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TITOLO TESI

Effect of short-term suboptimal temperature storage
to assist large-scale production of two dipterans:
Exorista larvarum (L.) and *Bactrocera tryoni* (Froggatt)

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Esame finale anno 2018

To my parents

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Summary

Efficient rearing techniques providing high-quality insects are essential for pest control strategies entailing mass rearing and release in field, such as augmentative biological control and Sterile Insect Technique (SIT). Storage at suboptimal temperatures is a valuable procedure for prolonging the developmental time of insects and thus increasing the efficiency of insect rearing. The advantages that this procedure offers include a more flexible rearing schedule, the possibility to overcome periods of low production and the synchronization of field releases during pest outbreaks. Methods for storage of two model fly species, *Exorista larvarum* (L.) (Diptera: Tachinidae) and *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae), were studied in the present thesis by investigating the best conditions for an efficient storage and the consequences for fly quality.

Native to the Palearctic region, *E. larvarum* is a parasitoid introduced, and now established, in the USA as a biological control agent of *Lymantria dispar* (L.). The possibility to store the tachinid eggs at suboptimal temperatures once placed on artificial medium was first evaluated. Results showed that storage of the tachinid fly is possible to create a useful reserve of immatures, but the quality of the resulting females can be compromised. In addition, a study was performed with the aim of creating a useful stockpile of *E. larvarum* 1-day old puparia for use in small- or large-scale rearing programs. Pupal stage was efficiently prolonged by the storage treatments but female flies displayed a fitness reduction in some cases.

Bactrocera tryoni (Queensland fruit fly, Q-fly) is an endemic phytophagous species that represents a serious biosecurity challenge for Australia, attacking many commercial fruit and vegetable crops. The possibility to create a useful reserve of Q-fly eggs by placing them on a gel-based diet and by storing them at different suboptimal temperatures

was investigated. The preimaginal development was efficiently prolonged, but detrimental effects on biological parameters were observed. The following study evaluated the effects of suboptimal temperature storage on 1-day old Q-fly pupae for use in SIT programs. Survival and quality of the resulting adults were assessed and negative effects, of various intensity, were shown on standard and non-standard quality control parameters.

Statement of Originality

I certify that the work in this thesis entitled "**Effect of short-term suboptimal temperature storage to assist large-scale production of two dipterans:** *Exorista larvarum* **(L.) and** *Bactrocera tryoni* **(Froggatt)**" has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Maurizio Benelli

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1. Introduction

1.1 Insect mass rearing

The rearing of insects has long been essential for research and practical purposes (Smith, 1966). Although considerable effort is generally required at the commencement of rearing programs, great advances have been made over recent decades in the rearing of many insect taxa. Reliable supplies of healthy insects were initially needed as models for research and teaching (Needham et al. 1937). As an example, the easily reared vinegar fly *Drosophila melanogaster* Meigen has been the most widely used organism for biological research in genetics, physiology and life history evolution (Jennings 2011). The rearing of tsetse flies and mosquitoes, as well as other insect vectors of animal pathogens, have greatly benefited medical and veterinary disciplines (Singh et al. 1974; Mews et al. 1977). Similarly, agricultural sciences have required the development of efficient rearing methods for phytophagous insects to underpin the mechanisms of interaction with their host plants (De Moraes et al. 1988; Awmack and Leather 2002). As advances in entomology have deepened understanding of the fundamental aspects of insect rearing, the small-scale production for research purposes naturally progressed also into a large-scale production (Morales-Ramos et al. 2014), due to the utility of numerous insect species through their products or services (Lokeshwari and Shantibala 2010).

Historically, insects have been cultured to obtain commercial products such as silk (*Bombyx mori* L.), honey (*Apis mellifera* L.), cochineal dye (*Dactylopius coccus* Costa*)* and lac (*Kerria lacca* Kerr), or even as a source of food themselves for animals, including humans (Rumpold and Schlüter 2013). In addition to the demands of such commercial development of insect products, the pest management industry is increasingly demanding efficient mass rearing techniques that provide high-quality and cost-effective insect

production for use in Integrated Pest Management (IPM) programs (DiTomaso et al. 2017). The extensive use of pesticide-based control strategies has been responsible for causing imbalances in the population levels of insect pests (outbreaks) and also for contributing to ecosystem contamination (Hajek 2004). As an alternative, biological based control strategies, such as augmentative biological control or Sterile Insect Technique (SIT), are becoming increasingly important. In both cases, the final aim is the control of the target pest by releasing large numbers of insects into the environment. The development of large-scale rearing facilities and processes requires multidisciplinary effort and significant economic investment to develop the rearing technology, build adequate infrastructure, and train staff.

Large-scale mass rearing of insects takes place in biofactories - large multiroom facilities, with controlled environment chambers, where the production chain assumes the characteristics of an industrial process (Cáceres et al. 2012) (Figure 1.1). Facility operations are performed sequentially and, despite some mechanisation, trained personnel are required for each phase of the rearing process, such as diet preparation, insect multiplication and management, quality assurance, packing and shipping (Leppla and Ashley 1978). Generally, the rearing procedures are species-specific and require knowledge of the insect's biology, population genetics, natural distribution and nutritional requirements. This information is needed in order to establish a genetically suitable founding population, establish the optimal environmental conditions (temperature, relative humidity and photoperiod) and provide the best food substrate (plant material or artificial diet) for rearing. In the traditional (tritrophic) rearing procedure of the entomophagous insects, a suitable prey (or host) organism must be provided. In turn, prey/hosts also need to be cultured and fed (Thompson 1999). Quality control of mass-reared insects is essential for the market competitiveness of the associated

industrial enterprises (Leppla and Ashley 1989). However, the concept of quality is defined according to the purpose of the insect production and the assessment criteria may vary (Grenier 2009).

1.1.1 Mass rearing for biological control programs

Mass rearing and release of large numbers of arthropod natural enemies is the cornerstone of augmentative biological control programs (King 1993; van Lenteren 2000). The goal of these programs is to increase and reinforce natural enemy populations in the environment (inoculative releases) or the control of pest populations by inundating them with natural enemies as "biological insecticides" (inundative releases).

The first pioneer study using the augmentative biological control was set in pear orchards where green lacewings were released to control outbreaks of mealybugs (Doutt and Hagen 1949). After nearly 70 years, sales of natural enemies for augmentative biological control continue to grow. Indeed, the biological control industry that gradually emerged over recent decades (National Research Council 1996) is now undergoing accelerated development. In 2003, approximately 85 companies worldwide were rearing and selling more than 125 species of natural enemies on a large-scale, with a predominant availability in European Countries (van Lenteren 2003). In the following years, efficient biological control agents continued to be discovered and up to 230 species became commercially available (van Lenteren 2012). Specifically referring to entomophagous insects, at least 15 species of predatory insects and 23 parasitoids are marketed and released throughout the USA (Leppla and Johnson 2011).

Entomophagous insects, to be used for lowering the population level of a target pest species, include predators and parasitoids (King et al. 1985) (Figure 1.2). The most common predatory insects for use as natural enemies belong to the families of Coccinellidae (Coleoptera) for control of aphids, scales, mealybugs, thrips and whiteflies,

Chrysopidae (Neuroptera), such as *Chrysoperla carnea* Stephens, and Cecidomyiidae (Diptera), such as *Aphidoletes aphidimyza* (Rondani) for control of aphids. Furthermore Anthocoridae (Hemiptera), such as *Anthocoris nemoralis* F., are routinely marketed for control of psyllids. Among parasitoids, popular marketed options include a number of Hymenoptera Terebrantia, such as braconids (e.g., *Aphidius colemani* Viereck) to control a variety of aphid species, aphelinids (e.g., *Encarsia formosa* Gahan and *Eretmocerus* spp.) for control of whiteflies in greenhouses and trichogrammatids (*Trichogramma* spp.) for control of lepidopteran pests. A list of the most important invertebrate natural enemies used in augmentative biological control is provided in Table 1.1.

Dipteran species only account for a small portion of the natural enemies used in commercial augmentative biocontrol programs (Figure 1.3), in part because the role of dipteran parasitoids in biological control has often been undervalued in comparison to hymenopterans (Feener and Brown 1997). Following mass rearing in state-funded facilities, experimental releases of parasitoid flies have been trialled, especially tachinids, against a number of agricultural pests, although production in commercial biofactories has rarely occurred (Dindo and Grenier 2014). The role of parasitoid flies in IPM strategies could be better investigated if more effective technology for commercial rearing becomes available. A list of parasitoid flies released as natural enemies in biological control programs is provided in Table 1.2.

One of the most difficult challenges for successfully rearing entomophagous insects is the need to rear their prey or host as food source. Indeed, in these situations there is only one product that generates profits, while the company faces costs for producing two different species (Van Driesche 1996). Similarly, the expense of providing biological hosts for phytophagous insects can be a severe constraint on commercial viability of rearing programs. Given the complexity of also rearing host material, many natural prey

or plant hosts are replaced with artificial diets. Accordingly, the cost-effectiveness of rearing predators and parasitoids is greatly influenced by rearing technology. Artificial diets could, however, be developed for direct rearing of entomophagous insects, in order to simplify production and lower the costs (Grenier 2009). However, the nutritional value of these food sources is often lower than live prey or hosts, and this often results in the reduction of productivity and quality (Grenier and De Clercq 2003; Riddick 2009). The quality of the mass-reared insects depends not only on their food but also on other factors, including the genetic structure of their colonies and the physical rearing conditions (Bigler 1989).

Sustainability is a key concept for a modern IPM that includes biological control programs. In a free-market economy, mass-reared natural enemies can be considered as an alternative to other pest control technologies. In order to become available on the market and also sustainably produced, production costs of biofactories should be minimized.

1.1.2 Mass rearing for Sterile Insect Technique programs

The environmentally sustainable SIT is being applied as a component of Area-Wide Integrated Pest Management (AW-IPM) programs against several major insect pests, including screwworms, fruit flies, moths and mosquitoes (Dyck et al. 2005a) (Figure 1.4). With SIT, massive numbers of high-quality male insects are sterilised, commonly through irradiation, and released into the field to induce reproductive failure in females of wild populations (Knipling 1955). Repeated releases lead to population control and, theoretically, eradication under some conditions. As a biological-based control method, SIT is species-specific and reduces risks related to the introduction of new organisms into the environment. Since SIT relies on mass rearing facilities for the supply of large

numbers of insects ([Table 1.3](#)), the investment required for developing these facilities can only be supported by large industrial or governmental organisations (Dyck et al. 2005b).

