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Storage at suboptimal temperature of puparia of *Exorista larvarum* (L.), a tachinid larval parasitoid of Lepidoptera

Master thesis

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Statuory declaration

I hereby declare on oath that I have created the present master thesis without any outside help and without the use of any sources and aids other than those specified, and that I have indicated as such passages taken from the sources I used either verbatim or in substance.

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Index

1. Abstract

Exorista larvarum (L.) (Diptera: Tachinidae) is a parasitoid generally known as an antagonist of lepidopterous defoliators of forest and agricultural interest. This beneficial entomophagous insect can be easily reared *in vivo* and *in vitro,* on artificial media. The *in vivo* rearing technique is generally perfomed by adopting a factitious host, such as *Galleria mellonella* (L.) (Lepidoptera: Pyralidae). Storage at low temperatures is a useful technique to extend insects' developmental time and thus elongate their shelf-life. Immobile stages, including puparia, are usually more tolerant to the cold exposure. In the present study, the effects of storage at 15 °C (for four weeks) on *in vivo* produced *E. larvarum* puparia were tested and compared with puparia stored at 26 °C. The results showed some decrease in adult quality parameters, namely longevity and fecundity, however, the storage treatment (15 °C) proved effective for extending the parasitoid development. According to the different purposes of insects' mass-rearing, a certain reduction in quality can be accepted in favour of a more flexible rearing schedule.

Key words: tachinid, storage, parasitoid puparia, suboptimal temperature, *Exorista larvarum*

2. Introduction

2.1. General remarks on the parasitoid *Exorista larvarum* (L.):

Exorista larvarum (L.) is a dipteran polyphagous larval endoparasitoid particularly known as antagonist of Lepidoptera. This gregarious species is a member of the Tachinidae family, which includes about 8,500 parasitoid species described all over the world and is the largest group of non-hymenopteran parasitoids (Michalkova *et al*. 2009; O'Hara 2013). The greater numbers of the hosts of tachinid flies are herbivorous insects. In general, parasitoid activity has two favourable effects at the community level, namely a control of the populations of pest species and, as a consequence, an indirect decrease of their feeding damage on plants. The effectiveness of the parasitoidism, however, depends on different factors, such as the size of the host population, also the size of the parasitoid population and the environmental circumstances (O'Hara 2008).

2.1.1. Origin

This Palaearctic tachinid fly is distributed from Europe, to northern Africa, and certain Asian regions (Cerretti and Tschorsnig 2010). It has also become established in North America during the 20th century, when it was introduced there to control *Lymantria dispar* (L.) (the gypsy moth), which is a lepidopterous defoliator species causing serious damages in the forests, introduced to North America from Europe in the XIX century (Kennis and Lopez Vaamonde 1998).

2.1.2. Hosts

Among the approximately 45 natural host species of *E. larvarum* (Hafez 1953; Herting 1960), many lepidopterans of forest interest have been recorded, such as *L. dispar*, *Malacosoma neustria* (L.), *Tortrix viridana* L., *Hyphantria cunea* (Drury), *Dendrolimus pini* L. (Herting, 1960; Delrio *et al*., 1983; 1988). This tachinid is also an antagonist of some agricultural noctuid pest species such as *Mamestra brassicae* (L.) (Sannino and Espinosa, 1999), *Autographa gamma* (L.) and *Lacanobia oleracea* (L.) (Cerretti and Tschorsing 2010). Moreover, other lepidopterous species, not attacked in nature, may be suitable for the development of *E. larvarum*; one of these is the wax moth *Galleria mellonella* (L.), which has been used as a

laboratory host for this tachinid in the laboratory of Entomology of the University of Bologna since 1993 (Mellini *et al.* 1993).

2.1.3. Biology and life cycle

Exorista larvarum has a developmental life cycle which consists of an egg stage, three larval instars, a pupal stage (protected by a puparium, the hardened shell of the third instar larva) and an adult stage (O'Hara 2008; Mellini 1991) (Figure 1). Hafez (1953) and Michalkova *et al.* (2009) described the biology of *E. larvarum.* Females lay macrotype eggs (the average size of which is ca. 0.6 mm in length) directly on the host cuticle with the help of their substitutional ovipositor (Mellini 1991). Eggs are oval shaped and white in colour. They turn to pale yellow during embryonic development (Hafez 1953).

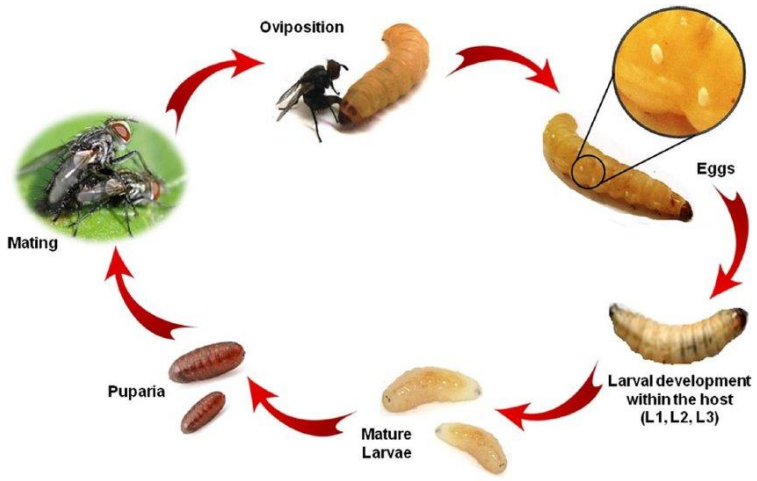

Figure 1. Life cycle of *E. larvarum* Source: Dindo and Nakamura (2018) (drawn By M. Benelli)

The first instar larvae hatch after 2-3 days at 26 °C and have to penetrate the host integument with their strong and well-developed cephalopharyngeal skeleton and special body musculature (Valigurová *et al.* 2014). Soon after penetration, they form a primary integumental respiratory funnel, constructed from the modified host integument and host haemocyte action. This funnel leads to the surface of the host, and allows the parasitoid larva (which presents a pair of spiracles on the last abdominal segment) to stay in contact with atmospheric oxygen from the beginning of its development. The larval penetration is indicated by a visible dark brown or black spot on the host surface, where the cuticle melanisation has occurred (Figure 2). The larvae use their saliva to soften the host skin and penetrate it with their pointed hook-like labrum (Mellini 1991). Larvae continuously develop

within the body cavity of the host and, at the beginning they feed on the haemolymph with the avoidance of damaging any vital organs. Very soon, however, the larvae will attack the host fat body and vital organs. In parallel the host stops moving and feeding and it is killed in 1-2 days after the egg hatching (O'Hara 2008; Valigurová *et al.* 2014; Depalo *et al.* 2010). Larvae develop independently of the host hormonal balance and physiology (Dindo 2011). Second instars larvae stay attached to the respiratory funnel by a second hook, constantly increasing in size and expanding in space within the host body. After moulting to the third instar, the parasitoids can freely move in the body cavity, and thanks to that feature, progressively nourish on host's interior tissue until it is available (Marchetti 2006; Valigurová *et al.* 2014). More than one parasitoid larva can successfully develop in one host (up to 5-6 in the factitious host *G. mellonella*) (Mellini and Campadelli 1997).

Figure 2. Melanisation of the host's cuticle; Private picture

After crawling away from the host by the third instar larvae, pupation occurs and puparium forms as the hardened shell of the last instar (Figure 3). In some cases, this phenomenon can take place within the host (O'Hara 2008) (Figure 4). The size of the puparia can be different according to their number obtained per host. Sex and other factors can influence it too (Hafez 1953).

Figure 3-4. Examples for pupation, out of the host and within the host; Private pictures

Adult emergence usually takes place 8 days after the pupation at 26 °C (Marchetti 2006). Previous studies carried out at the laboratory of Entomology of the University of Bologna demonstrated that the full development of *E. larvarum* (from egg stage to adult) takes 15-16 days at 26-27 °C (Marchetti 2006).

The newly-emerged adult sex can be easily distinguished according to pretarsi, which are firmly forked in case of the males and slightly divided in regard of the females (Hafez 1953) (Figure 5-6). Adults are mating immediately after emergence; the female pre-oviposition period can vary between 2-3 days in laboratory conditions at 26 °C, but in nature it can take 6 days when the temperature is lower, like in autumn (Hafez 1953; Dindo *et al.* 2007). Host location occurs by the identification of chemical signals, like host-induced plant volatiles. Visual cues, however, play a crucial role in host detection: for that reason, the motion signals of the host insects have very important features (Depalo *et al.* 2010; 2012). Females oviposit directly on the host cuticle by extending their ovipositor and attach an egg to the host laterally or to the dorsal surface (Mellini *et al.* 1994). The average of the eggs laid is 7 eggs per day by females during 20-25 days; most of the eggs are laid during the first 10 days following the beginning of oviposition (Hafez 1953; Dindo *et al.* 1999).

