of Variation } (CV = \frac{S}{\bar{X}} * 100\%)$$

and differences between groups analyzed by Kruskal-Wallis nonparametric ANOVA. Although Kruskal Wallis is based on ranks, data in the graphs are displayed as mean ± SE. Statistical procedures were carried out by the statistical software SPSS 20.0.0 (2011).

RESULTS

In the laboratory tests, parasitoid females accepted and successfully parasitized all the exposed *G. lineatum* eggs batches. After the storage treatment, the three tested development stages (immature larvae, mature larvae, and pupae) showed a different hatching rate. However no statistically significant differences were detected in adult emergence percentage among the twelve groups. Kruskal Wallis one-way ANOVA: $H = 19.60$; $df = 11$; $n = 177$; $P > 0.05$ (Fig. I). Conversely, adult emergence time was heavily affected by the different development stages and storage conditions. Statistically significant differences were in fact recorded among the groups (One-way ANOVA: $H = 188.83$; $df = 11,163$; $P < 0.0001$) (Fig. II). Moreover synchronization of parasitoid emergencies was equally strongly influenced by the storage treatments since marked statistically significant differences in the coefficients of variation were observed among the groups. Kruskal Wallis one-way ANOVA: $H = 110.12$; $df = 11$; $n = 175$; $P < 0.001$ (Fig. III). Although *O. pityocamapae* is reported to be a parthenogenetic species at the conditions tested in our experiments, few males hatched representing the 0.5% of the total number of emerged parasitoids.

DISCUSSION

Insect cold tolerance is highly variable among different life stages. Therefore a generalization of this pattern to genera or species is often complex since exceptions often occur. Accordingly, the determination of the most cold-tolerant parasitoid development stage plays a key role in the design of a cold storage protocol (Colinet & Boivin, 2011). Conservation of immatures is usually considered the best procedure and pupae are often regarded as the best stage when short-term storage is planned (van Lenteren & Tommasini, 2002). Evidence was indeed provided showing that pupae are more cold tolerant than adults, larvae or eggs (Jalali & Singh, 1992; Nakama & Foerster, 2001). Nonetheless, in other studies, adults were found to be more resistant to cold storage than juveniles (Bayram *et al*., 2005; Riddick, 2001). However, since egg-parasitoid pupae are immobile and protected by desiccation and handling inside their cocoon, they are often

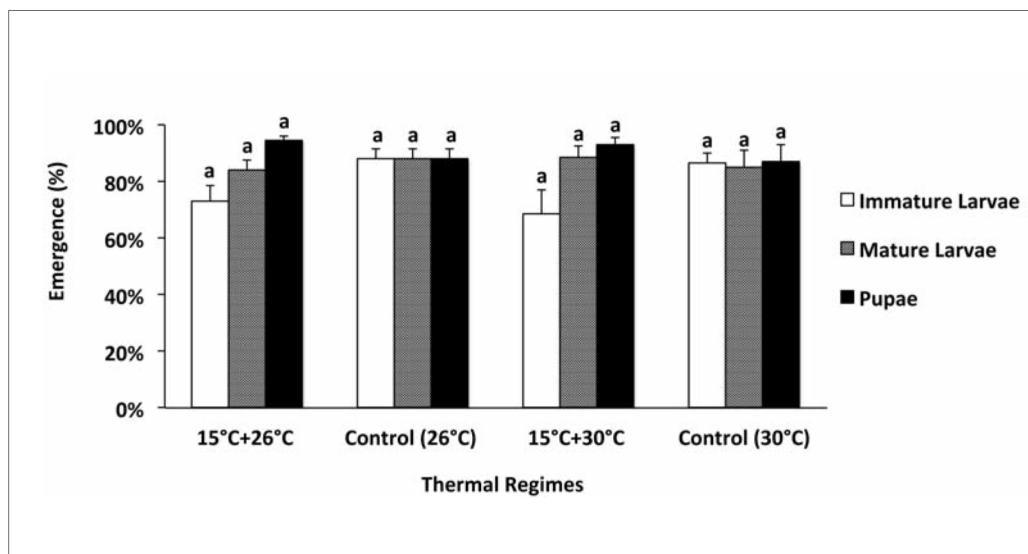

Fig. I – Percentage of emerged adults (mean ± SE) after storage of immatures at 15°C for 10 days. Bars sharing the same letter are not statistically different.

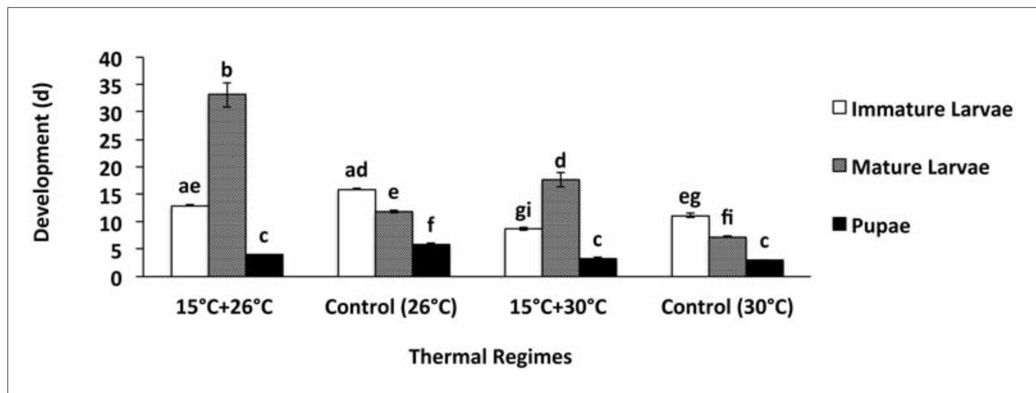

Fig. II – Adult emergence time (mean ± SE) after storage of immatures at 15°C for 10 days. Bars sharing the same letter are not statistically different.