The beginning of industrial insect rearing was marked by the rapid development of an eradication program for the New World Screwworm (NWS), *Cochliomyia hominivorax* (Coquerel), in the USA and Mexico through the use of SIT (Vargas-Terán et al. 2005). The NWS is a serious pest of livestock, with female flies laying eggs in sores and open wounds and larvae feeding on living tissues, thus debilitating their host animal and exposing them to infection. Laboratory techniques for culturing the NWS were initially developed using ground meat in 1936, a major breakthrough since, for the first time, an obligate parasite was reared without a live host animal. Since the official SIT program commencement in 1957, the rearing technique has been improved, with new diet developments and mechanisation (Taylor 1992). As a consequence, a remarkable fly production of 250-300 million screwworm flies was reached at the facility established in Tuxtla Gutierrez, Mexico. Gradually, NWS has been declared virtually eradicated from North America and Mexico and a state-of-the-art facility relocated to Pacora, Panama (Wyss 2000).

By adapting knowledge and methods proven effective for eradicating the screwworm, an innovative mass rearing program has been developed for using SIT against the Mediterranean fruit fly (Medfly), *Ceratitis capitata* (Wiedemann) (Enkerlin 2005). The Medfly is a major pest of fruit and vegetables in many regions globally. The primary mass rearing technique was originally developed in Seibersdorf, Austria, with substantial research carried out by the International Atomic Energy Agency (IAEA). The transfer of the rearing technology to rearing facilities, and its continuous improvement, achieved a production of 500 million pupae per week for irradiation and air distribution with aircraft. At present, there are many Medfly SIT programs underway globally

(Hendrichs et al. 2002), with at least 14 operative mass rearing facilities providing pupae for irradiation and release in field. The largest is located at El Pino, Guatemala, with a remarkable production of up to 5 billion flies per week (Tween 2002).

While SIT gained its reputation for "eradicating" insect pests (= reducing to zero their presence within an area, at least theoretically), it is important to recognize its potential for suppressing (= reducing to low levels of presence), containing (= prevent from expanding), and even preventing (= avoid introduction) insect pests in AW-IPM strategies (Hendrichs et al. 2005). The development of advanced mass rearing techniques will greatly benefit the future SIT programs and thus promote pesticide-free control strategies against a variety of insect pests (Parker 2005).

1.2 *Exorista larvarum* (L.)

1.2.1 General information

Exorista larvarum (L.) is a dipteran parasitoid belonging to Exoristinae, the most primitive subfamily within the family Tachinidae (Richter 1991) (Figure 1.5). This tachinid fly is a Palaearctic species, originally from Europe, northern Africa, and some Asian Regions. It has also become established in North America following introduction at the beginning of the 20th Century (Kenis and Lopez Vaamonde 1998). *Exorista larvarum* is a polyphagous and gregarious larval parasitoid that in nature attacks mostly lepidopterans. More than 45 different lepidopteran species have been reported as its natural hosts (Hafez 1953; Herting 1960), but recent host records appear to be incomplete or scarce. Kara and Tschorsnig (2003) and Cerretti and Tschorsnig (2010) have recorded host species present in Turkey and Italy, respectively. The host range varies according to the habitat and includes some well-known agricultural and forestry pest species, such as

Lymantria dispar (L.), *Hyphantria cunea* (Drury), *Dendrolimus pini* L. and several noctuid species.

Like all tachinids, the complete life cycle of this parasitoid includes an egg stage, three larval stages (L1, L2 and L3), a pupal stage and an adult stage (Mellini 1991). Eggs are macrotypical and ca. 0.6 mm long and 0.3 mm wide, oval-shaped and with colour turning from white, immediately after oviposition, to pale yellow as embryonic development progresses (Hafez 1953) ([Figure 1.5-c](#)). Dipteran parasitoids lack a true piercing ovipositor, therefore eggs are laid using a substitutional ovipositor composed of extensible posterior abdominal segments. Females display a direct oviposition strategy, meaning that the eggs are deposited directly on the host integument (Nakamura et al. 2013) ([Figure 1.5-b](#)). Mature females locate their hosts using chemical and visual cues (Depalo et al. 2012) and the eggs are mostly sticked on the dorsal or lateral surface of the host integument (Mellini et al. 1994). As an oviparous species, *E. larvarum* eggs are undeveloped when laid and they hatch after 2-3 days at 26 °C (O'Hara 2008).

First instar larvae are pyriform and transparent, with a length of ca. 0.7 mm and a width of ca. 0.25 mm. Immediately after egg hatching, the first instar larvae soften the host integument using their saliva and penetrate it using their pointed hook-like labrum. They then form a primary respiratory funnel that allows them to be in contact with atmospheric oxygen and grow rapidly (Michalková et al. 2009). The development of *E. larvarum* larvae is independent of host physiology and hormonal balance. First instar larvae feed on host haemolymph, avoiding harm to vital organs. When mature, they moult into L2 larvae increasing their size (ca. 3 mm long, 1.5 mm wide) and developing a second hook, but they still feed on body fluids. Contrarily, L3 larvae (ca. 8 mm long, 3 mm wide) move freely in the host body and quickly kill it by feeding on its organs until the food

source is exhausted (Marchetti 2006). When mature, the parasitic larva commonly abandons the host remains to pupate (Mellini et al. 1993) ([Figure 1.5-d](#)).

Pupation of *E. larvarum* takes place within the hardened skin of L3 larvae, the puparium. Pupae are exarate, featuring free appendages, with a variable size depending on various factors, including sex (male pupae are usually heavier) and the number of pupae per host (Hafez 1953). Newly formed puparia are yellow, but their colour turns to dark-red within a few hours ([Figure 1.5-e](#)). Studies performed at the Department of Agricultural and Food Sciences (DISTAL) (University of Bologna) have shown that under a regime of 27 °C the development from egg to adult takes on average 16 days (3 days for egg hatching, 6 days for larval development and 8 days for completing the pupal stage) (Marchetti 2006).

Newly emerged adults have a medium size (10-14 mm), they are black in colour with grey stripes, with the body covered in bristles ([Figure 1.5-a](#)). Sexual dimorphism makes males clearly identifiable by their distinctively forked pretarsi, which is less visible in females (Cerretti 2010). Adult flies mate soon after emergence. The pre-oviposition period varies, but generally takes 2-3 days under standard laboratory conditions (Dindo et al. 2007). Hafez (1953) observed that females lay an average of 7 eggs per day over 20-25 days and Dindo et al. (1999) showed that most eggs are laid during the first 10 days after sexual maturation. Similarly to other tachinids, in nature *E. larvarum* adults feed on sugary substances, such as nectar and honeydew. Their longevity is ca. 18 days for males and 21-22 days for females, with factors such as food source and environmental conditions influencing this parameter (Dindo et al. 1999).

1.2.2 Use in biological control programs

Its polyphagy and ease of rearing, both in vivo (= tritrophic system) and also in vitro (= directly on artificial media, in the absence of host), make *E. larvarum* a strong candidate for biological control programs against several economically important pest insects.

Herting (1960) considers *E. larvarum* the second most important enemy of the gypsy moth *L. dispar* in its native areas. This lepidopteran is an important defoliator of more than 300 species of deciduous and coniferous trees, with periodic outbreaks causing great economic damage to the forestry industry (Tobin et al. 2012). The gypsy moth has been introduced in the USA, but this voluntary introduction had caused devastating results, with several millions of acres defoliated. In the 20th Century, considerable efforts have been made to introduce entomophagous insects from Europe into North America to control *L. dispar*. While most of them failed to establish, *E. larvarum* was successfully introduced with sporadic inoculative releases, and eventually became established, providing a contribution to the control of *L. dispar* (Sabrosky and Reardon 1976; Kenis and Lopez Vaamonde 1998).

To date, the release of *E. larvarum* in forestry ecosystems in the USA is the only example of use of this tachinid in biological control programs. Nevertheless, laboratory research carried out at DISTAL has showed promising results for augmentative releases of *E. larvarum* for controlling noctuid pest species, such as *Xestia c-nigrum* L., *Pseudaletia unipuncta* (Haworth), *Peridroma saucia* (Hübner) (Simões et al. 2004), *S. littoralis* (Depalo et al. 2010) and *Mythimna unipuncta* (Haworth) (Depalo et al. 2012).

1.2.3 In vivo rearing technique

Knowledge of *E. larvarum* host range is required to develop efficient rearing techniques for this fly, both for research and for biological control. Natural hosts may not be easily cultured, due to their characteristics (e.g., the presence of urticating hairs on the larval

integument), requirement of peculiar environmental conditions (which may be poorly known), lack of suitable artificial diets, or even because they are expensive to rear (De Clercq 2008). Therefore, the natural hosts are routinely replaced with factitious hosts for in vivo rearing, meaning that these hosts would not be attacked in nature, but in laboratory conditions they can sustain *E. larvarum* development.

The greater wax moth *Galleria mellonella* (L.) has been used for years at DISTAL as a suitable and easy-to-rear factitious host for *E. larvarum* (Campadelli 1988) ([Figure 1.5-b, c](#)). The host is reared using an artificial diet and kept in complete darkness at 30 ± 1 °C and 65 ± 5% RH (Campadelli 1987). When adopting the in vivo rearing technique, adults of *E. larvarum* (50-70 individuals) are kept in plexiglass cages (40 x 30 x 30 cm) in a controlled environment room at 26 ± 1 °C, 65 ± 5% RH and 16:8 LD (the standard rearing conditions). They are provided distilled water, sugar cubes and cotton balls soaked in a honey-water solution. Once a week, ca. 3 mature *G. mellonella* larvae per female are inserted into the cage. After 40-60 minutes, 4-6 eggs (the optimal number for parasitoid production) are usually laid on each larva (Mellini and Campadelli 1996). The larvae with eggs are retrieved and placed in plastic cages (24 x 13 x 8 cm) in the same controlled environment room until puparium formation. Depalo et al. (2010) evaluated also the acceptance and suitability of *S. littoralis* for *E. larvarum* in the laboratory. This noctuid species has been sporadically recorded as a natural host of the tachinid in Egypt (Hafez et al. 1976; Assal and Koilab, 1984). In Depalo's study, however, puparia yields were lower in comparison with the use of *G. mellonella*.

In all tachinid parasitoids, parasitization success depends not only on the host species, but also on other factors, including the number of eggs laid per host and the host age (Dindo and Grenier 2014). Superparasitism is very common in *E. larvarum*, but this

phenomenon should be limited to optimize production. Lower adult size has been observed following a reduction in space per individual in the host (Baronio et al. 2002).

1.2.4 In vitro rearing technique

Biological control programs based on the release of parasitoid species have encouraged the development of in vitro rearing techniques by replacing their natural, or factitious, hosts with an artificial medium (Grenier and De Clercq 2003).