Figure 5-6. *E. larvarum* male with his forked pretarsi; and a female adult with slightly divided pretarsi, Private pictures

Like in the majority of tachinids, in nature adults feed on sugar based materials (nectar, honeydew). The longevity of the adults depends on different factors such as the temperature, and the available food source. In general females stay alive approximately 18-22 days under laboratory conditions, although they may live longer (Hafez 1953; Dindo *et al*. 2002, 2004). In the laboratory, the adults usually feed on honey and water solutions, lamp sugar or pollen (Dindo and Grenier 2014).

2.2. Importance of *E. larvarum* as an applied biological control agent

The members of Tachinidae can be applied in classical and augmentative biological control programs. Yet, the potential of these beneficial enemies as antagonist of target insect pests is still underestimated and misunderstood. Difficulties may arise from the scarcity of knowledge about the biology, behaviour and ecology of both the tachinids to be used as biological control agents and the target insect pests. Also, the rearing techniques and shipping methods of tachinids flies are relatively poorly known in comparison with hymenopteran parasitoids (Dindo and Grenier 2014).

Exorista larvarum is a good biocontrol candidate against forest lepidopterous defoliators. One of the most important target pest is *L. dispar* (native in Eurasia), impairing deciduous and evergreen species. This defoliator damages more than 300 species of trees. The pest was introduced from Europe into the USA in the end of the 19th century (O'Hara 2008). To date, however, *E. larvarum* has been used as a biological control agent against *L. dispar* only

in inoculative releases in the northern United States, where it has become established (Sabrosky and Reardon 1976).

The potential of *E. larvarum* as an antagonist of forest and agricultural insect pests deserves to be better exploited. This parasitoid has positive features, among which, it can be efficiently reared *in vivo* and *in vitro* (on artificial media, without the host) too (Dindo and Grenier 2014). As emphasised by Greathead (1986) the success of biological control with entomophagous insects (classical and, especially, augmentative) is largely based on the availability of efficient techniques for producing the parasitoids or predators involved. Due to that, at the University of Bologna, extensive research is focusing on the mass production of this species (on artificial media as well) (Dindo and Grenier 2014; Dindo *et al.* 2016). Their results of laboratory studies are encouraging regarding the possible augmentative releases of *E. larvarum* against noctuid populations of agricultural interest, such as *S. littoralis* (Boisduval) (Depalo *et al.* 2010) or *M. unipuncta* (Haworth) (Depalo *et al.* 2012) and also including *Agrotis segetum* Schiffermüller, *Prodenia litura* F. (Hafez, 1953) and *M. brassicae* (L.) (Sannino and Espinosa 1999).

2.3. General remarks on *Galleria mellonella*, a factitious host of *E. larvarum*:

For laboratory rearing alternative hosts are frequently used, instead of natural hosts, because of the easier rearing requirements. *Galleria mellonella* (L.) (Lepidoptera, Pyralidae) (the greater wax moth) is a factitious host for several parasitoid insects and it is a more convenient host for *E. larvarum* as well (Mellini *et al.* 1993). In consideration of agricultural crop protection and forestry ecosystem management and also for biodiversity conservation, the possibility to mass rear parasitoids using *G. mellonella* as a laboratory host is very important. Therefore, the greater wax moth has a particular role for entomological application (Roversi *et al*. 2007). The greater wax moth larvae are less problematic (they do not show an active defence) compared to the natural hosts; some of them (e.g. *L. dispar*) have dangerous urticating hairs (Bonnot *et al.* 1984). This species is one of the most commonly used insects in the mass production of many biological control agents (not only parasitoids): for instance it has proven to be suitable for the rearing of at least 21 other tachind species and also for the production of entomopathogenic nematodes (Grenier 1986; Hala *et al.* 2012). It has also been used in model studies to inquire into host-pathogen or

host-parasitoid interactions, and into invertebrate immune systems (Roversi *et al.* 2007). It has favourable properties such as the relatively simple nutritional needs, ecological adaptation and developmental characteristics (Miyata *et al.* 2003; Mylonakis *et al.* 2005).

2.3.1. Distribution and damage

In nature, *G. mellonella* is widely distributed from Asia to northern Africa, Great Britain, and some parts of Europe, northern America, and New Zealand; it can be found almost everywhere, where beekeeping is adopted (Kwadha *et al.* 2017). It seriously damages field-based honeybee hives and stored bee wax and other hive products (Metwally *et al.* 2012).

2.3.2. Biology and life cycle

Galleria mellonella undergoes four different life stages of development, such as egg, larva, pupa, and adult, this insect has a typical holometabolic life cycle (Figure 7). The whole development from egg to adult may take weeks or months depending on the available food sources, competition in between the individuals, temperature and other biotic and abiotic factors (Nielsen *et al.* 1979).

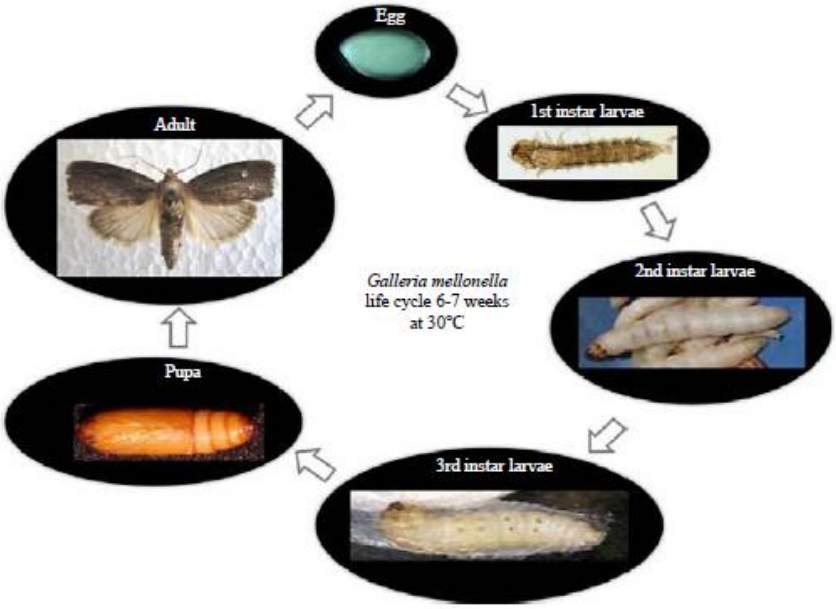

Figure 7. Life cycle of *G. mellonella* Source: Abidalla (2018)

Adult females are capable of laying eggs in a relatively short time after emergence and mating. Female moths oviposit 50-150 eggs in clusters in less detectable places as cracks or crevices in the hive: this oviposition mode raises the survival of larvae. Larval hatching occurs

after some days (3-30 days depending on environmental conditions) after the eggs deposition, the newly-hatched larvae immediately looking for food source (Williams 1997; Shimanuki *et al.* 1981; Charriere *et al.* 1999). Usually, the larvae feed on honey, pollen and even brood, (cannibalism may happen), but, under laboratory conditions, they can be reared also on different artificial diets, containing honey wax and some cereal compounds (Williams 1997). Younger larvae feed more intensively than last instars. They go through 7-10 moulting stages, which takes 28 day to 6 months (usually 7 stages in the lab, at 30 °C); the silk production is specific for all larval stages until pupation, but only the last instars form a cocoon (Nielsen *et al.* 1979). During the pupal stage the insect is immobile and not feeding; this stage takes 1-9 weeks (Williams 1997; Paddock 1918).

2.4. Production of parasitoids: general remarks with special reference to *E. larvarum*

The mass rearing of parasitoids can be traced back to the 1940s. The motivation was to make these biological control agents easily available and, in the end, more preferable as pest control agents in comparison with mechanical, chemical and other methods. For this purpose, an important aim was (and is) to establish more and more efficient and economic rearing techniques. The commercialisation of natural enemies started during the 20th century (van Lenteren and Tommasini 2003).

Nowadays, different mass production techniques are available. A procedure is to rear the natural enemy *in vivo*, on its natural target host species, which, in turn, needs food (either plant or an artificial diet). Otherwise, sometimes this is not possible, because of the high cost of the host rearing (especially labour costs) or undesirable risk of infections or diseases. For that fact alternative hosts, if they are available, can provide a solution, because their rearing (still an *in vivo* rearing technique) is generally easier and more economic (van Lenteren and Tommasini 2003; Dindo and Grenier 2014).

Another possibility is to culture the parasitoids directly on artificial media, without a host (*in vitro* rearing technique). This may be considered as a cheaper solution and also needs less space. It was especially regarded as a very promising solution a few decades ago, and some hymenopterous and tachinid species (including *E. larvarum*) were successfully reared to adult on artificial media (Thompson 1999; Dindo and Grenier 2014). However, this technique can cause quality limitations and problems. Rearing of parasitoids on artificial media may,

therefore, be considered useful as an emergency solution, in case of discontinuity problems (i.e. scarcity or even lack of hosts). The use of artificial diets is more common as a technique of pest (host) species production (van Lenteren and Tommasini 2003; Grenier *et al.* 1994).

