Low-temperature storage of Exorista larvarum puparia as a tool for assisting parasitoid production

Low-temperature storage of *Exorista larvarum* puparia as a tool for assisting parasitoid production

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Abstract

Tachinid parasitoids deserve to be better exploited as natural enemies of insect pests. The development of efficient mass-rearing techniques for these entomophagous insects may encourage their use in biological control programs. *Exorista larvarum* (L.) (Diptera: Tachinidae) is a Palearctic species, which has been introduced in northern America for the control of the gypsy moth, *Lymantria dispar* (L.). The potential use of this polyphagous parasitoid of Lepidoptera against other forest and agricultural pests encourages research aimed at the improvement of techniques for its rearing, including protocols for storage at low temperature. This technology allows a certain degree of control over the speed of development of the stored insects and assists mass-rearing facilities that have to match production with demand, especially during field outbreaks of pests. With the aim of developing storage protocols for *E. larvarum*, we investigated the effects of storage for 1–4 weeks of 1-day-old puparia at 15 °C. Lower temperatures (5 and 10 °C) and longer storage periods were excluded following the outcome of preliminary experiments. Parasitoid emergence and quality control parameters of the female flies obtained from the stored puparia were evaluated. In addition, female lipid body reserves were measured. The temperature of 15 °C proved to be suitable for all the durations tested, although some detrimental effects were observed following storage (e.g., lower longevity and fecundity). Our findings may prove useful to increase the flexibility of *E. larvarum* colony management.

Introduction

Augmentative biological control programs depend upon releasing mass-reared natural enemies into the environment for controlling population levels of pest organisms (van Lenteren, 2000). The production of entomophagous insects, in large numbers and of high- quality, is one of the fundamental pillars for the success of this control strategy.

As biological control is a component of integrated pest management programs (Kogan, 1998), insect mass-rearing factories are in competition with other companies offering alternative pest management solutions. Despite the incredible advances in insect mass-rearing technologies made in recent decades (Morales-Ramos et al., 2014), rearing insects continues to represent a challenge and offers both difficulties and opportunities. In this context, the industrial scale production of high-quality entomophagous insects must be profitable, hence the rearing costs have to be minimized and the predators and parasitoids must be delivered on time in the field or greenhouses, especially during the outbreak of their prey or hosts (van Lenteren, 2012).

Except for species which can enter an induced diapause (Vallo et al., 1976; Quednau, 1993), living insects have a fairly short shelf life. Storage at low temperatures (below the optimal rearing temperature) is a valuable tool for prolonging the shelf life of insects (Leopold, 2007). This procedure usually involves the placement of immature stages of the natural enemies between 4 and 15 °C (van Lenteren & Tommasini, 2003), with the aim of slowing their metabolic activities while having the minimum detrimental effect on their survival and quality. This technology, often called 'cold storage', is a great convenience for mass-rearing companies that need to match their production with the demand from clients, which is difficult to predict and

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depends mostly on the pest population levels in the field (Leopold, 1998; Chen et al., 2008). Moreover, the continuous culture chain of entomophagous natural enemies can also face periods of low production, either due to the difficulties encountered when rearing their prey or hosts, or to equipment failure or temporary unavailability of trained personnel. Regardless of the storage period, a reduction in fitness of stored insects is commonly observed and represents a serious drawback that must be considered (Colinet & Boivin, 2011; Rathee & Ram, 2018).

Despite their secondary importance in terms of number of species compared to hymenopterans (Feener & Brown, 1997; Stireman et al., 2006), dipteran parasitoids have shown potential for regulating phytophagous insect populations in applied biological control (Dindo, 2011). Fly parasitoids with potential for mass-rearing for use against insect pests belong to the families of Tachinidae, Sarcophagidae, Phoridae, Cryptochaetidae, and Bombyliidae (Eggleton & Belshaw, 1992). Tachinidae comprises 8 500 worldwide described species and represent the largest and most significant family of non-hymenopteran parasitoids for use in biological control programs (O'Hara, 2013).

Several examples can be found in literature of the use of tachinids in classical and augmentative biological control (Dindo & Grenier, 2014). Among other species, three parasitoids of lepidopterous sugarcane borers – i.e., *Lixophaga diatraeae* (Townsend) in Cuba (Medina, 2002; Massó Villalón, 2007), and *Lydella minense* (Townsend) and *Paratheresia claripalpis* van der Wulp in Brazil, Colombia, and Peru (van Lenteren & Bueno, 2003) – have been reared for biological control purposes at an industrial-scale level. The development of efficient rearing techniques for these and other species, also including storage at low temperatures, may reduce the rate of failure in biological control programs already in place and encourage the development of new programs involving tachinid parasitoids.