Exorista larvarum is one of the most promising tachinid parasitoids to be cultured in vitro, with potential for production on a mass rearing scale (Dindo and Grenier 2014). The first artificial medium for this tachinid was developed by Mellini et al. (1993) and it was mainly composed of bovine serum and homogenate of *G. mellonella* pupae. Subsequent trials were carried out in an attempt to simplify the preparation of the medium and eliminate any host components. As a result, an effective medium comprising of skimmed milk, yeast extract, chicken egg yolk and sucrose was developed by Mellini and Campadelli (1996). The medium gave adult yields (43-44%, calculated on the eggs placed in culture) and puparial weights similar to those obtained in vivo. Nutritionally, the tachinid (which is polyphagous) showed some adaptability, but further development was required in terms of physical properties of the media. In order to prevent larval drowning, a physical support was needed., such as a gelling agent (agar) or cotton (Dindo et al. 2003). Furthermore, to avoid mould contamination, the medium is routinely supplemented with gentamicin and the eggs disinfected through alcohol washings (Mellini and Campadelli 1999). As alternative insect media, tissue culture media were developed by Bratti and Coulibaly (1995) while veal homogenate (as main ingredient) was adopted by Dindo et al. (1999).

Direct oviposition onto media has not yet been achieved for *E. larvarum*, but this goal is desirable for continuous in vitro rearing since it eliminates the need to rear the

parasitoid hosts. The in vitro rearing technique of this tachinid fly currently entails removing eggs from previously superparasitized host larvae. Captive females have, however, been observed to lay eggs throughout the rearing cage (Mellini et al. 1994). The out-of-host laid eggs can be retrieved and cultured on artificial medium (Dindo et al. 2007; Marchetti et al. 2008).

1.3 *Bactrocera tryoni* (Froggatt)

1.3.1 General information

Bactrocera tryoni (Froggatt), the Queensland fruit fly (Q-fly), is a dipteran belonging to the subfamily Dacinae, which includes tropical fruit flies within the family Tephritidae (Drew and Romig 2013) (Figure 1.6). Q-fly is endemic to Australia, but while it was originally distributed in Queensland and northern New South Wales, it is now widely established in Australia's east coast and some inland areas (Figure 1.7), as well as some Pacific Islands (Dominiak and Daniels 2012; Dominiak and Mapson 2017). Anthropogenic climate change is likely to influence Q-fly distribution in Australia, highliting that long-term vigilance is needed to prevent further range expansion of this species (Sultana et al. 2017).

The complete life cycle of *B. tryoni* consists of an egg stage, three larval stages (L1, L2 and L3), a pupal stage and an adult stage. Immature stages and preimaginal development were described in detail by Exley (1955), Anderson (1962; 1963) and Elson-Harris (1988). Eggs are ca. 0.91 mm long and 0.23 wide, elongate, elliptical and glistening white in colour. Following oviposition into fruits, egg hatching generally occurs within 2-3 days at 25 °C (Yonow et al. 2004). First instar larvae are pale yellow and display a moderately sclerotized cephalopharyngeal skeleton, with small mouth hooks utilised for eating and digging into the fruit pulp. Second instar larvae continue feeding. They are

characterized by the presence of large preapical teeth on their hooks and medium sclerotized posterior spiracles (Figure 1.6-b). When mature, they moult into L3 larvae reaching the size of ca. 8-11 mm in length and 1.2-1.5 mm in width, displaying large heavily sclerotized mouth hooks with elongate, clearly defined, posterior spiracles. Mature L3 larvae abandon their fruit by jumping into the soil beneath the host plant, where they burrow and pupate. The larval developmental period varies according to temperature, larval density and host plant species. Generally, it takes between 5 and 31 days (Meats 1984; O'Loughlin et al. 1984; Fletcher 1987). Queensland fruit fly pupae are protected within the puparium which is barrel-shaped and with a colour turning from pale yellow to reddish-brown in the hours following pupation (Figure 1.6-c). Puparia have a variable size and display visible, but not functional, spiracles at one end. Pupal developmental time varies and lasts ca. 11 days at 26 °C (Resilva and Pereira 2014).

Adult Q-flies are medium size flies (6-8 mm long), red-brown coloured with characteristic yellow markings (Drew 1989) (Figure 1.6-a). Their wings are transparent, marked with brown areas, with a wing expanse of 10-12 mm. Further morphological traits are described by Plant Health Australia (2016).

Post-teneral nutrition plays a key role in reproductive development and sexual performance of adult Q-flies (Weldon and Taylor 2011; Taylor et al. 2013). Natural food sources for tephritids include nectar, extra-glandular secretions, fruit juices, bird faeces and bacteria (Drew and Yuval 1999). Under laboratory conditions and with a "sucrose + protein" diet, the percentage of mating males and females reached the peak in 8 to 11 days after emergence (Perez-Staples et al. 2007b).

Queensland fruit flies mate only at dusk for a period of ca. 30 minutes, when males aggregate on foliage, release pheromone and emit a courtship song by beating their wings (Tychsen and Fletcher 1971). After mating, females store the sperm within their two

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spermathecae and enter a phase of sexual refraction (Perez-Staples et al. 2007a). Oviposition occurs in daytime. Females locate host plants attracted by olfactory and visual stimuli and lay eggs by piercing the fruit cuticle using their ovipositor (Pritchard 1969; Fowler 1977). Egg production per female per day varies, reaching a peak of 100-120 and oviposition of fertile eggs can last for more than 7 weeks (Barton-Browne 1957; Clarke et al. 2011). Adult Q-fly longevity varies according to several factors, including genetic structure, environmental conditions, especially temperature, and food availability (Bateman 1967).

1.3.2 Management options and use in SIT programs

Bactrocera tryoni is by far the most economically damaging insect pest of Australia, causing direct loss of production and inducing premature fruit drop in numerous commercial fruit and vegetable crops (Hancock et al. 2000). It has been estimated that AUS $432 million is the total export value of the top 25 commodities susceptible to Q-fly damage in the country. Furthermore, this pest threatens AUS $1 billion worth of products traded domestically (Chen et al. 2016). The cost for fruit fly surveillance and management must be also taken into account and Plant Health Australia (2009) estimated a total of AUS $128.7 million spent in Australia between July 2003 and June 2008 for these purposes. With the aim of developing sustainable solutions to the problem, a national action plan is periodically implemented (NFFS committee 2017).

Queensland fruit fly management is currently undergoing profound changes. Regulatory authorities have recently restricted the use of organophosphate insecticides, dimethoate and fenthion, on which growers have relied for decades (Reynolds et al. 2017). Alternative insecticide formulations are listed online (APVMA 2017), but their effectiveness is questionable. Dominiak and Ekman (2013) provide a comprehensive

review of the past and present fruit fly chemical control, including the use of repellents, bait sprays, cover sprays, fumigants and dips for postharvest treatments.

As part of the standard toolkit for Q-fly management and monitoring, lure and kill techniques are becoming widespread options (El-Sayed et al. 2009). Among these, the Male Annihilation Technique (MAT) and Protein Bait Sprays (PBS) have shown promising results. MAT adopts the principle of reducing the density of males in fruit fly populations to such a low level that mating is minimised. This involves the deployment of bait stations that contain a male lure combined with an insecticide. In the case of *B. tryoni*, cue-lure (4-(4-acetoxyphenyl)-2-butanone) is the most common and effective lure utilised for MAT (Bateman et al. 1973; Dominiak et al. 2003) due to the strong responsiveness of males and stability of the lure compound (Drew 1989). In contrast, PBS relies on the fact that male and female Q-flies require protein to reach sexual maturation, therefore a protein source mixed with an insecticide formulation can be used to attract and kill both sexes (Lloyd et al. 2010; Balagawi et al. 2014). Lure and kill techniques, also using female lures, chromotropic traps or artificial fruits, and the use of Q-fly natural enemies are reviewed by Clarke et al. (2011). Q-fly management and eradication options have also been recently evaluated by Stringer et al. (2017).

Sterile Insect Technique, which entails weekly field releases of millions of sterile insects, is destined to become the major control strategy against the Q-fly, especially considering the human health concerns caused by pesticide applications. The first general principles for an economic mass rearing of Q-flies, their irradiation, transport and release were formulated ca. 50 years ago (Monro and Osborn 1967), following successful trials with field releases of sterile flies in western New South Wales between 1962 and 1965 (Andrewartha et al. 1967). Research on Q-fly SIT was then implemented during the following years and a Fruit Fly Exclusion Zone (FFEZ) was established in south-eastern

Australia, with the aim of eradicating any outbreaks of *B. tryoni* within the area and also preventing introduction from the outside (Meats et al. 2003) (Figure 1.7). To guarantee an adequate number of Q-flies for SIT, a mass rearing facility was built in 1996 at Elizabeth Macarthur Agricultural Institute (EMAI) (Menangle, NSW, Australia) (Fanson et al. 2014) (Figure 1.8-a).

Due to the difficulties of defending against massive and persistent fruit fly pressure, the FFEZ has now been formally abandoned. On the other hand, the fruit fly SIT programs are undergoing substantial revision with the aim of increasing their efficiency and cost-effectiveness (PBCRC 2015). For this purpose, a "SITplus" consortium has been recently established, putting together a strategic, coordinated and national approach with a 5-year AUS $45 million research and development partnership, in which Macquarie University is involved (Hort Innovation 2017). At the same time, a ambitious new national SIT facility has been built in Port Augusta (South Australia) (Figure 1.8-b), set to produce 50 million sterile male Q-flies a week by 2019 and clearly mark a new era for SIT in Australia.

Generally, Q-fly control must be applied in areas where this pest is present to ensure a high-quality horticultural production, thus permitting growers to meet the strict requirements for market access, both domestic and international (Jessup et al. 2007). Sterile Insect Technique, in combination with MAT and PBS, has been found to be a key tactic in eradication programmes against a number of fruit fly species (Suckling et al. 2016). Its efficacy and the possibility of combination with other control strategies in area wide programs (Vreysen et al. 2006), make SIT the most promising approach for suppressing or eradicating Q-fly populations.

1.3.3 Rearing techniques

A method for culturing the Q-fly under laboratory conditions was first described by Myers (1952), although Allman (1938) successfully bred *B. tryoni* in uncontrolled environment rooms. In his experiments, Myers kept adult Q-flies in glass cages at 22-24.5 °C and 62-68% RH and remarked on the importance of a dusk period before darkness for mating. Eggs were collected inserting an apple in the cages and the larval medium was composed of paw paw paste, banana paste, peeled orange and honey. Since then, the Q-fly rearing technique has been refined to achieve higher yields and uniformity in terms of speed of development and survival of the flies. For this purpose, Bateman (1967) adopted 24.5-25.5 °C and 65-85% RH as rearing conditions and a larval rearing medium mainly composed of dehydrated carrot and brewer's yeast, derived from a modification of the medium of Finney (1953). Transitioning from the small-scale rearing for research purposes towards a large-scale rearing needed for SIT, a method for Q-fly mass-production was first proposed by Monro and Osborn (1967).