2.4.1. *In vivo* and *in vitro* rearing techniques

For *in vivo* production we need to successfully rear the host insects, generally on artificial diets in replacement of their natural food, to make the rearing less expensive and safer from infections and diseases as well. In rearing of parasitoids (many of which are antagonist of Lepidoptera) lepidopterous larvae are used in many cases (King and Leppla 1984; Cohen 2004). It is important to have knowledge about the parasitoid and host biological, physiological and behavioural traits to rear the parasitoid successfully. For *E. larvarum* rearing at the laboratory of Entomology (DISTAL; University of Bologna, Italy) *G. mellonella* is applied as a factitious host. Larvae are less dangerous (without urticating hairs) than the natural hosts and it is also relatively easy to rear them on artificial diet (Bonnot *et al.* 1984). Details on the *in vivo* rearing procedure of *E. larvarum* on *G. mellonella* applied at the Entomology area of DISTAL are given in the Material and Methods section.

Rearing conditions of natural enemies are considered as effective when they are similar to their natural environmental conditions (van Lenteren and Tommasini 2003). However, as stated above, in some cases *in vitro* techniques on artificial media can be also effective and may be helpful to overcome emergency situations, such as host shortage (Dindo and Grenier 2014). *Exorista larvarum* has been successfully reared on different artificial media composed of crude components; with yields of fecund adults approaching those usually obtained *in vivo* (Dindo and Grenier 2014). As an example, Dindo *et al.* (2006) compared of the results obtained on two artificial media composed of crude components with *in vivo* (host-reared) technique on *G. mellonella*. In that experiment, the pupal yield and the puparial weights of *E. larvarum* were even significantly higher on the media then on alternative host. The developmental time of both genders was, however, faster in the host than on media. Field tests showed that the overall quality of the *in vitro* reared parasitoid was lower compared with that of the *in vivo* reared ones (Dindo *et al.* 2002).

2.4.2. Storage procedures

Methods of natural enemies storage assists decision making of delivery time in mass-production programs, since demand for field releases can be unpredictable, also considering variable weather conditions at the time of release (van Lenteren and Tommasini 2003). Leopold (2007) emphasised the importance of entomophagous insect storage at low temperatures in order to extend their shelf-life. According to the duration of the treatment, storage can be classified as short- or long-term, and can be applied to any developmental stage (both immature and adult stages). Cold storage tolerance can be influenced by a variety of factors, including diet (natural prey or host vs. artificial diet) (Coudron 2007). Other factors affecting natural enemies' mass-production and release to be considered are their life cycle diversity, their host or prey selection, and their physiological or developmental stage when released in the field (Leopold 1998). For these reasons, quality control of mass-reared insects is fundamental; the combination of quality traits has high impact on the parasitoids effectiveness under field conditions and must be taken into account also in the view of storage (Bigler 1994).

Cold storage protocols are not always available for mass-reared natural enemies. However, the possibility to store these beneficial insects in order to produce useful stockpiles, meet market requirements and increase the flexibility of the rearing schedule is highly desirable for production companies (van Lenteren and Tommasini 2003). Most natural enemies can be stored only for a short time. Commonly, they are stored as immatures at temperatures between 4 and 15 °C. Even if storage is applied for few weeks, negative impacts on fitness can be detected (van Lenteren and Tommasini 2003). To store beneficial insects in their diapausing stage is also a possibility and has been tested; this method requires really precise regulatory cues and preconditioning; the majority of these kinds of studies are not introduced to the daily practice, because of the insects' high mortality rates (Tauber *et al.* 1993; Leopold 1998).

In the case of *E. larvarum*, a previous study performed at the Department of Agricultural and Food Sciences (DISTAL; University of Bologna, Italy), showed that suboptimal temperature storage at 15 or 20 °C of the parasitoid reared *in vitro* from the egg stage was possible for a short term (Benelli *et al.* 2017). However, some quality parameters (such as adult emergence) of the reared flies were negatively affected by the thermal treatments experienced during the rearing process.

3. Research question

The purpose of the present study is to improve the rearing technique and mass-production of *E. larvarum*, by testing suboptimal temperature storage at 15 °C for a longer period compared to previous studies performed at the Department of Agricultural and Food Sciences (DISTAL; University of Bologna, Italy). Specifically, the present thesis investigates the effects of storage for 4 weeks of *E. larvarum* puparia.

The questions to be answered are the following:

- How many adults per treatment will emerge? What is their sex ratio?
- How long does it take for completing pupal development?
- What is the longevity of the resulting females?
- Are emerged females capable of laying eggs?
- How many F1 puparia will form?
- How many adults will emerge from the F1 puparia?

According to the current knowledge, some hypothesis can be identified for this research:

- Some quality parameters can be negatively affected by the storage at low temperature (Leopold 1998).
- In the first two weeks no emergence will occur at 15 °C (Benelli 2017).

4. Materials and methods

4.1. Rearing of *Exorista larvarum*:

The maintenance of the stock colony of *E. larvarum* at the Department of Agricultural and Food Sciences (DISTAL; University of Bologna, Italy) started in 1992 and was renewed in 2004 with adults emerged from *L. dispar* and *Hyphantria cunea* (Drury) (Lepidoptera: Erebidae) larvae collected in the field near Bologna and Modena (Emilia Romagna, northern Italy) (Depalo *et al.* 2010). *Exorista larvarum* adults are kept in 40 x 30 x 30 cm plexiglas cages under "standard conditions", namely at a temperature (T) set at average 26 ± 1 °C and relative humidity (RH) of (65 ± 5 % RH). The insects are kept in a controlled incubator room with provided photoperiod of 16:8 (L: D) hrs (Dindo *et al.* 1999). During the experiments the colony consisted of two adult cages. The parasitoids were continuously reared *in vivo* by using *G. mellonella* larvae as factitious hosts. The diet of adult flies consisted of sugar cubes, pollen, yeast extract and water provided in drinking cups with water-soaked cotton (Figure 8).

Figure 8. A cage from the standard colony with water cup, food, puparia and adults, Private picture

For the standard colony maintenance, up to 2-5 parasitization procedures were performed weekly, by exposing last instar larvae of *G. mellonella* to flies (however younger larvae can also be suitable for them) (Baronio *et al.* 2002). According to the number of flies 40-70 host larvae were inserted into the adults' cage. Exposure occurred after about 5 days from the beginning of fly emergence: the females were, therefore, mated, since in *E. larvarum* mating

occurs soon after emergence (Mellini *et al*., 1994). Before exposure, the drinking troughs, pollen, yeast and sugar cubes, as well as the remaining puparia were removed from the cage (Figure 9). The alternative hosts were removed after 3-4 parasitoid eggs were visible on their cuticle: this is the optimal number per host, to avoid excessive superparasitism and enhance the survival of the new individuals (Mellini and Campadelli 1997; Baronio *et al.* 2002) (Figure 10). The following step was to collect the host larvae with eggs and to remove the silk from the cage of the fly colony. The larvae with eggs were placed together in a separate plastic box (24 x 13 x 8 cm) without food and left in the same rearing room until puparia formed. The following days after parasitization the silk produced by the host had to be removed to facilitate the emergence of parasitoid third instars from the host remains and their pupation. A new cage was established each week with the collected puparia from the previous parasitization procedures. Sometimes, the older flies were gathered together with younger in order to maintain about 50-70 individuals per cage.

Figure 9-10. Parasitisation for the stock colony, and collected larvae with eggs, Private pictures

4.2. Rearing of *Galleria mellonella*:

Galleria mellonella colony was reared on artificial diet developed by Sehnal (1966) and modified by Campadelli (1987) under controlled conditions in a cabinet set at 30-32 °C ± 1 °C, 65±5 % RH and in complete darkness. A number of 9-10 boxes (24 x 13x8 cm) of larvae of different, continuous ages were maintained. Three times a week the larvae were fed with grinded diet. The requirements of the amount of diet were different according to the developmental stages (younger larvae need more diet). Three boxes of fertile adult moths were kept for egg producing reasons; they laid the eggs on filter papers, which were collected each week. The eggs which were not used were stored at 4 °C in a refrigerator.

Artificial diet preparation was made monthly. The diet contained corn flour, white flour, and wholemeal flour, milk powder, lyophilised brewer's yeast; the ingredients were mixed together in a big pot; solid wax and liquid glycerine were put in two other pots, and two bottles of honey were prepared in advance. All of the ingredients were placed in a special oven set at 100 °C for 3-4 hours. This is a method to disinfest the materials and to melt the solid ingredients. In a special mixing machine all components were mixed until they got mash texture; before the whole dollop became rigid it was mounted to a plastic tray and cut into smaller pieces ("cubes") (Figure 11). When the diet cubes were cold they became solid and some of the cubes were grinded in a shredding machine. The grinded diet was placed for 24 h into a freezer (-24 °C), and the following day it was unfrozen and was used for larval feeding. The remaining diet was stored in a fridge set at 4-5 °C until further use.