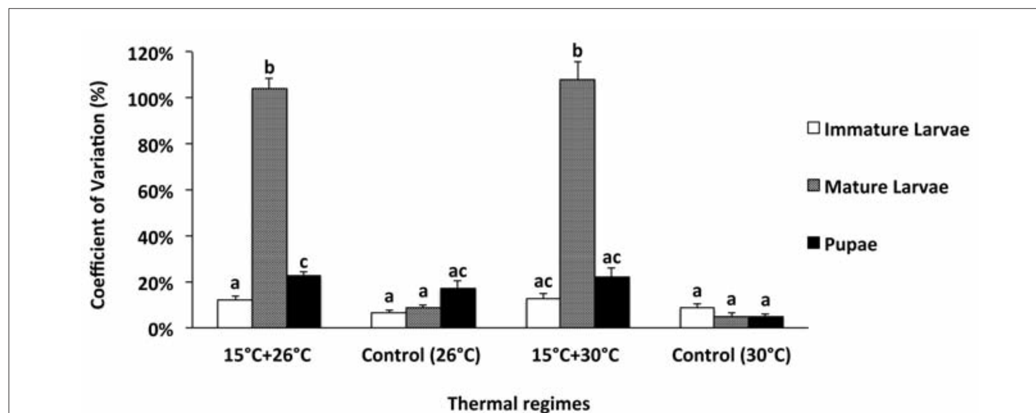

Fig. III – Synchronization of emergencies measured as coefficient of variation (mean ± SE) after storage of immatures at 15°C for 10 days. Bars sharing the same letter are not statistically different.

selected for conservation. That being said, adequate storage protocols should be carefully organized because, pupae, though theoretically inactive, are yet a metabolically very active stage. In fact, during this phase larval tissues undergo histolysis to be subsequently reorganized in the adult shape (COLINET & BOIVIN, 2011).

Our results generally agree with the trend described by VAN LENTEREN & TOMMASINI (2002). Pupae indeed showed the highest emergence percentage among juvenile stages. On the other hand, immature larvae presented the lowest hatching rate with a reduction in adult emergence up to 20%. However the high variability in the emergence percentages of larvae, particularly for those transferred to 30°C after storage, can partially account for the lack of statistically significant differences among the hatching rates of all the groups. Nevertheless, overall results showed that speeding parasitoid development after the treatment did not affect *O. pityocampae* emergence.

In the context of low temperature preservation, the storage of dormant stages was found to be useful for conserving parasitoid species whose cold hardiness is enhanced by diapause induction (BOIVIN, 1994). ANDERSON & KAYA (1974) observed that in *Ooencyrtus ennomus* Yoshimoto the induction of diapause was facultative and occurred at the end of larval development soon before defecation and pupation. The same Authors noted that the length of chilling of *O. ennomus* diapausing larvae affected adult emergence time, synchronization of emergencies and mortality. In fact, days required for hatching increased when short time chilling was applied. Similarly, synchronization of parasitoid hatching was lower after short chilling periods, while mortality increased when the length of chilling decreased (ANDERSON & KAYA, 1975).

Likewise, the diapause of *O. pityocampae* mature larvae hosted in egg masses of *T. pityocamapa* was investigated by BATTISTI *et al.* (1990). As in *O. ennomus,* larvae were chilled at 5°C for different time lags, but this time a gradient of 2°C/day was applied before and after the treatment. The main outcomes showed again an increased survival when cooling periods increased while mortality affected up to 50% of the parasitoid larvae after host masses had been cooled for a short period (7days). A reduction of the time required for post diapause development and a higher synchronization of adult hatching were also observed with progressively longer cooling intervals (BATTISTI *et al.*, 1990).

Our findings showed that immature larvae and pupae had a similar adult emergence time when compared with their respective controls either for post-storage temperature set at 26°C or 30°C. Conversely, treated mature larvae presented on average a much longer development compared with their controls. This phenomenon may be due to the onset of diapause in

accordance with what was reported for *O. ennomus* and *O. pityocampae* stored at 5°C (ANDERSON & KAYA, 1974; BATTISTI *et al.*, 1990). Moreover, as expected, juveniles exposed to 30°C after treatment, had generally a faster development compared with those transferred to 26°C. Nonetheless the adult emergence time of mature larvae held at 26°C after the storage, almost doubled that of mature larvae maintained at 30°C after the same treatment. This difference is not explainable in terms of discrepancies between synchronization of emergencies, since this parameter resulted to be equivalent in both groups; it should be conversely related to the different temperatures at which diapause was interrupted. As previously mentioned, *O. pityocampae* mature larvae are reported to interrupt diapause only above 28°C (MASUTTI, 1964). However, in the present experiment, after 10 days storage at 15°C, diapause was equally interrupted by exposing parasitized egg batches to 26°C, though this process resulted in longer hatching times for developing larvae. On the other hand, the high coefficients of variation recorded for mature larvae, underline that the synchronization of adult hatching in this group is lower than that observed for the other juvenile stages that underwent storage. This phenomenon is likely to be a direct consequence of the onset of diapause in mature larvae. In fact, the conditions tested in our trial could have induced diapause in only a certain percentage of parasitoids, leading thus to a marked variability in hatching times. Under the conditions examined, pupae, because of the high emergence rates and high synchronization of adult hatching, appear to be the best stage for short-time storage. Finally, the emergence of some parasitoid males at the conditions tested in our trial requires further investigation in order to evaluate the role played by endosymbiotic bacteria in altering *O. pityocampae* sex ratio.

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