In general, while rearing conditions have been changing slightly over time, the great improvements for an efficient Q-fly rearing method have been made in terms of larval medium. Starting in 1996, the facility at EMAI has been mass producing *B. tryoni* on a lucerne chaff-based medium (Figure 1.9-c) achieving high-quality flies and good production levels (Dominiak et al. 2008), in replacement of more expensive diets containing more costly bulking agents such as granulated carrots (Figure 1.9-b) or wheat bran (Finney 1953; Hooper 1987). The potential use of liquid diets for Q-fly rearing has also been evaluated by substituting bulking agents with inert substrates, such as synthetic sponges (Khan 2013). While liquid diets have shown potential as economical alternatives to classical solid diets, they have shown some inconvenience, including the separation of their components, fermentation and the regular replacement of the supporting substrates.

The need of an economical and practical mass rearing of Q-flies for use with SIT has led to further research in diet formulations. To date, remarkable achievements have been shown by developing gel-based media for larval rearing (Moadeli et al. 2017) ([Figure 1.9-a](#)) that have already been adopted for use in the newly built SIT facility in South Australia. In this case, agar has been added to existing liquid media formulations providing improved pupal recovery (up to 99%) and adult fly quality.

1.4 Suboptimal temperature storage

Biological control and SIT programs involve the industrial-scale production of insects in large biofactories, where the rearing process takes place in controlled environment rooms set at the standard rearing conditions. Precisely, this means that the optimal level of temperature, relative humidity and photoperiod are adopted to meet the requirements of each cultured species. Despite all being important parameters to be controlled, insects are poikilothermal, and therefore temperature has significant influence on their metabolism and development (Logan et al. 1976; Ikemoto 2005).

A period of time spent at a temperature that is lower than the optimum can provide a valuable method for prolonging the developmental time of insects during their rearing process (Leopold 1998). Studies on this subject started at the beginning of the 20th century, but their aim was mainly focused on investigating the effect of low temperature on insect biology and the possibility to use it for disinfesting fruits (Fuller 1906; Back and Pemberton 1916; De Ong 1921). Low-temperature treatments remain a very important post-harvest control strategy against a number of insects attacking fruit crops, including tephritid flies (e.g., Castro et al. 2016). In particular, they are routinely part of quarantine security protocols for diminishing the risk of accidentally introducing pests through the importation of agricultural products (Follet and Naven 2006; De Lima et al. 2017). It was during the 1930s that the application of suboptimal temperatures started to

be exploited also as an aid for managing arthropod rearing (King 1934; Hanna 1935; Lebedyanskaya et al. 1936). This eventually became a technology, often called "cold storage".

The term "cold storage" can be found in literature referring to a number of different techniques, which include the preservation of insect embryos at extremely low temperatures (-196 °C) (cryopreservation) or the use of low temperatures in combination with changes in the photoperiod for inducing dormancy in some insect species (Leopold 2007). We here refer to cold storage, or suboptimal temperature storage when temperatures are closer to the optimum, as the method which proved useful for increasing the shelf life of reared insects, making their production more efficient, flexible, and ultimately reducing the rearing costs, both on a small or large-scale. Although small-scale rearing (e.g., research laboratories) also benefits from the ability to maintain a reserve of flies, cold storage is a great convenience and potential economic value for pest control strategies that entail mass rearing and release of insects: cold storage enables synchronisation of field releases according to the presence of the target (McDonald and Kok 1990; Venkatesan et al. 2000) and facilitates shipment (Cáceres et al. 2007).

In order to reduce the metabolic rate and development of insects for storage, insects are generally placed at temperatures above 0 °C and often ranging between 4 and 15 °C (van Lenteren and Tommasini 2003). However, low-temperature may be deleterious and injurious if not optimised and so the investigation of the physiology of cold tolerance remains an important area of biological research (Zachariassen 1985), with many applications. Generally, insects exposed to suboptimal temperatures above 0 °C may experience chilling injury, resulting in a reduction of fitness. This type of injury differs from the freezing injury that occurs when the storing temperatures are below 0 °C and cause physical damage due to ice crystal formation within the tissues (Block 1990). In

both cases, mortality represents the extreme consequence of the treatments. Overall, the capacity to tolerate cold treatments depends on a series of exogenous and endogenous factors, as reviewed by Colinet and Boivin (2011). These factors and the fitness-related traits that may be influenced by cold storage are listed in Table 1.4. Dietary changes also proved to be an important factor affecting insect cold tolerance (Coudron et al. 2007).

When developing suboptimal temperature storage protocols for use in the insect rearing industry, there is a need to strike a good compromise between the benefits and the drawbacks (potentially reduced quality, cost) of this technology.

1.5 Thesis objective

The objective of this thesis is to test whether the use of a short-term suboptimal temperature storage during a specific preimaginal stage (or stages) of two dipteran species may delay their development while having the minimum impact on biological parameters and quality of the resulting adults. The results obtained may be helpful in setting the stage for developing protocols for mass rearing facilities that produce these insects for use in biological control or SIT programs. Cold storage benefits include the ability to overcome periods of low production, the establishment a stockpile of insects while waiting for their release in field, preserve them during shipment, decrease in production costs and increase the overall flexibility of the rearing process.

Exorista larvarum was chosen as a model biological control agent to be studied in this thesis. The promising results obtained in the laboratory investigating the use of this fly for the control of several lepidopteran pest species, as well as the possibility to be easily mass-reared in vivo or in vitro, made *E. larvarum* a highly suitable candidate for my research.

In a first study (Chapter 2), I evaluated the possibility of storing *E. larvarum* eggs at suboptimal temperatures once placed on artificial medium in rearing plates. I applied the

temperature treatment in combination with the in vitro rearing technique. This rearing method showed potential for the retrieval of eggs laid out of host that captive females oviposit on the cage surfaces. Placing them on artificial media is the only way to prevent their loss. By storing eggs on media at suboptimal temperatures it could be possible to create a useful reserve of flies for use in colony maintenance. However, as a first step, I tested eggs removed from previously parasitized hosts.

In a second study (Chapter 3), I evaluated the possibility of prolonging the pupal stage of *E. larvarum* by storing puparia obtained with the in vivo rearing technique at suboptimal temperatures. This study may be useful for utilisation of the tachinid in biological control programs. In the case of mass rearing in facilities, there may be situations in which fly emergence needs to be delayed before field releases with minimum impact on fly performances, for example when the pest insect to be controlled is scarce or in an unsuitable stage, or when there is unfavourable weather. Both studies on *E. larvarum* were performed in the Department of Agricultural and Food Sciences at the University of Bologna (Bologna, Italy).

Bactrocera tryoni, the Q-fly, was chosen as model insect pest targeted by a SIT program to be studied in this thesis. This endemic tephritid represents a serious biosecurity challenge for Australia, attacking many commercial fruit and vegetable crops and causing, as a consequence, economic damages to producers and strict market access regulation. Currently, mass rearing of this species takes place in facilities for use with SIT, as part of an Area Wide Management plan. As a result, massive numbers of sterile males are released in field for suppressing Q-fly infested areas. An efficient, flexible and cost-effective mass rearing technique will assist the current Q-fly SIT program, therefore studies on the storage on *B. tryoni* are justified.

In a first study (Chapter 4), I evaluated the possibility of storing Q-fly eggs at suboptimal temperatures in Petri dishes in a gel-based diet. By creating baseline knowledge on Q-fly cold tolerance, this kind of investigation is useful for creating a stockpile of immatures that can be brought back at the optimal rearing temperature when needed for continuing their life cycle at regular speed.

In a second study (Chapter 5), I evaluated the possibility of prolonging the pupal stage of *B. tryoni* with the aim of increasing its shelf life while minimising the impact on quality of flies for use in SIT programs. During mass rearing in SIT facilities, a delay in fly emergence is desirable under some circumstances, for example when the field release of sterile males must be synchronised with outbreaks of wild populations, or when there is the need to release a massive number of flies (when the demand does not match the pace of the weekly production). Furthermore, mechanical failure of irradiation equipment may also require fly emergence to be delayed while waiting for repair. Both studies on *B. tryoni* were performed in the Department of Biological Sciences at Macquarie University (Sydney, Australia).

This thesis has been developed in the framework of a cotutelle agreement between the University of Bologna and Macquarie University.

1.6 Thesis structure

This thesis is written adopting a "thesis by publication" format and it is composed of six chapters. The structure of the thesis is briefly summarized below.

Chapter 1 (this chapter), reviews the importance of mass rearing as part of insect pest control programs, introduces the two model dipteran species adopted in my studies, the use of suboptimal temperatures for storing insects and the objectives of this thesis.

Chapter 2 is entitled "Effects of storage at suboptimal temperatures on the in vitro-reared parasitoid *Exorista larvarum* (Diptera: Tachinidae)" and has been published in the

Journal of Economic Entomology (Volume 110, Issue 4, 1st August 2017, Pages 1476-1482). DOI 10.1093/jee/tox169.

Chapter 3 is entitled "Storage at suboptimal temperature of *Exorista larvarum* (Diptera: Tachinidae) puparia for mass rearing programs" and has been prepared for submission to *Entomologia Experimentalis et Applicata*.

Chapter 4 is entitled "Effects of storage at suboptimal temperatures of Queensland fruit fly eggs" and has been prepared for submission to *Journal of Applied Entomology*.

Chapter 5 is entitled "Suboptimal temperature storage of Queensland fruit fly pupae for mass rearing programs" and has been prepared for submission to *Journal of Pest Science*.

Chapter 6 draws general conclusions and also discusses possible future research.