Figure 11. Diet preparation, Private pictures

4.3. Experiment description:

The experiments included a preliminary test and a main experiment which consisted of two treatments (including the control treatment). To achieve data which could be compared with those collected in a previous study by Benelli (2017), we tested the survival rate and the capacity to complete the development of *E. larvarum* puparia stored 'ad libitum' (preliminary test) or for 4 weeks (main experiment) at 15 °C. This temperature previously showed to be suboptimal for the short term storage of *E. larvarum* eggs (Benelli *et al*., 2017)

and puparia - the latter up to 3 weeks, tested by Benelli (2017). In the main experiment some quality parameters of the adult *E. larvarum* females that had emerged from puparia stored at 15 °C for 4 weeks were compared with control puparia always maintained under the standard conditions (at 26 °C). These quality parameters could give an indication about the possibility to use the flies emerged from the stored puparia as biological control agents or even for making the colony maintenance more flexible.

4.3.1. Treatments and procedure

Exorista larvarum puparia to be stored at the experimental conditions were obtained from the stock colony available at the laboratory of Entomology and selected within a weight range of 35-55 mg (Dindo et al., 2006). Prior to storage at 15 °C, regardless of the storage period, puparia were kept under the standard rearing temperature of 26 °C for 24 hrs to allow completion of the first crucial developmental period.

A preliminary test was conducted to assess the possibility of 1-day old puparia to complete their development and lead to adult emergence when continuously ('ad libitum') stored at 15 °C. For this purpose, 10 puparia were maintained at this suboptimal temperature and 10 control puparia were kept at 26 °C. Shortly after the third week of storage (21 days), puparia stored at 15 °C began to give origin to adults. Puparia that did not produce adults within the fourth week were kept under observation at 15 °C for additional 2 weeks, but all of them resulted in being non-viable. For this reason, four weeks (28 days) were selected as a maximum storage period to be investigated in the main experiment.

Following the results of the preliminary test, our main experiment was designed to investigate the capacity of *E. larvarum* puparia to survive and complete development when stored at 15 °C for 28 days (= 4 weeks) (1) compared with puparia kept at the standard temperature of 26 °C from their formation (controls) (2). For each of the two tested treatments, four replicates were carried out, each consisting of 10 puparia placed in a plastic cup (6-cm diameter) with no lid. The plastic cups were transferred into small plexiglass cages (20x20x20 cm) and located either inside an incubator set at 15 ± 1 °C, 65 ± 5 % RH (1) or in the same rearing chamber of the laboratory stock colony (set at 26 ± 1 °C, 65 ± 5 % RH) (2). Storage conditions were continuously monitored by data loggers (RHT10, Extech, USA). Puparia were daily inspected for detecting adult emergence. At the end of the storage

period of 28 days, puparia stored at 15 °C which had not emerged as adults were brought back at 26 °C, for safe (i.e. to check a possible parasitoid adult emergence, which never occurred). Newly-formed adults were counted and sexed and female flies were selected for the quality control assessment. In treatment 1) emergence occurred before the storage period completion (within the incubator) and female flies were transferred under standard rearing conditions. Male flies were discarded.

4.3.2. Female quality control

Immediately after emergence, female flies were individually placed in small plexiglass cages of the same type described above and provided a standard diet of water, sugar cubes and a cotton ball soaked in honey/water solution. Subsequently, they were paired with a male fly from the laboratory colony, which had emerged in the same timeframe (48 hrs). When more than one female emerged from the same replicate within 24 hrs, they were placed in the same cage and paired with an equal number of males (Figure 12).

After 3 days from pairing (preoviposition period, see Mellini et al. 1994 and Dindo et al. 2007), the first host larvae were exposed to the females. Specifically, after removing adult food from the cages, 3 mature *G. mellonella* larvae per female were inserted into the cages. After allowing females to oviposit for 1 hr, host larvae were collected and the eggs on their integument counted. Host larvae with eggs were kept in small plastic cups (similar to those used during the storage period, but with a lid) under the standard rearing conditions (26 ± 1 °C) until parasitoid pupation (Figure 13). Newly-formed F1 puparia and F1 adults were counted. The parasitization procedure described above was repeated daily for 10 days (or until female death, if this occurred before the 10th day). In fact, *E. larvarum* females lay most eggs during this period of time (Dindo et al. 1999).

Figure 12-13. Paired experimental females in small cages (left); Plastic cups with parasitized larvae (right), Private pictures

4.3.3. Parameters evaluated

The observed parameters during the experiment were the following:

1. **Adult emergence** (%) calculated on the original puparium number (=10);
2. **Pupal development** (days), calculated separately for males and females, from the day on which puparia were obtained until adult emergence;
3. **Sex ratio** (% of females), calculated on the total number of adults obtained;
4. **Female adult longevity** (days), calculated from emergence until death;
5. **Fecundity** (F1 eggs/female), this parameter was calculated according to Dindo et al. (1999; 2006). In detail, the number of F1 eggs per female laid on host larvae (e = number of F1 eggs/number of alive females) was calculated daily. The single e-values were then added to determine the mean number of F1 eggs laid on the larvae within the 10 days of exposure (= E10). The total F1 eggs laid within 10 days by all the females of each treatment were also calculated;
6. **F1 puparium yield** (%), calculated on the total number of eggs laid by females;
7. **F1 adult emergence** (%); calculated on the number of F1 puparia obtained.

4.3.4. Statistical analysis

Means and standard error were calculated. The statistical analysis was performed with STATISTICA software for Windows (StatSoft. Inc., Version 2010). The level of significance was set at 0.05 (P) for each statistical measure. For analysing the data variability first we used Levene's Test for Homogenity of Variance. If the test justified it, One-Way ANOVA was applied and in case of not homogenous variances a non-parametric analysis we used (Kruskal-Wallis test). Before the analysis percentage values were transformed by using arcsine transformation (with ASN$\sqrt{\%/100}$ formula) (Zar, 1984).

5. Results

In the present study, we aimed at the improvement of the rearing technique of *E. larvarum*, also considering possible applications in mass-rearing. Specifically, we have selected the suboptimal temperature of 15 °C and a longer storage period (4 weeks) for *E. larvarum* puparia compared to those already tested in previous studies (Benelli 2017).

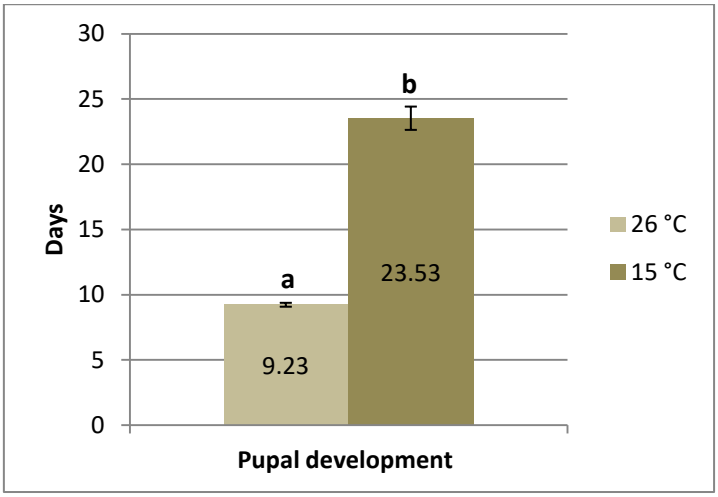

Figure 14. Effect of storage of puparia at 15 °C on the pupal development of *E. larvarum.* The development time was calculated from puparium formation to adult emergence. The mean values are indicated by the columns (± standard error). Different letters above the columns indicate significant results. See text for statistics.

In general, the obtained results showed that pupal development was significantly affected by the low temperature. Control flies (stored at 26 °C) completed pupal development in about 9 days, while flies stored at 15 °C needed up to 23.5 days (Kruskal-Wallis non parametric test; H = 5.398; P = 0.0202; Figure 14). The first adults emerged between the third and the fourth week of storage, and the remaining puparia died during the storage period. After restoring puparia at 26 °C when the storage period was completed, no adults emerged.

Based on the microscopic images, it is visible that some of the not emerged puparia contained underdevelopped individuals or adult dead shortly before emergence (Figure 15).

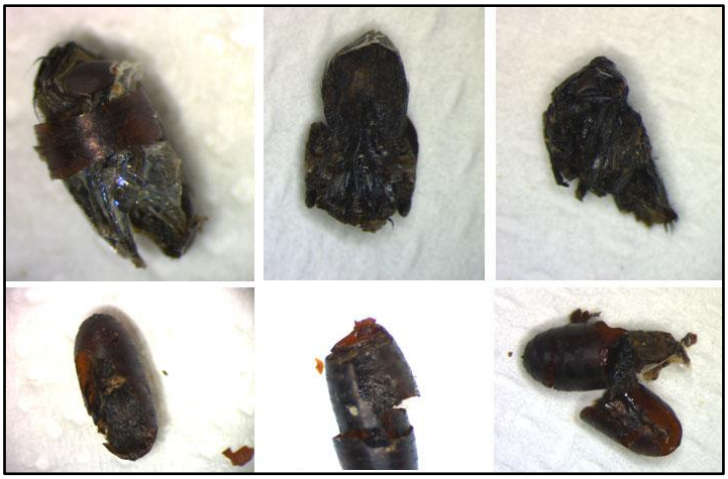

Figure 15. High resolution microscopic images on opened puparia without emergence. Private pictures.