Exorista larvarum (L.) (Diptera: Tachinidae) is a gregarious larval parasitoid recorded as a natural enemy of several lepidopteran pests of forest and agricultural interest, including the gypsy moth, *Lymantria dispar* (L.), the cabbage moth, *Mamestra brassicae* (L.), and other noctuid species (Cerretti & Tschorsnig, 2010; Depalo et al., 2012). It is widely distributed in the Paleartcic region and it has also been introduced against the gypsy moth into the northern USA, where it has established (Sabrosky & Reardon, 1976). The life cycle of *E. larvarum* has been described in the laboratory adopting the natural noctuid host *Spodoptera* (= *Prodenia*) *litura* (Fabricius) (Hafez, 1953) and the factitious pyralid host *Galleria mellonella* (L.) (Mellini et al., 1993; Michalková et al., 2009). Eggs are laid on the host larva cuticle. The newly hatched maggots penetrate the host body, where they build primary integumental respiratory funnels, which allow them to breathe atmospheric air from the beginning of their development, and thus grow rapidly. This parasitoid does not show a developmental synchrony with its host, which is quickly killed. Pupation generally occurs outside the host larval remains (Mellini et al., 1993; Dindo, 2011).

Also due to the rather simple life cycle, its gregariousness and polyphagy, *E. larvarum* can be reared in a relatively easy way (Dindo & Grenier, 2014). The availability of various mass-rearing techniques for this parasitoid makes it a good candidate to be produced at an industrial scale and released against target pest species. Indeed, this tachinid showed good potential, in terms of yield and quality, when reared in vivo, particularly on the factitious host *G. mellonella* (Mellini et al., 1993), or in vitro, using artificial media with or without host components (Bratti & Coulibaly, 1995; Dindo et al., 2006, 2016). However, considering the variety of host taxa that *E. larvarum* can attack, any deliberate introduction of this parasitoid in the environment must meet current regulations to avoid negative consequences, such as population declines on non-target insect species (Boettner et al., 2000).

Protocols for storage at low temperatures of *E. larvarum* are scant at present. Following the experiments performed by Benelli et al. (2017), where *E. larvarum* eggs were stored on artificial medium at suboptimal temperatures (15 or 20 °C), we planned an investigation to verify whether storage of the tachinid puparia at low temperatures may prolong pupal development while having minimum impact on the fitness of the resulting adults (Easwaramoorthy et al., 2000). As outlined by van Lenteren & Tommasini (2003), the pupal stage is suitable for short-term storage of insects. At present, little is known about the biology of *E. larvarum* in natural environments and how this species overwinters. The scant information available, however, suggests that, as in many polyphagous tachinids displaying non-synchronized development with their hosts (Mellini, 1991), also *E. larvarum* may overwinter in different stages, depending on climate and host availability. For instance, Hafez (1953) showed that in Egypt *E. larvarum* overwinters in any stage and does not enter a quiescence phase, despite a prolonged development. Conversely, Dowden (1962) reported that in the northern USA *E. larvarum* overwinters as immature larva within an overwintering host, the brown-tail moth *Euproctis chrysorrhoea* (L.). As *E. larvarum* is recorded in a wide geographical range, including temperate to cold regions, it is likely that it may also overwinter as puparium in the soil, as many tachinids do (Mellini, 1991), although this information is unconfirmed at present. Storage of puparia at low temperature is thus justified.

In the view of possible utilization of low-temperature storage protocols for mass rearing of tachinid flies, our study on *E. larvarum* is motivated by the potential usefulness of this species against several insect pests. Moreover, *E. larvarum* is a non-target species during insecticide spraying in agricultural ecosystems, therefore its production may prove useful for investigations on the side effects of chemical control on non-target insects that are commonly present in the environment (Marchetti et al., 2009; Francati & Gualandi, 2017). On the basis of preliminary experiments, we focused on the use of 15 °C as a storing temperature, investigating the effects of storage for 28 days in a first experiment, and the effects of storage for 7, 14, or 21 days in a second experiment. As stated by Sehnal (1991), any temperature below that to which a species is best adapted may be regarded as 'cold'. Similar storage periods were tested by Gross & Johnson (1985) for the puparia of the tachinid *Archytas marmoratus* (Townsend).