1.7 List of figures

Figure 1.1 – The mass rearing facility "El Pino" located in Guatemala. It represents an example of large facility where an insect species, the Mediterranean fruit fly, is produced at an industrial-scale level for use in a pest control strategy, the Sterile Insect Technique (source: Cáceres et al. 2012).
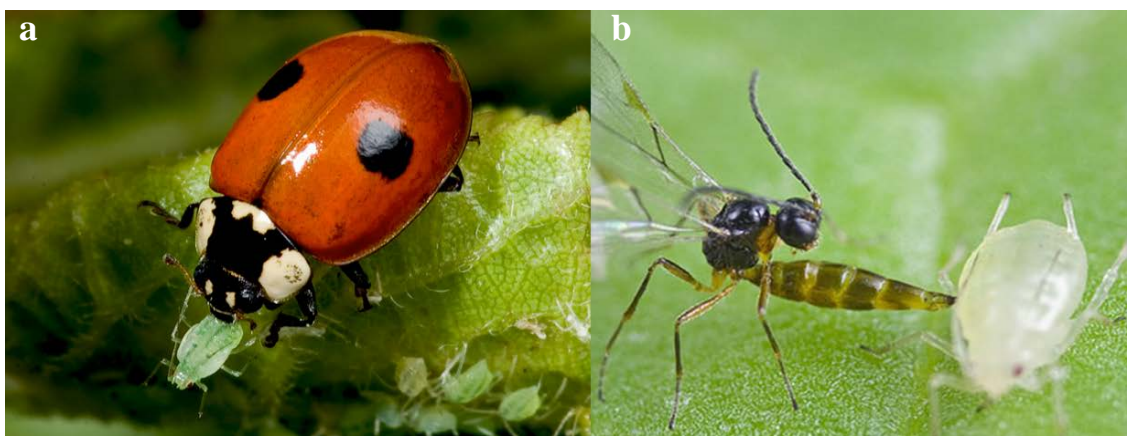
Figure 1.2 –Two entomophagous insect species commonly mass-reared in biofactories for use in biological control programs: (a) the predator *Adalia bipunctata* L. (two-spot ladybird) (source: http://www.treknature.com); (b) the parasitoid *Aphidius colemani* Viereck (source: http://shop.agrimag.it).
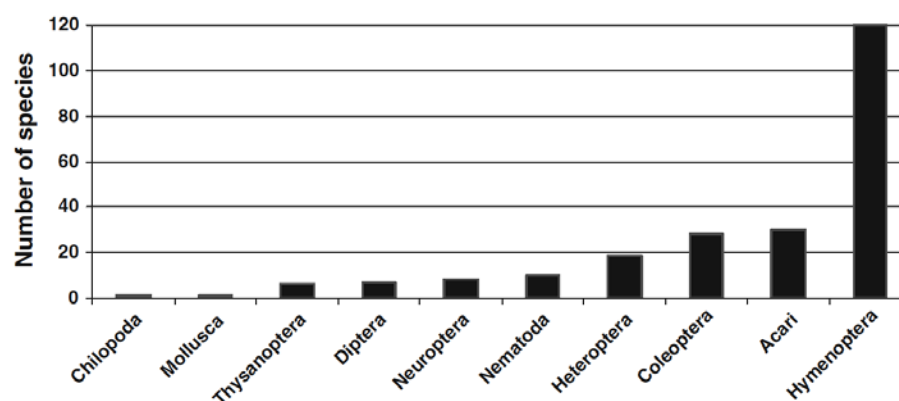
Figure 1.3 – Taxonomic groups providing natural enemies used in commercial augmentative biological control from 1900 to 2010 (source: van Lenteren 2012). Dipteran species only account for a small portion of the total number of natural enemies released in field, most of them were hymenopterans.
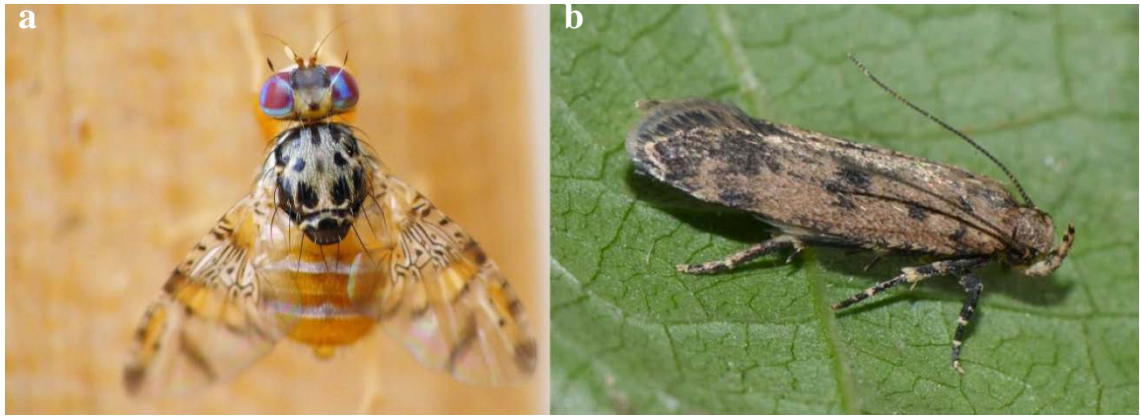
Figure 1.4 –Two phytophagous insect pests mass-reared in biofactories for use in Sterile Insect Technique programs: (a) the fruit fly *Ceratitis capitata* (Wiedemann) (Medfly) (source: http://www.globalspecies.org); (b) the moth *Pectinophora gossypiella* (Saunders) (pink bollworm) (source: http://www.nbair.res.in).
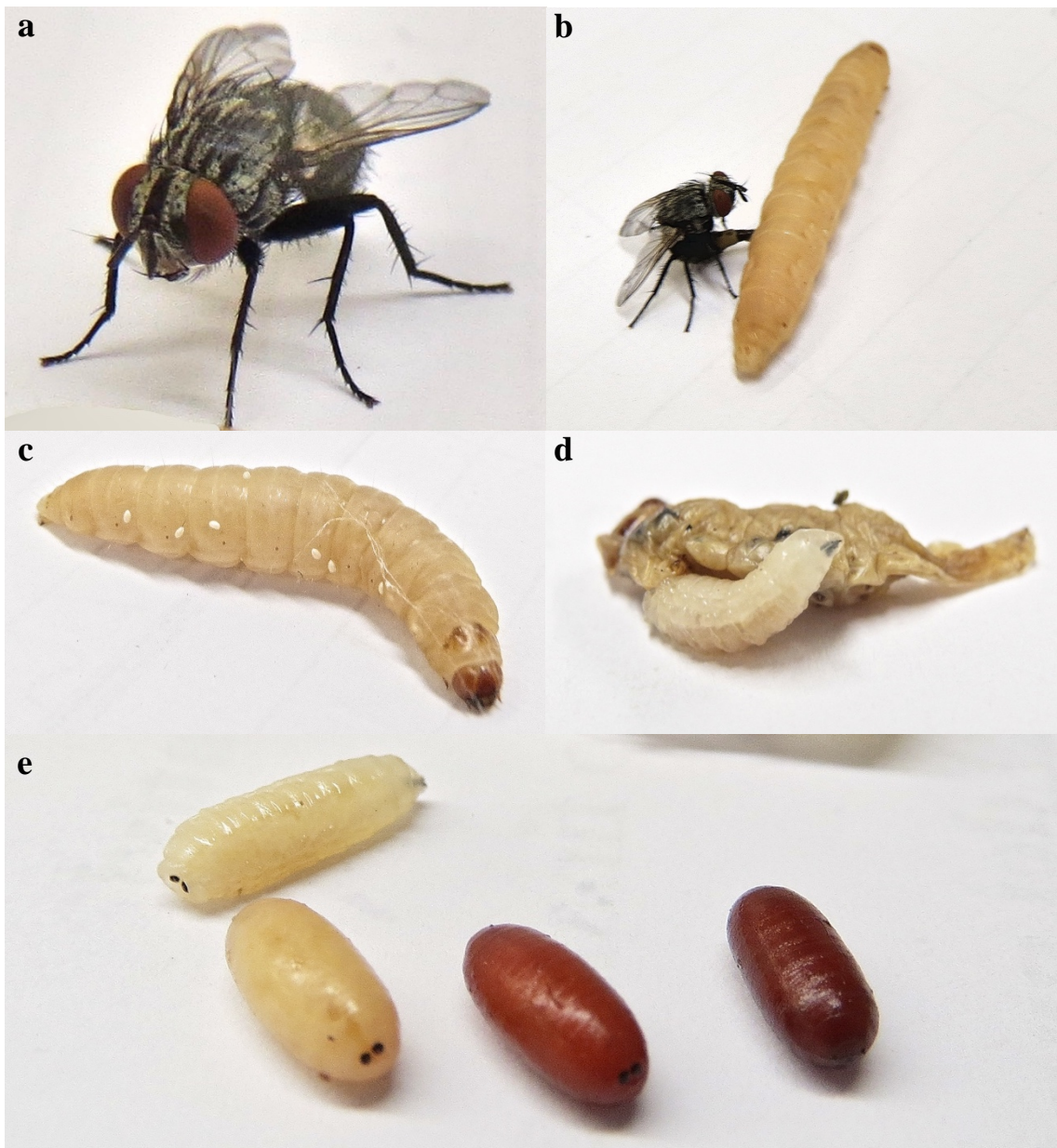
Figure 1.5 – The tachinid parasitoid *Exorista larvarum* (L.) in vivo-reared: (a) adult female; (b) sexually mature female ovipositing on the factitious host *G. mellonella*; (c) particular of the eggs laid on *G. mellonella* integument; (d) a mature third instar larva of *E. larvarum* is abandoning the host remains; (e) puparium formation. All photos were taken by the author.
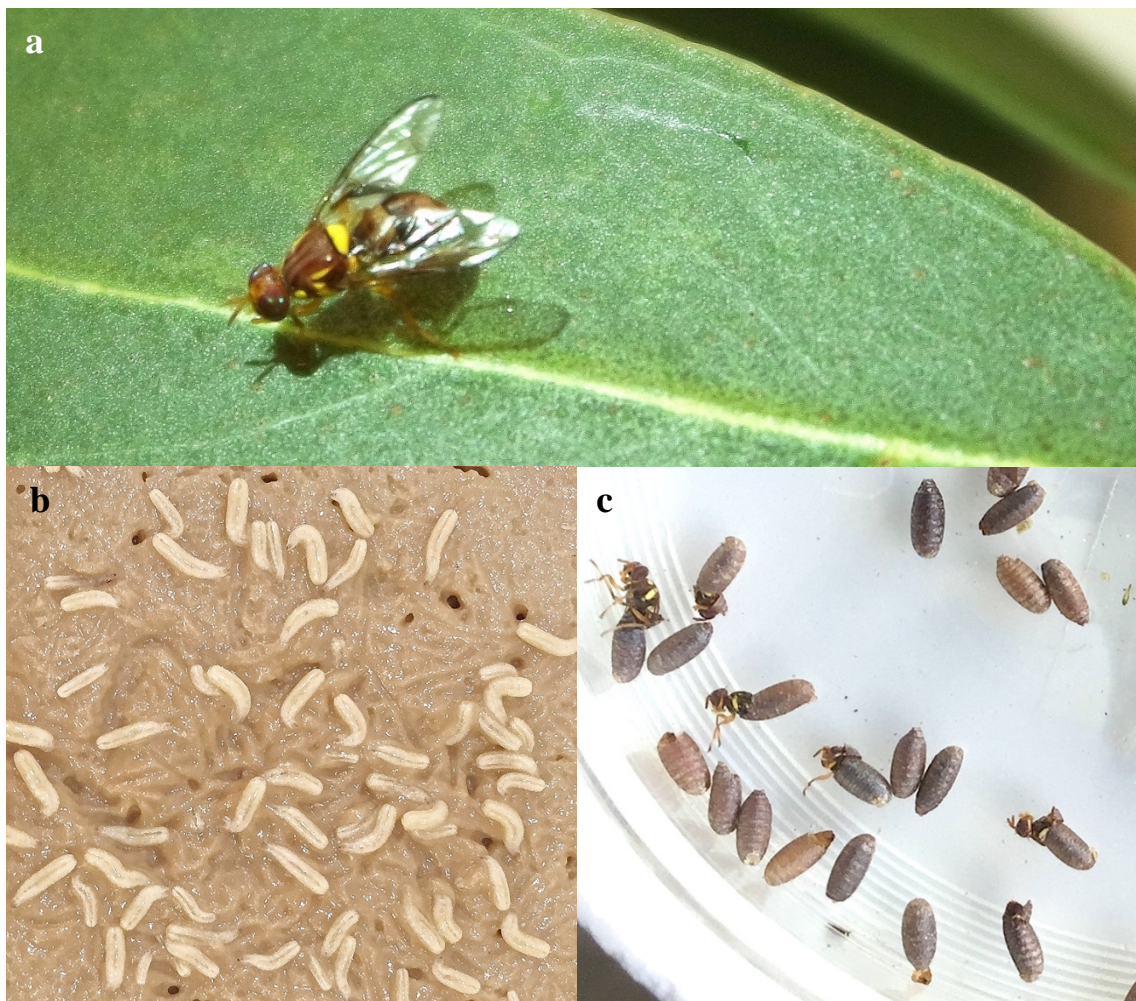
Figure 1.6 – The tephritid fly *Bactrocera tryoni* (Froggatt) (Queensland fruit fly): (a) adult male on foliage; (b) second instar larvae; (c) puparia and emerging adults. All photos were taken by the author.
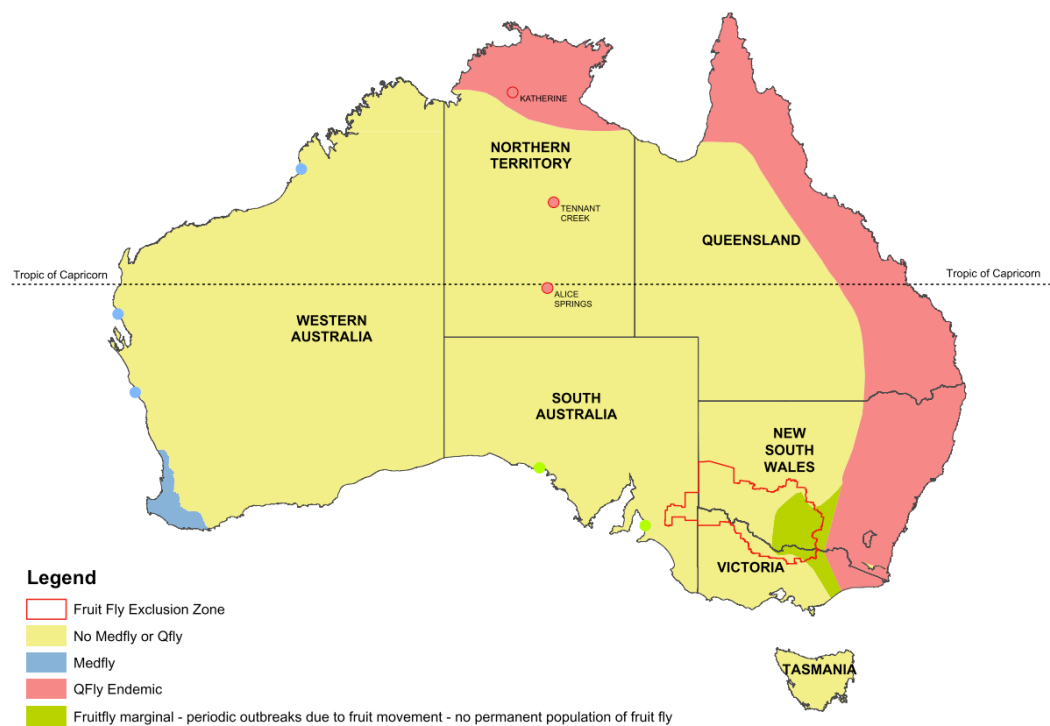
Figure 1.7 – Distribution of Queensland fruit fly and Mediterranean fruit fly in Australia according to Dominiak and Daniels (2012). The Fruit Fly Exclusion Zone (area within the red line) is no longer in place.