Adult emergence rate and sex ratio were not significantly affected by storage temperature (Table 1). Female development took more days than in case of the males at both temperatures (this was consistent with the findings of previous work, as summaraized by Dindo and Grenier, 2015) . Under standard conditions male development took approximately 9 days and for females it took around 10 days. Otherwise, during the suboptimal storage males reached the adult stage in about 22-23 days, whereas females emerged after about 24-25 days. For both sexes, therefore, the pupal development was about two weeks longer at 15°C than at 26°C. The difference between the development of males and females under 26 °C was about one day, and at 15 °C that was two days.

TABLE 1**.** Effect of storage at suboptimal temperature (15 °C) of *Exorista larvarum* puparia for 4 weeks on adult emergence, pupal development and sex-ratio. Number of replicates = 4. Within a column, means followed by the same letter are not significantly different (P<0.05; Kruskal-Wallis non parametric test).

Treatment	Adult emergence (%)[1]	Pupal development (males) (days)[2]	Pupal development (females) (days)[2]	Sex ratio (= % females)[3]
26 °C (Control)	75±2.887 a	8.84±0.099 a	9.67±0.236 a	42.86±7.180 a
15 °C (4 weeks)	72.5±7.5 a	22.27±0.832 b	24.51±0.968 b	55.55±3.929 a
H (N=8)	0.089	5.398	5.398	2.108
P	0.7645	0.0202*	0.0202*	0.1465

[1] percentages calculated on the puparium number

[2] calculated from puparium formation to adult emergence

[3] percentages calculated on the number of emerged adults

Storage at 15 °C negatively influenced the longevity of the emerged females, which was almost halved when compared with control flies (One-Way ANOVA; F ($df_{1,6}$) =7.3203; P = 0.0353; Figure 16). Similarly, the number of eggs laid by the stored females and F1 puparium yield was significantly reduced, approximately a 50% reduction was found (Table 2). F1 adult emergence did not differ among puparia stored at the low temperature and control puparia kept at 26 °C.

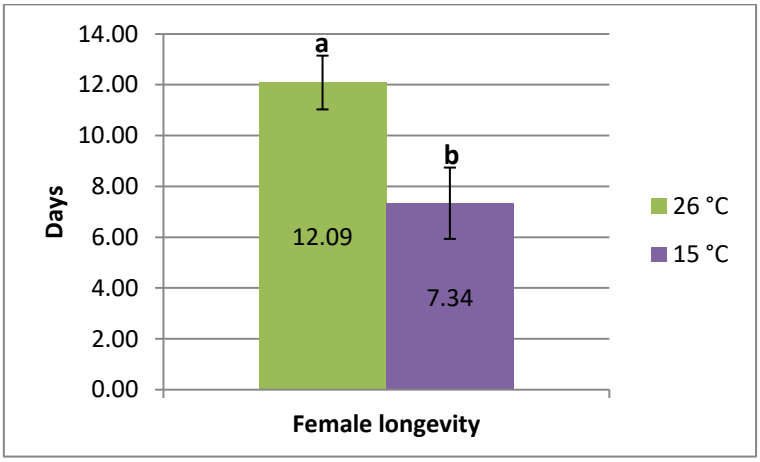

Figure 16. Effect of storage of puparia at 15 °C on female longevity of *E. larvarum.* The mean values are indicated by the columns (± standard error). Different letters above the columns indicate significant result. [Female number per replicate = 2 (1st), 3 (2nd), 5 (3rd), 3 (4th) for the controls and 5 (1st),4(2nd), 4 (3rd), 3 (4th) for 15°C]. See text for statistics.

TABLE 2. Effect of storage at suboptimal temperature (15 °C) of *E. larvarum* puparia for 4 weeks on different quality parameters of the resulting females: female longevity, F1 eggs/female laid in 10 days from the beginning of oviposition, F1 puparium yield and F1 adult emergence. Host larvae were exposed to female flies from the 4th day from emergence. Number of replicates = 4. Within a column, means followed by the same letter are not significantly different (*P*<0.05; One-Way ANOVA).

Treatment	F1 eggs/female in 10 days (E10)[1]	F1 puparium yield (%)	F1 adult emergence
26 °C (Control)	90.46±10.696 a	21.98±2.103 a	84.87±3.462 a
15 °C (4 weeks)	42.88±12.336 b	9.66±1.713 b	92.86±7.143 a
F (df1,6)	8.493	21.1879	2.848
P	0.0268*	0.0037*	0.1425

[1] Female number per replicate = 2 (1st), 3 (2nd), 5 (3rd), 3 (4th) for the controls and 3 (1st),3(2nd), 3 (3rd), 2 (4th) for 15°C

6. Discussion

Effective production of biological control agents requires efficient mass-rearing techniques at competitive costs (van Lenteren 2012). The role of tachinid parasitoids in applied biological control programs has been sometimes underestimated; they receive less attention than hymenopteran parasitoids, on which more research has been carried out (Dindo and Grenier 2014). However, examples of successful classical and augmentative biocontrol programs wich involved fly release can be found in literature (Grenier 1988; Shendage and Sathe 2016). For example, lepidopterous sugarcane borers have been succesfully controlled by *Lixophaga diatraea* in the Southern USA, Central and South America. This tachinid has also potential to be introduced in new Regions producing sugarcane, such China and other Asian Regions (Dindo and Grenier 2014). Another example is our target species, *E. larvarum*, which was successfully introduced in North America against *L. dispar* in the 20th century along with other parasitoid species (Kennis and Lopez Vaamonde 1998). This fly has also shown potential to control other forest defoliators and agricultural lepidopterous pest species, like *S. littoralis* (Boisduval) (Depalo *et al.* 2010) or *M. unipuncta* (Haworth) (Depalo *et al.* 2012) and also other noctuid species, such as *Xestia c-nigrum* (L.), *Peridroma saucia* (Hübner), *Pseudaletia unipuncta* (Haworth) (Simões *et al.* 2004). This beneficial entomophagous parasitoid can be mass-produced both *in vivo*, in the presence of a host, and *in vitro* on artificial media, although, as already emphasised, the latter technique is not competitive with the *in vivo* one, but it may be useful in emergency cases (Benelli *et al.* 2017; Dindo *et al.* 1999).

Storing immature stages of insects at low temperatures provides the possibility to increase the shelf-life by delaying their development and proves also useful as a better tool to adapt at their delivery time according to the demand. In addition, storage increases the overall flexibility of the rearing and, at the same time, can reduce the rearing costs (Glenister and Hoffmann 1998). In his study, Benelli *et al.* (2017) stored *E. larvarum* eggs on artificial media under suboptimal temperatures for short periods. Even though some detrimental effects on quality were observed, suboptimal temperature storage proved effective in delaying the parasitoid development thus making its rearing more flexible. A quality reduction of the produced flies was noticed; however, quality is not an absolute concept.

According to the results obtained in the present study, we demonstrated that storage at 15 °C for 4 weeks can increase the shelf-life of *E. larvarum* puparia., However, the low temperature storage caused a reduction in the quality of the resulting females. As reported by Leopold (1998), these findings are generally observed in studies of this kind. Mills and Nealis (1992) also noticed similarities concerning their cold storage research on puparia of the tachinid *Aphantorhaphopsis* (= *Ceranthia*) *samarensis* (Villeneuve), which could be stored approximately at 8 °C until 8 months.

In conclusion, some quality reductions can be acceptable in favor of other requirements, such as the optimal timing schedule by elongation of the development.

Cold storage is a promising technology for insects' mass-rearing, since it can assist the releases of biocontrol agents both in greenhouses and open field. As previously underlined, its benefits are numerous and include a more flexible schedule for releases and the facilitation of shipment procedures. In the view of better implementing the protocols already developed for *E. larvarum*, further research is needed to understand the mechanisms of cold storage tolerance of this parasitoid species. Furthermore, future studies will aim at reducing the impact that storage at suboptimal temperatures has shown on the quality of the stored parasitoid (for instance by modifying the parasitoid or host food).