In general, the lack of effective industrial mass-rearing technologies for most tachinid flies has been considered as one of the reasons for failures that have occurred with field releases of these parasitoids in biocontrol strategies (Dindo & Grenier, 2014). Therefore, the development of an efficient rearing technique for *E. larvarum*, including protocols for their storage at low temperature, may contribute in making this species a more suitable candidate for future production and utilization in applied biological control programs.

Materials and methods

Insects

A stock colony of *E. larvarum* was kept in the Entomology Laboratory of the Department of Agricultural and Food Sciences (DISTAL, University of Bologna, Italy), using *G. mellonella* as factitious host for its continuous in vivo rearing. The colony was started in 2004 and augmented in 2010 with adults that had emerged from *L. dispar* and *Hyphantria cunea* (Drury) larvae collected in the field in the provinces of Modena (44°10′49″N, 10°38′54″E) and Forlì-Cesena (44°13′21″N, 12°2′27″E) (region Emilia Romagna, northern Italy). Adult flies were kept in Plexiglas cages (40 × 30 × 30 cm, with 50–70 individuals per cage) in a controlled environment room set at 26 ± 1 °C, 65 ± 5% r.h., and L16:D8 photoperiod (standard conditions). The temperature of 26 °C was selected as standard on the basis of previous experience with the rearing of other tachinid species (Baronio & Campadelli, 1979; Bratti et al., 1992; Dindo et al., 2003). Adult diet consisted of sugar cubes, cotton balls soaked in a honey-and-water solution, and water in drinking cups with soaked cotton.

For colony maintenance, parasitization was performed once a week by introducing three mature *G. mellonella* larvae per tachinid female into the cages (Bratti & Coulibaly, 1995). Host larvae were removed when they displayed 3–4 eggs on their integument. They were placed in a separate plastic box (24 × 13 × 8 cm) in the same controlled environment room until parasitoid larvae emerged and puparia formed. The *G. mellonella* colony was kept separately in a controlled environment cabinet set at 30 ± 1 °C, 65 ± 5% r.h., and complete darkness, using the artificial diet developed by Campadelli (1987) for larval rearing.

For all experiments, newly formed *E. larvarum* puparia (weight range 35–55 mg) were selected from the laboratory colony and maintained at the standard temperature of 26 °C for the first 24 h, to allow the newly formed pupae to complete their first crucial period of development (Tsiropoulos, 1972). Subsequently, the puparia to be stored at low temperature were transferred to the experimental conditions, whereas control puparia were maintained at 26 °C.

Preliminary experiments

To assess the ability of *E. larvarum* puparia to complete their development and emerge as adults after short-term storage at 5 and 10 °C, 10 puparia were maintained at each of these temperatures and 10 control puparia were kept at 26 °C. After 21 days of storage, the cold-treated puparia were returned to the standard temperature, but none of them completed the pupal development after an additional 21 days, resulting in 100% mortality. Conversely, 100% of the control puparia emerged as adults. For this reason, and also considering the studies carried out with *E. larvarum* eggs (Benelli et al., 2017), we selected 15 °C as a lower-bound low temperature to be studied.

To assess the ability of the pupae to complete their development and emerge as adults when puparia were stored at 15 °C (65 ± 5% r.h., complete darkness), 10 puparia were maintained at 15 °C and 10 control puparia were kept at 26 °C (control) continuously. Shortly after the 3rd week of storage (21 days), puparia stored at 15 °C began to emerge as adults. Puparia that did not produce adults within the 4th week were kept under observation at 15 °C until week 6, but none of these emerged. For this reason, 4 weeks (28 days) was selected as the maximum storage period to be investigated in the main experiments.

Storage at 15 °C for 28 days

We tested the capacity of *E. larvarum* pupae to survive, complete development, and produce high-quality adults when the puparia were stored for 28 days at 15 or at 26 °C (control). For each of the two treatments, four replicates

were carried out, each consisting of 10 puparia placed in a plastic cup (6 cm diameter) with no lid. The total number of puparia was thus 40 per treatment. The cups containing the puparia were in turn placed in small Plexiglas cages (20 × 20 × 20 cm) (one per treatment) and located inside an incubator set at either 15 ± 1 °C or 26 ± 1 °C, both at 65 ± 5% r.h. and L16:D8 photoperiod. Temperature and relative humidity were monitored by RHT10 data loggers (Extech, Nashua, NH, USA).

Puparia were inspected daily for adult emergence. Newly emerged adults were counted and sexed, and female flies were selected for quality control. Male flies were discarded. At the end of the storage period of 28 days at 15 °C, puparia that did not produce an adult were returned to 26 °C for 2 weeks to check for the possibility of delayed parasitoid emergence (which never occurred) and finally dissected.