Figure 1.8 – Queensland fruit fly mass rearing facilities in Australia: (a) facility at Elizabeth Macarthur Agricultural Institute (EMAI) in Menangle (New South Wales) (author's photo); (b) newly built SITplus facility in Port Augusta (South Australia) (source: http://www.pir.sa.gov.au).

Figure 1.9 – Different larval media for rearing the Queensland fruit fly: (a) gel-based medium with eggs on top; (b) granulated carrot-based medium; (c) lucerne chaff-based medium. All photos were taken by the author.

1.8 List of tables

Biological control agent	Family	Target(s)	No. of countries where used	Year of first use
1. *Amblyseius swirskii*	Phytoseiidae	Whiteflies, thrips, mites	>20	2005
2. *Aphidius colemani*	Braconidae	Aphids	>20	1991
3. *Aphidoletes aphidimyza*	Cecidomyiidae	Aphids	>20	1989
4. *Dacnusa sibirica*	Braconidae	Leafminers	>20	1981
5. *Diglyphus isaea*	Eulophidae	Leafminers	>20	1984
6. *Encarsia formosa*	Aphelinidae	Whiteflies	>20	1926
7. *Macrolophus pygmaeus (=nubilis)*	Miridae	Whiteflies	>20	1994
8. *Neoseiulus cucumeris (=Amblyseius cucumeris)*	Phytoseiidae	Thrips	>20	1985
9. *Phytoseiulus persimilis*	Phytoseiidae	Mites	>20	1968
10. *Steinernema feltiae*	Steinernematidae	Sciarids	>15	1984
11. *Aphidius ervi*	Braconidae	Aphids	>15	1996
12. *Orius laevigatus*	Anthocoridae	Thrips	>15	1993
13. *Cryptolaemus montrouzieri*	Coccinellidae	Coccids, pseudococcids	>15	1989
14. *Galeolaelaps aculeifer (=Hypoaspis aculifer)*	Laelapidae	Sciarids	>15	1996
15. *Feltiella acarisuga (=Therodiplosis persicae)*	Cecidomyiidae	Mites	>15	1990
16. *Leptomastix dactylopii*	Encyrtidae	Pseudococcids	>15	1984
17. *Stratiolaelaps miles (=Hypoaspis miles)*	Laelapidae	Sciarids	>15	1995
18. *Aphelinus abdominalis*	Aphelinidae	Aphids	>10	1992
19. *Heterorhabditis bacteriophora*	Heterorhabditidae	Coleopterans	>10	1984
20. *Heterorhabditis megidis*	Heterorhabditidae	Coleopterans	>10	1990
21. *Neoseiulus californicus (=Amblyseius californicus)*	Phytoseiidae	Mites, thrips	>10	1985
22. *Eretmocerus eremicus*	Aphelinidae	Whiteflies	>10	1995
23. *Eretmocerus mundus*	Aphelinidae	Whiteflies	>10	2001
24. *Episyrphus balteatus*	Syrphidae	Aphids	>10	1990
25. *Trichogramma evanescens*	Trichogrammatidae	Lepidopterans	>10	1975

Table 1.1 – Invertebrate natural enemies routinely used in augmentative biological control programs ranked by number of countries in which each is used (source: van Lenteren 2012).

Family	Parasitoid Species	Target Insect	Type of Biological Control	Country of Application
Tachinidae	*Cyzenis albicans*	*Operophtera brumata*	C	Canada
	Aphantorhaphopsis samarensis	*Lymantria dispar*	C	Canada and Northern USA
	Exorista larvarum			Northern USA
	Parasetigena sylvestris			
	Blepharipa pratensis	*Lymantria dispar* and *Nygmia phaerrhoea*	C and A	
	Compsilura concinnata			
	Lydella jalisco	*Eoreuma loftini*	C	USA (Texas)
	Archytas marmoratus	*Helicoverpa zea* *Spodoptera frugiperda*	A**	Southern USA
	Lixadmontia franki	*Metamasius callizona*	C	Southern USA (Florida)
	Ormia depleta	*Scapteriscus* spp.	C	
	Lydella minense *Paratheresia claripalpis*	*Diatraea* spp.	C and A	Brazil, Colombia, and Peru
	Lixophaga diatraeae	*Diatraea saccharalis* and other lepidopterous sugarcane borers	C and A	Southern USA, West Indies islands, South America, and Southeast Asia
			A	Cuba
		Chilo infuscatellus and other lepidopterous sugarcane borers	A**	China (Guangxi)
	Trichopoda pennipes	*Nezara viridula*	C	Different countries
	Trichopoda giacomelli		C	Australia
	Bessa remota	*Levuana iridescens*	C	Fiji islands
Sarcophagidae	*Agria housei*	*Choristoneura fumiferana*	C	Canada
Cryptochaetidae	*Cryptochaetum iceryae*	*Icerya purchasi*	C	USA (California) Mauritius Israel
	Cryptochaetum jorgepastori	*Paleococcus fuscipennis*	C	Israel
Phoridae	*Pseudacteon* spp.	*Solenopsis* spp.	C	Southern USA

*There are also additional examples in different regions of the world.
**At experimental level.

Table 1.2 – Parasitoid flies released as natural enemies in classical (C) or augmentative (A) biological control programs (source: Dindo and Grenier 2014).