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8. Appendix

8.1 List of Figures:

8.2. Table index:

8.3. List of abbreviations

no. or nr. – number

pup. – puparia

G. larvae – *Galleria* larvae (*Galleria mellonella*)

EM – Emergence

Avr – Average

8.4. Original data tables

I.	Parasitisation (date)	Puparia obtained on (date)	Storage start (date)	Puparia (no.)	Weight range (mg)	Storage finished on (date)	Days of storage	Emergence start (date)	Male (no.)	Female (no.)	Total (no.)
26°C 1.	16.okt	23.okt	24.okt	10	35-55	–	–	01.nov	5	2	7
15°C-1.	16.okt	23.okt	24.okt	10	35-55	21.nov	28	15.nov	4	5	9
15°C-2.	16.okt	23.okt	24.okt	10	35-55	21.nov	28	15.nov	4	4	8
26°C 2.	13.nov	22.nov	23.nov	10	35-55	–	–	30.nov	5	3	8

	Emergence of 26°C -1.			
Pup. obtained on	Puparium	Sex	Date	pup.stage
23.okt	1	Male	01.nov	9
23.okt	2	Male	01.nov	9
23.okt	3	Male	01.nov	9
23.okt	4	Male	01.nov	9
23.okt	5	Male	01.nov	9
23.okt	6	Female	01.nov	9
23.okt	7	Female	01.nov	9
	8	–	–	
	9	–	–	
	10	–	–	
		Avr.:		9.00

	Emergence of 15°C-1.			
Pup. obtained on	Puparium	Sex	Date	pup.stage
23.okt	1	Male	15.nov	23
23.okt	2	Male	15.nov	23
23.okt	3	Male	15.nov	23
23.okt	4	Male	16.nov	24
23.okt	5	Female	17.nov	25
23.okt	6	Female	17.nov	25
23.okt	7	Female	18.nov	26
23.okt	8	Female	18.nov	26
23.okt	9	Female	19.nov	27
	10	–	–	
		Avr.:		24.67

	Emergence of 26°C -2.			
Pup. obtained on	Puparium	Sex	Date	pup.stage
22.nov	1	Male	30.nov	8
22.nov	2	Male	30.nov	8
22.nov	3	Male	01.dec	9
22.nov	4	Male	01.dec	9
22.nov	5	Male	01.dec	9
22.nov	6	Female	01.dec	9
22.nov	7	Female	02.dec	10
22.nov	8	Female	02.dec	10
	9	–	–	
	10	–	–	
			Avr.:	9.00

	Emergence of 15°C-2.			
Pup. obtained on	Puparium	Sex	Date	pup.stage
23.okt	1	Male	15.nov	23
23.okt	2	Male	15.nov	23
23.okt	3	Male	16.nov	24
23.okt	4	Female	17.nov	25
23.okt	5	Male	18.nov	26
23.okt	6	Female	18.nov	26
23.okt	7	Female	19.nov	27
23.okt	8	Female	19.nov	27
	9	–	–	
	10	–	–	
			Avr.:	25.13

26 ˚C-1.

CAGE/EM	CAGE DATE	NO. FEMALES	NO. MALES	NO. PAR.	DATE	G. LARVAE	EGGS LAID	TOTAL EGGS LAID	PUP. OBTAINED	ADULTS OBTAINED
1	01.nov	2	2	1	04.nov	1 2 3 4 5 6	7 9 3 1 3 6	29	5	4
1	01.nov	2	2	2	06.nov	1 2 3 4 5 6	6 1 4 5 14 6	36	10	9
1	01.nov	2	2	3	07.nov	1 2 3 4 5 6	3 0 8 5 1 4	21	5	5
1	01.nov	2	2	4	08.nov	1 2 3 4 5 6	5 4 4 4 3 2	22	7	7
1	01.nov	2	2	5	09.nov	1	8	26	4	3

						2	3			
						3	9			
						4	1			
						5	3			
						6	2			
1	01.nov	2	2	6	10.nov	1	4	25	5	4
						2	5			
						3	2			
						4	0			
						5	11			
						6	3			
1	01.nov	2	2	7	11.nov	1	5	25	5	5
						2	1			
						3	5			
						4	1			
						5	8			
						6	5			
1	01.nov	1	2	8	13.nov	1	6	14	4	4
						2	4			
						3	4			
1	01.nov	1	2	9	14.nov	1	4	12	4	4
						2	8			
						3	0			
1	01.nov	1	2	10	15.nov	1	1	9	3	3
						2	1			
						3	7			
							SUM:	219	52	48

Life				
Female	Emergence	Death	Total	
1	01.nov	13.nov	12	
2	01.nov	18.nov	17	
				12.88235294
				90.17647059
				128.8235294
				109.5

15 °C-1.

CAGE/EM	CAGE DATE	NO. FEMALES	NO. MALES	NO. PAR.	DATE	G. LARVAE	EGGS LAID	TOTAL EGGS LAID	PUP. OBTAINED	ADULTS OBTAINED
1	17.nov	1	1	10	20.nov	1	0	3	0	0
						2	1			
						3	2			

3

Life			
Female	Emergence	Death	Total
1	17.nov	21.nov	4

3

14.85714286

37.14285714

AVERAGE 18.33333333

CAGE/EM	CAGE DATE	NO. FEMALES	NO. MALES	NO. PAR.	DATE	G. LARVAE	EGGS LAID	TOTAL EGGS LAID	PUP. OBTAINED	ADULTS OBTAINED
2	18.nov	2	2	1	21.nov	1	0	7	1	1
						2	0			
						3	1			
						4	4			

						5	0		
						6	2		
2	18.nov	2	2	2	22.nov	1	5	10	2	2
						2	0			
						3	0			
						4	3			
						5	1			
						6	1			
2	18.nov	1	1	3	23.nov	1	2	13	4	4
						2	1			
						3	10			
2	18.nov	1	1	4	24.nov	1	3	8	0	0
						2	4			
						3	1			
2	18.nov	1	1	5	25.nov	1	0	14	1	1
						2	7			
						3	7			
						SUM:		52	8	8

Life			
Female	Emergence	Death	Total
1	18.nov	23.nov	5
2	18.nov	27.nov	9

7.42857143 average number of eggs laid by each female counting all the parasitisation that it has done

14.8571429 average number of eggs laid by female 1

37.1428571 average number of eggs laid by female 2

15°C-2.

CAGE/EM	CAGE DATE	NO. FEMALES	NO. MALES	NO. PAR.	DATE	G. LARVAE	EGGS LAID	TOTAL EGGS LAID	PUP. OBTAINED	ADULTS OBTAINED
1	18.nov	1	1	1	21.nov	1 2 3	4 4 2	10	2	2
1	18.nov	1	1	2	22.nov	1 2 3	4 3 4	11	3	3
1	18.nov	1	1	3	23.nov	1 2 3	5 1 2	8	1	0
1	18.nov	1	1	4	24.nov	1 2 3	5 5 1	11	0	0
1	18.nov	1	1	5	25.nov	1 2 3	5 0 4	9	0	0
1	18.nov	1	1	6	27.nov	1 2 3	5 0 2	7	1	1
1	18.nov	1	1	7	28.nov	1 2 3	1 0 6	7	0	0
1	18.nov	1	1	8	29.nov	1 2 3	4 0 7	11	1	0
1	18.nov	1	1	9	30.nov	1	5	14	1	1

						2	4			
						3	5			
						1	0			
1	18.nov	1	1	10	01.dec	2	1	2	0	0
						3	1			
						Sum:		90	9	7

Life			
Female	Emergence	Death	Total
1	18.nov	04.dec	16

	90
	40.28571429
	100.7142857
Average	77

CAGE/EM	CAGE DATE	NO. FEMALES	NO. MALES	NO. PAR.	DATE	G. LARVAE	EGGS LAID	TOTAL EGGS LAID	PUP. OBTAINED	ADULTS OBTAINED
						1	2			
						2	1			
						3	1			
2	19.nov	2	2	1	22.nov	4	1	6	0	0
						5	0			
						6	1			
						1	3			
						2	3			
2	19.nov	2	2	2	23.nov	3	3	20	3	3
						4	1			
						5	6			

						6	4			
2	19.nov	2	2	3	24.nov	1 2 3 4 5 6	6 1 7 1 6 9	30	3	2
2	19.nov	2	2	4	25.nov	1 2 3 4 5 6	4 7 4 6 7 5	33	6	3
2	19.nov	1	2	5	27.nov	1 2 3	0 7 0	7	0	0
2	19.nov	1	2	6	28.nov	1 2 3	1 5 7	13	0	0
2	19.nov	1	2	7	29.nov	1 2 3	3 2 4	9	0	0
2	19.nov	1	2	8	30.nov	1 2 3	1 3 4	8	0	0
2	19.nov	1	2	9	01.dec	1 2 3	3 0 3	6	0	0

						1	3			
2	19.nov	1	2	10	02.dec	2	6	9	0	0
						3	0			

Sum: 141 12 8

Life			
Female	Emergence	Death	Total
1	19.nov	27.nov	8
2	19.nov	07.dec	18

10.07142857
40.28571429
100.7142857

26 °C-2.