To assess female quality, all female flies were maintained under 26 ± 1 °C, 65 ± 5% r.h., and L16:D8 photoperiod. They were individually placed in a small Plexiglas cage (20 × 20 × 20 cm) and provided water, sugar cubes, and a cotton ball soaked in honey-and-water solution. Immediately after emergence, each female was paired with a male fly from the laboratory stock colony, which had emerged in the same timeframe (within 48 h). When two females (or more) of the same replicate emerged within 24 h, they were placed together, in the same cage, and paired with an equal number of males from the stock colony. As the pre-oviposition period lasts about 3 days (Mellini et al., 1994; Dindo et al., 2007), the first host exposure took place on the 3rd day after pairing. Adult food was removed and three mature *G. mellonella* larvae per female were introduced into the cages. After 1 h, the host larvae were collected and the eggs laid on their integument were counted. Until parasitoid pupation, host larvae where kept under the standard conditions in small plastic cups, similar to those used for storing puparia, but covered with lids with small ventilation holes. Newly formed F1 puparia and F1 adults were counted. As *E. larvarum* females lay most eggs during the first 10 days of oviposition (Dindo et al., 1999), this parasitism procedure was repeated daily for 10 days, or until death if female flies died in this timeframe.

Storage at 15 °C for 7, 14, or 21 days

We verified the capacity of *E. larvarum* pupae to survive and complete development when puparia were stored at 15 °C for 7 (I), 14 (II), or 21 days (III). We also verified whether short-term stored puparia could produce high-quality adults, in comparison with those kept at the standard temperature of 26 °C (control). The methods were the same as described in the previous experiment, but each of the four replicates per treatment consisted of five puparia and the total number of puparia was, thus, 20 per treatment. For treatments I, II, and III, once the storage period at 15 °C was completed, puparia were returned to 26 °C within the plastic cups in the cages.

Also in this experiment, puparia were inspected daily for adult emergence. Newly emerged adults were counted and sexed, and one female fly per replicate per treatment was selected for the quality control test, which was performed as described for the previous experiment.

Adult lipid body reserves were also measured to assess adult fly quality, as lipid reserve depletion during storage has been found to be correlated with insect survival and longevity (Colinet et al., 2006). For this purpose, the excess female flies, which were not utilized for the quality control test, were frozen at −20 °C within 24 h of their emergence. Flies, individually placed in 5-ml glass tubes, were first dried in an oven at 60 °C for 48 h. When flies were dried, the glass tubes were immediately placed in a desiccator flask containing dry silica gel granules and the dry weight of the samples was measured with an AT21 microscale balance (Mettler Toledo, Columbus, OH, USA). Chloroform (Sigma-Aldrich, Milan, Italy) was added to the samples (1 ml per fly) using a pipette and a plastic plug applied to the tubes. Chloroform was used as a moderately polar solvent for the extraction (Raubenheimer et al., 2007; Ponton et al., 2011, 2015). The solvent was replaced every 24 h until flies completed 72 h in contact with it. At the end of the extraction period, the solvent was discarded and the samples were dried again at 60 °C for 48 h. Their weight was recorded for a second time. The difference between the initial and final dry weight of the samples reflected the lipid fraction extracted by the solvent (mg) and was expressed as a percentage of the initial dry weight.

Life history parameters

In both experiments, the parameters assessed were: pupal development (number of days from puparium formation to emergence), adult emergence (%, calculated over the original puparium number), sex ratio (% of adult females, calculated over the total number of adults obtained), and female longevity (number of days from emergence). The number of F1 eggs laid on host larvae per live female was calculated daily (e). The daily e-values were then summed across 10 days to determine the mean number of eggs laid by each female (E10), as an estimate of fecundity (Dindo et al., 1999, 2006). Additional parameters were: F1 puparium yield (%, calculated over the total number of eggs laid by females within the 10 days) and F1 adult emergence (%, calculated over the total number of F1 puparia). In the second experiment, also pupal development from the

puparia returned to 26 °C (days) and lipid content (%) of the female flies following low-temperature storage were assessed.

Statistical analysis

Data analysis was performed with the statistical program STATISTICA v.10.0 (StatSoft, 2010). Percentages were arcsine $\sqrt{x}$ transformed prior to analysis (Zar, 1984). Data were analyzed with one-way ANOVA after a Levene's test confirmed homoscedasticity of the variances of means. Tukey's honestly significant difference (HSD) or unequal n HSD tests were used to separate the means. In case of variance heterogeneity, non-parametric Kruskal-Wallis tests were applied, and means were compared pairwise with a Mann-Whitney U test. The independence of storage conditions on *E. larvarum* puparia and F1 puparium yield and F1 adult emergence was tested using 2 × 2 contingency tables and calculating χ^2 values. The significance threshold was set at 0.05 for all statistical procedures.