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Country	Location	Species	Weekly capacity (millions)
Fruit flies			
Peru	Lima	*Anastrepha fraterculus*	5–8
Mexico	Metapa	*Anastrepha ludens*	300
United States	Texas	*Anastrepha ludens*	140–160
Mexico	Metapa	*Anastrepha obliqua*	25–50
Japan	Okinawa	*Bactrocera cucurbitae*	70–200
Thailand	Pathumtanee	*Bactrocera dorsalis*	15–40
Philippines	Quezon City	*Bactrocera philippinensis*	10–15
Australia	Menangle	*Bactrocera tryoni*	20
Argentina	Mendoza	*Ceratitis capitata*	200[a]
Australia	Perth	*Ceratitis capitata*	5–15[a]
Brazil	Juazeiro	*Ceratitis capitata*	100[a]
Chile	Arica	*Ceratitis capitata*	30[a]
Guatemala	El Pino	*Ceratitis capitata*	2,000[a]
Israel	Sde Eliyahu	*Ceratitis capitata*	50[a]
Mexico	Metapa	*Ceratitis capitata*	500–600[a]
Peru	Lima and Piura	*Ceratitis capitata*	400–500[a]
Portugal	Madeira	*Ceratitis capitata*	50[a]
South Africa	Stellenbosch	*Ceratitis capitata*	10–12[a]
Spain	Valencia	*Ceratitis capitata*	500[a]
United States	Hawaii	*Ceratitis capitata*	200–250[a]
Moths			
Canada	Okanagan	*Cydia pomonella*	12–15
United States	Arizona	*Pectinophora gossypiella*	150–200
South Africa	Citrusdal	*Thaumatotibia leucotreta*	10–15
Screwworms			
Mexico	Tuxtla	*Cochliomyia hominivorax*	100–500
Panama	Panama City	*Cochliomyia hominivorax*	100–150
Tsetse			
Tanzania	Tanga	*Glossina austeni*	60–100[a]
Ethiopia	Kaliti	*Glosssina pallidipes*	1,000
Burkina Faso	Bobo-Dioulasso	*G. palpalis gambiensis*	40–50[a]

Table 1.3 – Mass rearing facilities supplying insects for use in Sterile Insect Technique programs in the world ([a]sterile male-only production, otherwise 50% sterile males and 50% sterile females) (source: Resh and Cardé 2009). *Bactrocera tryoni* is now mass-reared also in a new facility located in Port Augusta (Australia).

Factors of variability		Fitness consequences
Exogenous factors	*Endogenous factors*	Development time and patterns
		Lifespan
Temperature	Mass and body reserves	Mortality
Duration of exposure	Life-history strategy	Fecundity
Rate of cooling or heating	Nutrition	Oviposition period
Gradual acclimation	Mode of reproduction	Sex ratio
Rapid acclimation	Age/stage	Sterility
Acclimatization	Dormancy status	Mating behavior
Developmental temperature	Gender	Mobility and flight capacity
Constant or fluctuating cold exposure		Foraging behavior
Combined cold exposure		Parasitism
Humidity		Intergenerational effects
Photoperiod		F1 progeny biomass
Chemicals		Morphological alterations
Oxygen concentration		Beneficial effects
Handling		

Table 1.4 – List of exogenous and endogenous factors of variability that affect cold storage tolerance and fitness-related traits that may be affected by cold storage (source: Colinet and Boivin 2011).

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2. Effects of storage at suboptimal temperatures on the in vitro-reared parasitoid *Exorista larvarum* (Diptera: Tachinidae)

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2.1 Abstract

Exorista larvarum (L.) is a tachinid parasitoid native to the Palearctic region, known as an antagonist of lepidopterous defoliators. This species is suitable to be cultured in vitro, and yields of fecund adults, approaching those usually attained in host larvae, have been previously achieved on artificial media. Direct oviposition by *E. larvarum* on media has not yet been obtained, and the eggs for the in vitro rearing are routinely removed from parasitized host larvae. However, many eggs are usually laid throughout the cage by captive females and can be retrieved by placing them on artificial media. Storage at low temperatures provides a method for prolonging the development of insects and stockpile them when not needed immediately. We studied the effects of storage at 20 °C (for 5 days or until pupation) or 15 °C (for 5 days or until egg hatching) on the in vitro development of *E. larvarum.* Lower temperatures were excluded, because previous studies showed a strong negative impact on hatching when the eggs were stored at 5 °C or 10 °C. For the experiments, eggs were removed from hosts and placed on an artificial medium. The results suggested that it is possible to delay the development of the in vitro-reared *E. larvarum*, which eventually reached the adult stage, although some negative effects on fly quality (i.e., longevity and fecundity) were also observed. Because quality is not an

absolute concept, all the situations tested in this study could be appropriate according to the current requirements.

2.2 Introduction

Exorista larvarum (L.) (Diptera: Tachinidae) is a polyphagous larval parasitoid of Lepidoptera, well distributed throughout Europe, northern Africa, and several Asian regions (Cerretti and Tschorsnig 2010). It is well known as an antagonist of lepidopterous defoliators, and it has been used for inoculative releases to control the gypsy moth *Lymantria dispar* (L.) (Lepidoptera: Erebidae) in North America, where it has become established (Kenis and Lopez Vaamonde 1998). *Exorista larvarum* females lay macrotype eggs on the host cuticle, thus showing a direct oviposition strategy. The newly hatched larvae penetrate the host body and grow continuously until pupation (Michalkova et al. 2009).

The importance of parasitoids in biological control has encouraged the development of artificial media (in vitro rearing) to evade from the necessity of culturing their natural or factitious hosts (Morales-Ramos et al. 2014a). Several characteristics make *E. larvarum* particularly suitable to be cultured on artificial media. They include gregariousness, polyphagy, and the fact that the larvae develop independently of host hormonal balance (Dindo 2011). The complete in vitro development of this tachinid was obtained (at 26 ± 1 °C) on different artificial media composed of crude ingredients, with yields of fecund adults approaching those usually found using *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) as a factitious host (Dindo et al. 2006; 2016). However, direct oviposition by *E. larvarum* on artificial media has not yet been achieved, and the eggs utilized for the in vitro rearing are routinely removed from previously parasitized host larvae (Dindo and Grenier 2014).

At the Department of Agricultural and Food Sciences (DISTAL, University of Bologna, Italy), a stock colony of *E. larvarum* is currently maintained on *G. mellonella.* Despite the availability of host larvae, many eggs are usually laid throughout the cage and, as a rule, they are lost. Previous studies have, however, shown that the eggs laid out of host may be retrieved to rear *E. larvarum* by placing them on an artificial medium, with no decrease in adult production and fly quality compared with eggs removed from parasitized host larvae, at least for one generation (Dindo et al. 2007; Marchetti et al. 2008). This possibility may be useful for colony augmentation, especially when the host is scarce. The storage of the eggs, either removed from host larvae or laid out of host, may represent a further tool to facilitate the management of the parasitoid colony. Temperature plays a key role to successfully store insects (Leopold 1998; 2007). Natural enemies, including tachinids (on which, however, little information is available), are usually stored at temperatures between 4 °C and 15 °C (van Lenteren and Tommasini 2003; Dindo and Grenier 2014; Mahi et al. 2014). Depalo et al. (2007) showed that *E. larvarum* eggs, placed on agar, may be stored for a short period (5 days) at 20 °C or 15 °C. In both conditions, when the agar with eggs was restored at the standard rearing temperature of 26 °C, the egg hatching did not differ compared with the control eggs, maintained at 26 °C from the beginning of the experiment. Conversely, there was a dramatic reduction of the hatching rate when the eggs were stored at 5 °C or 10 °C. On this basis, the experiments described in this article were intended to study the effects of short-term storage at 20 °C or 15 °C on the in vitro development of *E. larvarum* and quality parameters of the resulting adult flies, with the final aim to contribute in making the rearing of this species more flexible and easy to manage. Parasitoid eggs removed from the host cuticle were used for the experiments, postponing the study with eggs laid out of host at a later date.

2.3 Materials and Methods

2.3.1 Insect colonies

A laboratory colony of *E. larvarum* was maintained at DISTAL on *G. mellonella* larvae. The tachinid adults were kept in plexiglass cages (40 x 30 x 30 cm) at 26 ± 1 °C, $65 \pm 5\%$ relative humidity (RH), and a photoperiod of 16:8 (L:D) h, and given water, sugar cubes, and cotton balls soaked in a honey and water solution. The colony was established in 2004 from adults which had emerged from *Hyphantria cunea* (Drury) (Lepidoptera: Erebidae) larvae collected in field in the province of Modena (44° 10′49″ N, 10° 38′54″ E, Emilia Romagna, northern Italy). *Galleria mellonella* larvae were reared on an artificial diet (Campadelli 1987) and kept at 30 ± 1 °C, $65 \pm 5\%$ RH and in complete darkness.

2.3.2 In vitro rearing procedure

In the experiments, the skimmed milk-based artificial medium developed by Mellini and Campadelli (1996) was used. The in vitro rearing of *E. larvarum* was performed in 24-well plastic rearing plates (Falcon, Corning Inc., NY). In each well, a piece of cotton (15±0.5 mg) was placed as a supporting substrate for the liquid medium (0.4 ml medium per well; Dindo et al. 2003). After flattening the medium-soaked cotton with a sterile glass rod, *E. larvarum* eggs were placed individually in each well. The newly laid eggs were removed from previously superparasitized *G. mellonella* larvae and transferred onto medium as described by Dindo et al. (2007). All operations were performed under aseptic conditions. Finally, the plates were sealed with Parafilm, wrapped with tinfoil, labelled and placed in incubators with the experimental conditions required for the tests. The in vitro rearing procedure adopted in the experiments is showed in Figure 2.1.

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2.3.3 Storage at 20 °C

The plates with eggs were placed in an incubator set at 20 °C and either transferred at the standard rearing temperature (26 °C) after 5 days (the longest egg storage time tested by Depalo et al. [2007]) (1) or maintained at 20 °C throughout the egg and larval development until pupation (= constant storage) (2). Control plates with eggs were constantly kept at 26 °C (3). In all treatments, the plates were maintained in complete darkness and at $65 \pm 5\%$ RH, except when they were removed for daily inspections, aimed at observing hatched eggs and larval development. The newly formed puparia were weighed, placed singly in glass tubes, and kept at 26 ± 1 °C, $65 \pm 5\%$ RH, and a photoperiod of 16:8 (L:D) h until emergence. The newly emerged adults were sexed. To test reproduction capacity, same-day emerged female flies were placed inside a plexiglass cage (20 x 20 x 20 cm) with an equal number of males obtained from the laboratory colony and maintained at the standard rearing conditions described above. Female longevity from emergence was recorded. After 3 days from pairing, mature *G. mellonella* larvae (three per female) were inserted in the cages and removed after 1 h (Dindo et al. 2016). The eggs (F1 eggs) laid on their integument were counted. The exposure of larvae to *E. larvarum* was performed daily until death of the parasitoid female. The parasitized *G. mellonella* larvae were placed in 6-cm-diameter plastic cups with small holes on the lid and maintained at 26 ± 1 °C, $65 \pm 5\%$ RH, and a photoperiod of 16:8 (L:D) h. Adult emergence (F1 adults) was recorded.