CAGE/EM	CAGE DATE	NO. FEMALES	NO. MALES	NO. PAR.	DATE	G. LARVAE	EGGS LAID	TOTAL EGGS LAID	PUP. OBTAINED	ADULTS OBTAINED
						1	12			
1	01.dec	1	1	1	04.dec	2	4	19	6	6
						3	3			
						1	10			
1	01.dec	1	1	2	05.dec	2	6	16	3	3
						3	0			
						1	0			
1	01.dec	1	1	3	06.dec	2	8	10	2	2
						3	2			

SUM: 45 11 11

Life			
Female	Emergence	Death	Total
1	01.dec	07.dec	6

45
107
107

Avr. 86.333333

CAGE/EM	CAGE DATE	NO. FEMALES	NO. MALES	NO. PAR.	DATE	G. LARVAE	EGGS LAID	TOTAL EGGS LAID	PUP. OBTAINED	ADULTS OBTAINED
2	02.dec	2	2	1	05.dec	1 2 3 4 5 6	3 7 5 6 3 5	29	9	8
2	02.dec	2	2	2	06.dec	1 2 3 4 5 6	1 4 4 7 7 8	31	11	8
2	02.dec	2	2	3	07.dec	1 2 3 4 5 6	6 3 4 15 0 1	29	6	2
2	02.dec	2	2	4	09.dec	1 2 3 4	4 6 15 9	48	10	8

						5	10		
						6	4		
2	02.dec	2	2	5	11.dec	1	2	35	9	9
						2	16			
						3	5			
						4	5			
						5	4			
						6	3			
2	02.dec	2	2	6	12.dec	1	3	27	7	6
						2	10			
						3	2			
						4	3			
						5	2			
						6	7			
2	02.dec	2	2	7	13.dec	1	3	15	7	7
						2	3			
						3	2			
						4	0			
						5	4			
						6	3			
							SUM	214	59	48

Life			
Female	Emergence	Death	Total
1	02.dec	14.dec	12
2	02.dec	14.dec	12

II.	Parasitisation (date)	Puparia obtained on (date)	Storage start (date)	Puparia (no.)	Weight range (mg)	Storage finished on (date)	Days of storage	Emergence start (date)	Male (no.)	Female (no.)	Total (no.)
15°C 3.	09.dec	18.dec	19.dec	10	35-55	16.jan	28	08.jan	2	4	6
15°C 4.	09.dec	18.dec	19.dec	10	35-55	16.jan	28	05.jan	3	3	6
26°C 3.	03.jan	11.jan	12.jan	10	35-55	–	–	20.jan	3	5	8
26°C 4.	03.jan	11.jan	12.jan	10	35-55	–	–	19.jan	4	3	7

	Emergence of 15°C -3.			
Pup. obtained on	Puparium	Sex	Date	pup.stage
18.dec	1	Male	08.jan	21
18.dec	2	Male	09.jan	22
18.dec	3	Female	10.jan	23
18.dec	4	Female	10.jan	23
18.dec	5	Female	11.jan	24
18.dec	6	Female	13.jan	26
	7	–	–	
	8	–	–	
	9	–	–	
	10	–	–	

	Emergence of 15°C -4.			
Pup. obtained on	Puparium	Sex	Date	pup.stage
18.dec	1	Male	05.jan	18
18.dec	2	Female	07.jan	20
18.dec	3	Male	08.jan	21
18.dec	4	Male	09.jan	22
18.dec	5	Female	10.jan	23
18.dec	6	Female	10.jan	23
	7	–	–	
	8	–	–	
	9	–	–	
	10	–	–	

Avr.: 23.17

Avr.: 21.17

Emergence of 26°C -3.				
Pup. obtained on	Puparium	Sex	Date	pup.stage
11.jan	1	Male	20.jan	9
11.jan	2	Male	20.jan	9
11.jan	3	Male	20.jan	9
11.jan	4	Female	21.jan	10
11.jan	5	Female	21.jan	10
11.jan	6	Female	21.jan	10
11.jan	7	Female	21.jan	10
11.jan	8	Female	21.jan	10
	9	–	–	
	10	–	–	

Emergence of 26°C -4.				
Pup. obtained on	Puparium	Sex	Date	pup.stage
11.jan	1	Male	19.jan	8
11.jan	2	Male	20.jan	9
11.jan	3	Male	20.jan	9
11.jan	4	Male	20.jan	9
11.jan	5	Female	21.jan	10
11.jan	6	Female	21.jan	10
11.jan	7	Female	21.jan	10
	8	–	–	
	9	–	–	
	10	–	–	

Avr.: 9.63

Avr.: 9.29

15 °-3.

CAGE/EM	CAGE DATE	NO. FEMALES	NO. MALES	NO. PAR.	DATE	G. LARVAE	EGGS LAID	TOTAL EGGS LAID	PUP. OBTAINED	ADULTS OBTAINED
1	10.jan	1	1	1	13.jan	1	0	0	0	0
						2	0			
						3	0			
1	10.jan	1	0	1	15.jan	1	0	0	0	0
						2	0			
						3	0			
						Sum		0	0	0

Life			
Female	Emergence	Death	Total
1	10.jan	16.jan	6

	0
	64
	58
Avr	40.66666667

CAGE/EM	CAGE DATE	NO. FEMALES	NO. MALES	NO. PAR.	DATE	G. LARVAE	EGGS LAID	TOTAL EGGS LAID	PUP. OBTAINED	ADULTS OBTAINED
2	11.jan	1	1	1	15.jan	1	5	15	0	0
						2	3			
						3	7			
2	11.jan	1	1	2	16.jan	1	2	14	0	0
						2	4			
						3	8			
2	11.jan	1	1	3	17.jan	1	1	11	0	0

						2	3			
						3	7			
						1	4			
2	11.jan	1	1	4	18.jan	2	5	18	0	0
						3	9			
							Sum	58	0	0

Life			
Female	Emergence	Death	Total
1	11.jan	19.jan	8

CAGE/EM	CAGE DATE	NO. FEMALES	NO. MALES	NO. PAR.	DATE	G. LARVAE	EGGS LAID	TOTAL EGGS LAID	PUP. OBTAINED	ADULTS OBTAINED
						1	2			
3	13.jan	1	1	1	16.jan	2	2	11	0	0
						3	7			
						1	0			
3	13.jan	1	1	2	17.jan	2	3	11	2	2
						3	8			
						1	4			
3	13.jan	1	1	3	18.jan	2	6	14	3	3
						3	4			
						1	8			
3	13.jan	1	1	4	19.jan	2	4	14	3	3
						3	2			

3	13.jan	1	1	5	20.jan	1 2 3	1 8 4	13	0	0
3	13.jan	1	1	6	22.jan	1 2 3	1 0 0	1	0	0
						Sum		64	8	8

Life			
Female	Emergence	Death	Total
1	13.jan	23.jan	10

64

15 °C-4.

CAGE/EM	CAGE DATE	NO. FEMALES	NO. MALES	NO. PAR.	DATE	G. LARVAE	EGGS LAID	TOTAL EGGS LAID	PUP. OBTAINED	ADULTS OBTAINED
1	07.jan	1	1	1	10.jan	1 2 3	0 0 0	0	0	0
1	07.jan	1	1	2	11.jan	1 2 3	5 1 3	9	0	0
1	07.jan	1	1	3	12.jan	1 2 3	0 4 3	7	0	0
1	07.jan	1	1	4	13.jan	1 2	4 6	14	2	2

						3	4			
1	07.jan	1	1	5	15.jan	1 2 3	10 4 1	15	0	0
1	07.jan	1	1	6	16.jan	1 2 3	1 5 3	9	2	2
1	07.jan	1	1	7	17.jan	1 2 3	2 2 7	11	2	2
1	07.jan	1	1	8	18.jan	1 2 3	0 0 5	5	0	0
1	07.jan	1	1	9	19.jan	1 2 3	0 0 1	1	0	0
							Sum	71	6	6

	71
	0
Avr	35.5

Life			
Female	Emergence	Death	Total
1	07.jan	20.jan	13

CAGE/EM	CAGE DATE	NO. FEMALES	NO. MALES	NO. PAR.	DATE	G. LARVAE	EGGS LAID	TOTAL EGGS LAID	PUP. OBTAINED	ADULTS OBTAINED
						1	0			
2	07.jan	1	1	1	10.jan	2	0	0	0	0
						3	0			
							Sum	0	0	0

Life			
Female	Emergence	Death	Total
1	10.jan	15.jan	5

26 °C-3.

CAGE/EM	CAGE DATE	NO. FEMALES	NO. MALES	NO. PAR.	DATE	G. LARVAE	EGGS LAID	TOTAL EGGS LAID	PUP. OBTAINED	ADULTS OBTAINED
						1	7			
						2	5			
						3	0			
						4	7			
						5	4			
1	21.jan	5	1	1	24.jan	6	0	69	16	15
						7	5			
						8	4			
						9	5			
						10	5			
						11	4			

						12	5			
						13	8			
						14	6			
						15	4			
						1	2			
						2	3			
						3	4			
						4	3			
						5	1			
						6	3			
						7	6			
1	21.jan	5	1	2	25.jan	8	2	46	19	15
						9	3			
						10	2			
						11	3			
						12	1			
						13	5			
						14	3			
						15	5			
						1	1			
						2	8			
						3	2			
						4	9			
1	21.jan	5	1	3	26.jan	5	3	101	21	19
						6	6			
						7	13			
						8	7			
						9	9			
						10	11			

						11	2			
						12	9			
						13	7			
						14	6			
						15	8			
1	21.jan	4	1	4	29.jan	1	5	103	19	17
						2	10			
						3	5			
						4	7			
						5	8			
						6	6			
						7	10			
						8	8			
						9	7			
						10	6			
						11	11			
						12	20			
1	21.jan	4	1	5	30.jan	1	2	60	13	9
						2	1			
						3	5			
						4	7			
						5	3			
						6	11			
						7	6			
						8	8			
						9	6			
						10	6			
						11	1			
						12	4			

1	21.jan	3	1	6	31.jan	1 2 3 4 5 6 7 8 9	6 3 2 0 2 4 5 4 4	30	6	6
1	21.jan	3	1	7	01.febr	1 2 3 4 5 6 7 8 9	4 7 2 1 4 11 5 8 5	47	5	5
1	21.jan	2	0	8	02.febr	1 2 3 4 5 6	3 2 5 10 4 4	28	2	2
1	21.jan	2	0	9	03.febr	1 2 3 4 5	0 4 1 3 5	17	0	0

						6	4			
1	21.jan	2	0	10	05.febr	1 2 3 4 5 6	5 0 4 2 3 5	19	0	0
		35					Sum:	520	101	88

Life			
Female	Emergence	Death	Total
1	21.jan	28.jan	7
2	21.jan	31.jan	10
3	21.jan	02.febr	12
4	21.jan	08.febr	18
5	21.jan	09.febr	19

14.85714286

148.5714286
148.5714286
44.57142857
74.28571429
104

Avr 104

26 °C-4.