Results

Storage at 15 °C for 28 days

Storage at 15 °C for 28 days significantly delayed the pupal development of *E. larvarum*. Control flies, kept at 26 °C, completed development in about 9 days, whereas flies stored at 15 °C completed emergence more than 2 weeks later, between the 3rd and 4th week of storage (Figure 1). Dissection of the remaining puparia, those that had not produced an adult after 4 weeks storage, indicated they were non-viable.

Adult emergence and sex ratio were not significantly affected by low-temperature storage (Table 1). Conversely, storage of puparia at 15 °C negatively influenced the longevity of the emerged females, resulting in an almost halved lifespan when compared with control flies. Similarly, fecundity (E10) was significantly reduced when puparia were stored at 15 °C, indicating approximately 48% reduction in the number of eggs laid during the first 10 days from the first host exposure (Table 1).

F1 puparium yield (calculated over the total number of F1 eggs laid) was higher for control flies than for flies stored at 15 °C for 28 days (21.6 vs. 9.0%; $\chi^2 = 36.97$, P<0.0001). Conversely, F1 adult emergence was similar for control (85.9%) and storage at 15 °C for 28 days (86.1%) ($\chi^2 = 0.001$, P = 0.98).

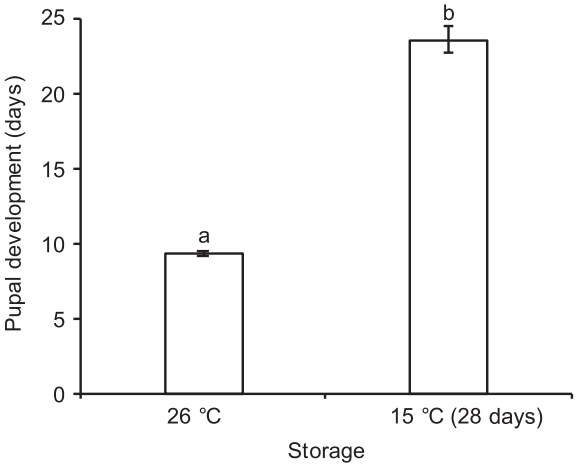

Figure 1 Mean (± SE; n = 4) pupal development duration (days) of *Exorista larvarum* 1-day-old puparia kept at 26 °C (control) or stored at 15 °C for 28 days. Each replicate consisted of 10 puparia. The different letters above the bars indicate a significant difference (Kruskal-Wallis test: H = 5.398, P = 0.020).

Table 1 Mean (± SE; n = 4) adult emergence (%), sex ratio (% females), longevity (days), and E10 (no. eggs laid in 10 days/female) following storage of *Exorista larvarum* puparia at 26 °C (control) or at 15 °C for 28 days. Each replicate consisted of 10 puparia (adult emergence, sex ratio) or a variable number of females as specified (longevity, E10)

Storage		Adult	Sex ratio[1]	Longevity	E10 (F1 eggs/female
Temperature (°C)	Duration (days)	emergence (%)	(% females)	(no. days)[2]	laid in 10 days)[3]
26		75 ± 2.89a	42.86 ± 7.18a	12.09 ± 1.06a	90.46 ± 10.70a
15	28	72.5 ± 7.5a	55.55 ± 3.93a	7.34 ± 1.40b	42.88 ± 12.34b
H (N)		0.0890 (8)	2.1080 (8)		
$F_{1,6}$				7.3203	8.4930
P		0.76	0.15	0.035	0.027

Means within a column followed by different letters are significantly different (Kruskal-Wallis test followed by Mann-Whitney U test, or one-way ANOVA followed by Tukey's HSD or unequal n HSD test: P<0.05).

[1]Calculated over the number of emerged adults.

[2]Number of females per replicate, from 1 to 4, was 2, 3, 5, and 3 (puparia stored at 26 °C), or 5, 4, 4, and 3 (puparia stored at 15 °C).

[3]Number of females per replicate, from 1 to 4, was 2, 3, 5, and 3 (puparia stored at 26 °C), or 3, 3, 3, and 2 (puparia stored at 15 °C).

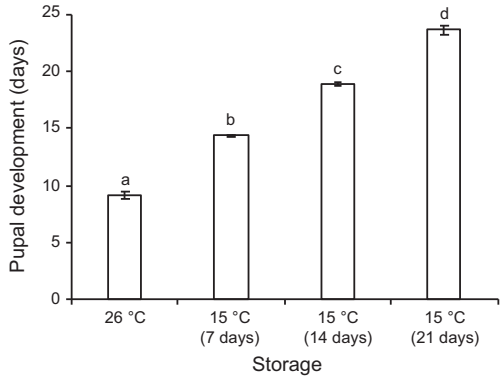

Figure 2 Mean (± SE; n = 4) pupal development duration (days) of *Exorista larvarum* 1-day-old puparia kept at 26 °C (control) or stored at 15 °C for 7, 14, or 21 days. When storage at 15 °C was completed, puparia were returned to 26 °C. Each replicate consisted of five puparia. Means capped with different letters are significantly different (one-way ANOVA: $F_{3,12} = 2190.34$, $P<0.0001$; followed by Tukey's HSD test: $P<0.05$).

Storage at 15 °C for 7, 14, or 21 days

Storage at 15 °C progressively and significantly delayed the pupal development of *E. larvarum* as the storage duration increased – maximum pupal development (about 2 weeks) was observed when puparia were stored at 15 °C for 21 days (Figure 2). Adult emergence did not occur during the storage at low temperature, but only after the puparia were returned to 26 °C. Nonetheless, development progressed during storage: the longer the storage, the shorter the time required for emergence once puparia were returned to the control temperature. Puparia stored at 15 °C for 7 days took 6.35 ± 0.12 days to emerge when returned to 26 °C, whereas those stored for 14 and 21 days took 3.89 ± 0.13 and 1.59 ± 0.43 days, respectively (one-way ANOVA: $F_{2,9} = 319.71$, $P<0.0001$, followed by Tukey's HSD test: $P<0.05$).

Adult emergence and sex ratio were not significantly affected by storage conditions (Table 2). Longevity was 23 days for the females emerged from control puparia and was shortened by storage at 15 °C. However, the reduction of longevity was significant only for the females that had emerged from the puparia stored at 15 °C for 7 days, compared with control flies (Table 2). Relative to the control, the number of eggs laid during the first 10 days from the first host exposure (E10) was significantly reduced when *E. larvarum* puparia were stored at 15 °C for 14 days, but the reduction was not significant when puparia were stored at 15 °C for 7 or 21 days (Table 2).

Female flies that emerged from control puparia laid eggs that resulted in the lowest F1 puparium yield (19%). The highest yield was obtained for females that emerged from puparia stored at 15 °C for 21 days (30%). F1 puparium yield differed significantly between the control vs. storage at 15 °C for 14 or 21 days, and between storage at 15 °C for 7 vs. 21 days (Table 3). F1 adult emergence was not significantly influenced by storage conditions (Table 4). The lipid content of the female flies emerging from stored puparia did not differ among treatments (Figure 3).

Discussion

The potential to store immature insects at low temperatures represents a powerful tool for increasing the

Table 2 Mean (± SE; n = 4) adult emergence (%), sex ratio (% females), longevity (days), and E10 (no. eggs laid in 10 days/female) following storage of *Exorista larvarum* puparia at 26 °C (control) or at 15 °C for 7, 14, or 21 days. When storage at 15 °C was completed, puparia were returned to 26 °C. Each replicate consisted of five puparia (adult emergence, sex ratio) or one adult female (longevity, fecundity)

Storage					
Temperature (°C)	Duration (days)	Adult emergence (%)	Sex ratio[1] (% females)	Longevity (no. days)	E10 (F1 eggs/female laid in 10 days)
26		95 ± 5a	52.5 ± 4.79a	23.0 ± 5.12a	170.75 ± 17.94a
15	7	85 ± 5a	52.5 ± 2.50a	6.75 ± 2.59b	120.50 ± 43.50ab[2]
	14	80 ± 8.17a	50 ± 10.21a	10.5 ± 2.72ab	61.50 ± 19.77b
	21	90 ± 5.77a	45 ± 2.89a	15.0 ± 0.41ab	105.75 ± 21.47ab
F		0.9937	0.3330	4.8310	4.5260
d.f.		3,12	3,12	3,12	2,10
P		0.43	0.80	0.020	0.030

Means within a column followed by different letters are significantly different (one-way ANOVA followed by Tukey's HSD or unequal n HSD test: $P<0.05$).

[1]Calculated over the number of emerged adults.

[2]Mean based on n = 2.