CAGE/EM	CAGE DATE	NO. FEMALES	NO. MALES	NO. PAR.	DATE	G. LARVAE	EGGS LAID	TOTAL EGGS LAID	PUP. OBTAINED	ADULTS OBTAINED
1	21.jan	3	3	1	24.jan	1	8	56	10	9
						2	10			
						3	9			
						4	8			
						5	5			
						6	7			
						7	2			
						8	2			
						9	5			
1	21.jan	3	3	2	25.jan	1	2	25	7	5
						2	2			
						3	4			
						4	4			
						5	8			
						6	2			
						7	1			
						8	0			
						9	2			
1	21.jan	3	3	3	26.jan	1	8	38	11	8
						2	5			
						3	6			
						4	3			
						5	2			
						6	1			
						7	6			

						8 9	4 3		
1	21.jan	2	2	4	29.jan	1 2 3 4 5 6	7 3 6 4 4 3	27	5	3
1	21.jan	1	0	5	30.jan	1 2 3	0 2 3	5	0	0
1	21.jan	1	0	6	31.jan	1 2 3	3 1 1	5	0	0
1	21.jan	1	0	7	01.febr	1 2 3	2 1 0	3	0	0
1	21.jan	1	0	8	02.febr	1 2 3	2 4 3	9	0	0
1	21.jan	1	0	9	03.febr	1 2 3	3 1 6	10	0	0
1	21.jan	1	0	10	05.febr	1 2 3	1 3 4	8	0	0
		17					Sum:	186	33	25

10.94117647

32.82352941
43.76470588
109.4117647

Life			
Female	Emergence	Death	Total
1	21.jan	28.jan	7
2	21.jan	30.jan	9
3	21.jan	06.febr	16

Avr. 62

Longevtity of females

Replication 1-2.	Emerence date	Mortality date	Total	
Treatmet 26°C/1	01.nov	13.nov	12	14.5
Treatmet 26°C/1	01.nov	18.nov	17	
Treatmet 15°C 4W	17.nov	20.nov	3	
Treatmet 15°C 4W	17.nov	21.nov	4	
Treatmet 15°C 4W	18.nov	23.nov	5	4.6
Treatmet 15°C 4W	18.nov	27.nov	9	
Treatmet 15°C 4W	19.nov	21.nov	2	
Treatmet 15°C ∞	17.nov	20.nov	3	
Treatmet 15°C ∞	18.nov	04.dec	16	11.25
Treatmet 15°C ∞	19.nov	27.nov	8	
Treatmet 15°C ∞	19.nov	07.dec	18	
Treatmet 26°C/2	01.dec	07.dec	6	
Treatmet 26°C/2	02.dec	14.dec	12	10
Treatmet 26°C/2	02.dec	14.dec	12	

Replication 3-4.	Emerence date	Mortality date	Total	
Treatmet 26°C /3.	21.jan	28.jan	7	
Treatmet 26°C /3.	21.jan	31.jan	10	
Treatmet 26°C /3.	21.jan	02.febr	12	13.2
Treatmet 26°C /3.	21.jan	08.febr	18	
Treatmet 26°C /3.	21.jan	09.febr	19	
Treatmet 26°C/4.	21.jan	28.jan	7	
Treatmet 26°C/4.	21.jan	30.jan	9	10.66667
Treatmet 26°C/4.	21.jan	06.febr	16	
Treatmet 15°C -3.	10.jan	12.jan	2	
Treatmet 15°C -3.	10.jan	16.jan	6	6.5
Treatmet 15°C -3.	11.jan	19.jan	8	
Treatmet 15°C -3.	13.jan	23.jan	10	
Treatmet 15°C -4.	07.jan	20.jan	13	
Treatmet 15°C -4.	10.jan	13.jan	3	7
Treatmet 15°C -4.	10.jan	15.jan	5	

Data for analysis

Treatment	Replicate	Pupal development (days)	n
26^{o}C 1.	1	9	7
26^{o}C 2.	2	9	8
26^{o}C 3.	3	9.63	8
26^{o}C 4.	4	9.29	7
15-4W 1.	1	24.67	9
15-4W 2.	2	25.13	8
15-4W 3.	3	23.17	6
15-4W 4.	4	21.17	6

Treatment	Replicate	Pupal development males (days)	n
26^{o}C 1.	1	9	5
26^{o}C 2.	2	8.6	5
26^{o}C 3.	3	9	3
26^{o}C 4.	4	8.75	4
15-4W 1.	1	23.25	4
15-4W 2.	2	24	4
15-4W 3.	3	21.5	2
15-4W 4.	4	20.33	3

Treatment	Replicate	Pupal development females (days)	n
26^{o}C 1.	1	9	2
26^{o}C 2.	2	9.67	3
26^{o}C 3.	3	10	5
26^{o}C 4.	4	10	3
15-4W 1.	1	25.8	5
15-4W 2.	2	26.25	4
15-4W 3.	3	24	4
15-4W 4.	4	22	3

Treatment	Replicate	Emergence rate (%) TRUE	Emergence rate (%) TRANSF
26°C 1.	1	70	56,7890892391009
26°C 2.	2	80	63,434948822922
26°C 3.	3	80	63,434948822922
26°C 4.	4	70	56,7890892391009
15-4W 1.	1	90	71,565051177078
15-4W 2.	2	80	63,434948822922
15-4W 3.	3	60	50,7684795164077
15-4W 4.	4	60	50,7684795164077

Treatment	Replicate	% of females TRUE	% of females TRANSF
26°C 1.	1	28.57	32,3106273048113
26°C 2.	2	37.5	37,761243907035
26°C 3.	3	62.5	52,238756092965
26°C 4.	4	42.86	40,8950486289265
15-4W 1.	1	55.55	48,1864821927874
15-4W 2.	2	50	45
15-4W 3.	3	66.67	54.73763605
15-4W 4.	4	50	45

% of female = sex ratio
calculated on the number of obtained adults (NOT on the original number of puparia = 10)

Treatment	Replicate	Fecundity (no. of eggs / female)	n
26°C 1.	1	109.5	2
26°C 2.	2	86.33333333	3
26°C 3.	3	104	5
26°C 4.	4	62	3
15-4W 1.	1	18.33333333	3
15-4W 2.	2	77	3
15-4W 3.	3	40.66666667	3
15-4W 4.	4	35.5	2

Treatment	Replicate	Longevity of females (days)	n
26°C 1.	1	14.5	2
26°C 2.	2	10	3
26°C 3.	3	13.2	5
26°C 4.	4	10.66667	3
15-4W 1.	1	4.6	5
15-4W 2.	2	11.25	4
15-4W 3.	3	6.5	4
15-4W 4.	4	7	3

Treatment	Total no. eggs laid	Total no. puparia obtained	Total no.adults obtained
26°C 1.	219	52	48
26°C 2.	259	70	59
26°C 3.	520	101	88
26°C 4.	186	33	25
15-4W 1.	55	8	8
15-4W 2.	231	21	15
15-4W 3.	122	8	8
15-4W 4.	71	6	6

Treatment	% puparia on eggs TRUE	% puparia on eggs TRANSF
26°C 1.	23.74	0.508923104
26°C 2.	27.03	0.546704902
26°C 3.	19.42	0.456396382
26°C 4.	17.74	0.434780981
15-4W 1.	14.54	0.391216753
15-4W 2.	9.09	0.306277369
15-4W 3.	6.56	0.258958337
15-4W 4.	8.45	0.294959344

Treatment	% adults on puparia TRUE	% adults on puparia TRANSF
26°C 1.	92.31	1.289761425
26°C 2.	84.29	1.163190454
26°C 3.	87.13	1.203851035
26°C 4.	75.76	1.055990372
15-4W 1.	100	1.570796327
15-4W 2.	71.43	1.006853685
15-4W 3.	100	1.570796327
15-4W 4.	100	1.570796327