Table 3 Effect of storage conditions on *Exorista larvarum* F1 puparium yield and 2 × 2 contingency table based on pairwise treatment comparisons (χ^2 values below the diagonal, P-values above the diagonal)

	Storage		Puparia produced		No puparia produced	
Treatment	Temperature (°C)	Duration (days)	n	%	n	%
A	26		129	18.9	554	81.1
B	15	7	49	20.3	192	79.7
C		14	67	27.2	179	72.8
D		21	127	30.0	296	70.0

	A	B	C	D
A		0.63	0.0059	<0.0001
B	0.24		0.074	0.0065
C	7.57	3.20		0.44
D	18.21	7.40	0.59	

flexibility of production in mass-rearing facilities and to better adapt it according to the demand (Glenister & Hoffmann, 1998; Leopold, 2000). On the other hand, this technology is often not available, and its development requires research efforts and implementation that has to be tailored to the requirements of the focal insect species. In our experiments, we demonstrated that by storing 1-day-old *E. larvarum* puparia at 15 °C for 21 days, pupal development can be prolonged more than 14 days when compared with puparia kept continuously at 26 °C, with no significant detrimental effects for any of the tested quality parameters. However, when storage was longer (up to 28 days), detrimental effects on the quality of female flies emerging from the puparia stored at 15 °C were observed. Even if puparia stored at 15 °C for 21 days took only 1.58 days on average to emerge once returned to 26 °C, this treatment appeared to be sufficient to mitigate the negative effects induced by the low temperature. Adult emergence was comparable to controls for each of the tested treatments in both experiments, meaning that the suboptimal temperature of 15 °C did not arrest the development during storage, but delayed it without lethal consequences for the parasitoid. As hypothesized, following the findings of Benelli et al. (2017), insects that adapt to low temperature maintain their vital functions (Hance et al., 2007). This observation is also supported by the finding that, when the puparia were stored for 7, 14, or 21 days, the longer the storage period at 15 °C, the shorter the time required for adults to emerge after puparia were restored to the standard temperature of 26 °C. This result is consistent with the findings of

Table 4 Effect of storage conditions on *Exorista larvarum* puparia and F1 adult emergence, and 2 × 2 contingency table based on pairwise treatment comparisons (χ^2 values below the diagonal, P-values above the diagonal)

	Storage		Adult production		No adult production	
Treatment	Temperature (°C)	Duration (days)	n	%	n	%
A	26		122	94.6	7	5.4
B	15	7	46	98.9	3	6.1
C		14	61	91.0	6	9.0
D		21	119	93.7	8	6.3

	A	B	C	D
A		0.86	0.35	0.77
B	0.03		0.57	0.97
C	0.89	0.32		0.50
D	0.09	0.001	0.46	

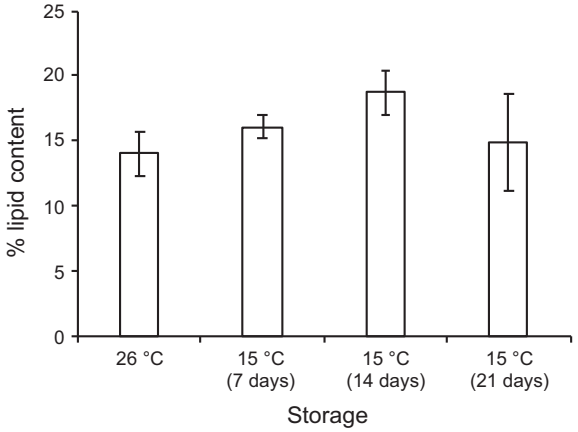

Figure 3 Mean (± SE) lipid content (%) of *Exorista larvarum* females emerged from puparia kept at 26 °C (control) or stored at 15 °C for 7, 14, or 21 days. When storage at 15 °C was completed, puparia were returned to 26 °C. The number of replicates, each consisting of one female, was 6 (26 °C), 5 (7 days 15 °C), and 4 (14 and 21 days 15 °C). Means were not significantly different (one-way ANOVA: $F_{3,15} = 0.9356$, $P = 0.45$).

Mills & Nealis (1992), who investigated cold-storage procedures for the puparia of the tachinid *Aphantorhaphopsis* (= *Ceranthia*) *samarensis* (Villeneuve).

When insect parasitoids are stored at temperatures below their optimum, the extension of their shelf life routinely results in a decrease in quality (van Baaren et al., 2006; Colinet & Boivin, 2011). As a general observation, when parasitoid immatures are exposed to low temperatures, adults that survive live for a shorter period (Rundle et al., 2004; Pandey & Johnson, 2005). In our case, storage at 15 °C only significantly shortened female longevity when the exposure lasted 7 or 28 days, but longevity reduction was less at intermediate storage periods (14 or 21 days). Our observations are in line with those of Foerster & Doetzer (2006). In their study, following a period of exposure to suboptimal temperature (15 or 18 °C), adult Scelionidae displayed a decrease in longevity once returned to the control temperature (25 °C). Fecundity of *E. larvarum* females was also negatively influenced by the low-temperature storage, but only when puparia were exposed to 15 °C for 14 or 28 days was the difference significant compared to control flies. As demonstrated by Levie et al. (2005), fecundity of female parasitoids that overcome storage at low temperature can be dramatically reduced. Exposure of *E. larvarum* puparia to 15 °C for 21 days led to adult fecundity comparable with the control, suggesting a non-linear relationship between the duration of the puparium storage period and fecundity. We cannot explain the non-linearity in responses to low-temperature for varying durations. Non-linearity has also been observed for some hymenopteran parasitoids, e.g., *Pteromalus puparum* L. and *Trichomalopsis sarcophagae* Gahan (McDonald & Kok, 1990; Lysyk, 2004). A more intuitive proportional reduction was observed for other stored parasitoids, such as the scelionid *Telenomus busseolae* Gahan (Bayram et al., 2005).

Beneficial effects following storage at low temperatures are less widely observed than detrimental effects; however, they do occur (Colinet & Boivin, 2011). The highest F1 puparium yields were obtained for those females that were stored at 15 °C for 14 or 21 days, surpassing even those obtained from control females. Similarly, Bernardo et al. (2008) found that adults of the parasitoid *Thripobius javae* (Girault) stored at low temperature for 10 days produced more progeny than unstored ones. We could not find an explanation for such apparently beneficial effects in the literature. In our second experiment, the F1 puparium yield may have been influenced by the number of eggs per host laid by parental control females. Excessive superparasitism is known to harm *E. larvarum* development (Mellini & Campadelli, 1997). Therefore, in our case we suggest a possible effect of our experimental conditions on the F1 puparium yield, rather than a beneficial effect of low-temperature storage. Conversely, when puparia were stored at 15 °C for 28 days, F1 puparium yield dropped from the control level to only 9.0%, stressing the importance of limiting the duration of low-temperature storage.

Energetic reserves may be critical to overcome low-temperature exposure and lipid reserve depletion during storage has been found to be correlated with insect survival and longevity (Colinet et al., 2006). We did not find any decline in *E. larvarum* female lipid content (fraction soluble in chloroform) when puparia were stored at 15 °C, regardless of the storage duration. Further studies investigating the effect of storing temperature will help to verify a possible effect of fat depletion during cold exposure on parasitoid fitness (Renault et al., 2003). Cold storage is also known to negatively influence fitness by inducing various physiological mechanisms including desiccation, dysfunction of the neuromuscular system, and hormonal balance alterations (Sehnal, 1991; Kelty et al., 1996; Ismail et al., 2010). Therefore, for *E. larvarum* the occurrence of one (or more) of these mechanisms, which are not related to lipid consumption, may be hypothesized.

Preliminary experiments excluded the possibility to store 1-day-old *E. larvarum* puparia at 5 and 10 °C. Our colony originated from Emilia Romagna, northern Italy; however, due to the extended distribution range of this parasitoid, different populations are expected to differ in adaptations to their environment, including resistance to low temperatures (Mellini, 1991). Moreover, since there were many

laboratory generations before experimental flies were sourced for our experiments, we cannot exclude a decrease in performance due to inbreeding depression and adaptations to continuous rearing at 26 °C. Our results, suggest that storage at 15 °C is suitable for increasing the flexibility of the parasitoid rearing. As a consequence, this procedure may prove useful for better scheduling field releases in biological control programs, and also for preserving flies during shipment and for small-scale rearing purposes (research laboratories). In terms of duration of attainable storage, all the tested exposures (1–4 weeks) may be adopted if a degree of fitness reduction is tolerable, depending on the purposes of the rearing (Grenier, 2009). In general, however, storage at 15 °C for 4 weeks proved to be more detrimental than a storage period of 3 weeks. Considering that pupal development was delayed to a similar degree (about 2 weeks), we recommend storage to be terminated before adult emergence to minimize detrimental consequences on parasitoid quality.

Further research on low-temperature storage of *E. larvarum* immatures is needed to better understand the underpinning physiological mechanisms taking place during storage. Additional factors to be considered may include pupal age and the possibility to store puparia following a period of acclimation, as a means to increase their cold hardiness (Hoffmann et al., 2003; Luczynski et al., 2007).

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