2.3.4 Storage at 15 °C

A preliminary trial was conducted to evaluate *E. larvarum* capacity to develop in vitro from egg to puparium at a constant temperature of 15 °C. Two 24-well plates with *E. larvarum* eggs on an artificial medium (48 eggs in total) were prepared as described above and constantly stored at the experimental temperature. Because most larvae died as

second instars and only one puparium (which resulted in one adult) was obtained, the main experiment was only focused on the storage of eggs. On the basis of these observations and also considering the trials carried out by Depalo et al. (2007) (where the hatching rate dropped following storage at 5 °C or 10 °C), we have set 15 °C as a lower-bound limit for storing *E. larvarum* eggs in our experiments. Our storage conditions comprised the following treatments: storage of plates with eggs at 15 °C for 5 days (1), or until the first egg of the plate hatched (2). In both treatments, the plates were then placed at the standard temperature of 26 °C and kept in darkness at 70% RH. Control plates with eggs were kept at 26 °C from the beginning (3). The experiment was conducted adopting the same procedure described for the previous one.

In both experiments, four replicates of 24 eggs each were carried out and temperature variability was ±1 °C for all treatments.

2.3.5 Parameters

The results were evaluated in terms of the following parameters: egg development period (from egg placement on media to hatching; days), hatching rate (%), female larval and pupal development period (days), male larval and pupal development period (days), puparium yield (%; based on the original egg number [= 24]), adult yield (%; calculated on the original egg number), female and male puparial weight (mg), sex ratio (% of females), and female longevity from emergence (days). 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 throughout female life span (= E), as an estimate of fecundity (Dindo et al. 2006). The percentage of eggs which produced an adult (= F1 adult yield) was also recorded.

2.3.6 Statistical analysis

Data were analyzed using the software STATISTICA 10.0 (StatSoft 2010). The significance level was set at 0.05 for all statistical procedures. Prior to analysis, the percentage values were transformed using an arcsine transformation (Zar 1984). A one-way ANOVA analysis was performed on data, followed by Tukey's HSD test when a significant difference occurred. In case of variance heterogeneity, the Kruskal-Wallis H non-parametric test was used, and means were compared pairwise with a Mann-Whitney U test.

2.4 Results

2.4.1 Storage at 20 °C

The duration of the egg development was significantly longer (of about 1-1.5 days) in both treatments with eggs stored at 20 °C compared with the control eggs (Figure 2.2). No significant difference was found for the hatching rate among the control parasitoids and those maintained at 20 °C either for 5 days or until puparium formation (Figure 2.3). Results for the larval and pupal development period were similar for females and males. Namely, the developmental period of the individuals stored at 20 °C until puparium formation was about 1 week or 5 days longer compared with the controls and the individuals stored for 5 days, respectively. For both sexes, the difference was significant among all treatments (Table 2.1).

The 5-day storage resulted in a significant reduction of the percentages of puparia and adult yields compared with the constant storage (until pupation). Both the 5-day and the constant storage were not significantly different from the controls for these parameters (Table 2.1).

The female and male puparial weight and the adult sex ratio did not differ significantly among the three storage conditions. The storage of *E. larvarum*, either for 5 days or until pupation, negatively, and significantly, affected the longevity of the resulting females. In both treatments, females lived about 5 days less than the control (Table 2.2).

The mean number of eggs laid by the females throughout their life span (F1 eggs) was dramatically (and significantly) lower after storing *E. larvarum* until puparium formation compared with the control females. No significant difference was found between the eggs laid by the females obtained from the 5-day-stored eggs and the other two treatments. For the parasitoids obtained from eggs and larvae stored until puparium formation, the F1 adult yield was lower compared with the other storage conditions. The difference was, however, significant only when compared with the 5-day storage (Table 2.2).

2.4.2 Storage at 15 °C

The duration of the egg development was significantly shorter (of about 4.5-6.5 days) for the control eggs compared with those stored either for 5 days or until the first egg of the plate hatched (Figure 2.4). The hatching rate was not significantly affected by the storage condition (Figure 2.5). The male and female larval and pupal development periods, the puparial and adult yields (Table 2.3), and sex ratio (Table 2.4) did not differ significantly among the three treatments. The control female puparia were significantly heavier than those obtained from eggs stored at 15 °C, either for 5 days or until the first egg hatched. No difference was found for male puparia. The female longevity, fecundity, and the percentage of emerged adults obtained from eggs (F1 adult yields) did not differ significantly among the three storage conditions (Table 2.4). It has, however, to be noted that the number of F1 eggs laid by the control females in this experiment was much lower compared with storage at 20 °C.

2.5 Discussion

Reliable and efficient mass rearing techniques are required to produce effective biological control agents at competitive costs (van Lenteren 2012; Morales-Ramos et al. 2014b). The role of tachinid parasitoids in applied biological control is, however, often underestimated, although some examples have been reported of the successful use of these beneficial insects in classical and augmentative biocontrol programs (Grenier 1988). The limited interest raised by tachinids as biocontrol agents has implied that also the studies so far conducted on their production techniques are relatively few, compared with those concerning hymenopterous parasitoids. Nevertheless, the availability of effective rearing procedures for tachinids may be important not only in the view of field release, but also on a small-scale, for studies concerning parasitoid biology as well as for applied research (Dindo and Grenier 2014). *Exorista larvarum*, which can be easily mass-produced both in vivo, using factitious hosts, and in vitro, on artificial media, is one of the tachinid species which deserves to be better exploited both as biocontrol agent and for research purpose. For example, it has been used as a model species in studies aimed at testing the side effects of pesticides on selected non-targets (Marchetti et al. 2009; 2012; Francati and Gualandi 2017). Studies aimed at further improving the rearing technique of this parasitoid are, therefore, justified.

Storage at low temperature provides a method to delay the development of insects and stockpile them when not immediately needed, thus permitting a more flexible rearing schedule while reducing the costs at the same time (Glenister and Hoffmann 1998). Coudron et al. (2007) discussed the possibility to improve the cold storage of different stages of entomophagous insects, through dietary change. Similarly to other topics dealing with parasitoid culture, also storage, both in the short and in the long run, has been especially studied for hymenopterans (van Lenteren and Tommasini 2003; Bayram

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et al. 2005; Lins et al. 2013; Mahi et al. 2014). Little data are available for tachinids and most of them are nor very recent. Fusco et al. (1978) successfully stored *Compsilura concinnata* (Meigen) (Diptera: Tachinidae), both as young larva in the host and as puparium, at 10-15.6 °C for 2-4 weeks. Leopold (1998) gave examples of tachinids of different species which were stored as puparia for 8 months in diapausing conditions, suggesting that cold storage may benefit from dormancy induction. *Archytas marmoratus* (Townsend) (Diptera: Tachinidae), a species showing an indirect oviposition strategy (e.g., the females lay eggs close to host and the first instars have to wait for a host to pass by) could be stored as a young maggot at 13 °C for a few days (Gross and Johnson 1985). Moreover, Campadelli (1983) showed that microtype eggs of *Pseudogonia rufifrons* (Wiedemann) (Diptera: Tachinidae), which were released by parasitoid females on the host food, remained viable at 4 °C for 60 days. In the present work, the availability of artificial media allowed the storage of in vitro-reared *E. larvarum* from the egg stage, an objective that had not been achieved yet for any parasitoid showing direct oviposition strategy. The results suggested that short-term storage of eggs and larvae of *E. larvarum* on artificial media is possible at 15 °C or 20 °C, e.g., lower temperatures than the standard one (26 ± 1 °C). Similarly to other studies (Lysyk 2004; Colinet and Hance 2010), by increasing the storage duration, the egg development was delayed (up to >9 days at 15 °C) without affecting the hatching rate. For this parameter, no significant difference was found between the stored eggs and the control eggs for any of the temperatures and storage periods tested. The larval and pupal development period was delayed for the parasitoids stored at 20 °C on the medium until puparium formation. Conversely, for the parasitoids moved to the standard temperature (26 ± 1 °C) after storage at 15 °C, the larval and pupal development period (calculated from egg hatching) was not significantly different compared with the controls. Therefore, egg storage at 15 °C, either for 5 days or

until the first egg of the plate hatched, resulted in a delayed egg hatching, but did not affect the subsequent parasitoid development. Storage at 20 °C or 15 °C did not significantly impact parasitoid puparial and adult yields in comparison with the controls that were always maintained at the standard temperature.

Storage of entomophagous insects at suboptimal temperatures is usually associated with a fitness reduction (van Lenteren and Tommasini 2003; Bayram et al. 2005). In the present study, the longevity of the females obtained from the stored eggs, their fecundity, and the F1 adult yield were affected by storage at 20 °C. In particular, the longevity was significantly lower compared with the control when storage occurred at 20 °C, either for 5 days or until pupation. Conversely, there was no significant adverse effect on these parameters when eggs were stored at 15 °C. Fitness and fecundity were usually found to be correlated with body size in tachinids (Bourchier 1991; Reitz and Adler 1995), but in our study, despite their lower puparial weight, *E. larvarum* females stored as eggs at 15 °C had a comparable fecundity with the control ones. This result is in line with the observations of Dindo et al. (2006), who did not find any correlation between the puparial weight of in vitro-reared *E. larvarum* females and their fecundity, and concluded that puparial weight alone cannot be considered a reliable enough quality assessment criterion.

The results obtained in the present study suggest that it is possible to store eggs (and larvae) of *E. larvarum* on artificial media, in the short-term, at a lower temperature than the standard one, with the advantage of a more flexible rearing schedule and easier transportation (which may occur under nonoptimal temperature). Given that the concepts of "quality" and "quantity" are not absolute, but depend on the objectives of the insect production (Grenier 2009), all the situations tested (i.e., egg storage at 15 °C or 20 °C for 5 days, egg storage at 15 °C until the first egg hatched, or in vitro development at 20 °C

from egg to pupation) may be applied according to the current requirements. A certain degree of difference between the parasitoids produced under the standard conditions and those stored at low temperature has, however, to be taken into account. In general, the quality of the entomophagous insects produced on artificial media or diets is lower in comparison with those reared conventionally, using the natural host or prey (Grenier and De Clercq 2003; Riddick 2009). For this reason, independently of storage conditions, we emphasize the importance of quality control, especially for the in vitro-reared parasitoids and predators (Leppla 2014).

Suboptimal temperature storage of *E. larvarum* eggs and larvae on artificial media needs to be further explored, as a helpful tool to overcome colony discontinuity problems, owing to host scarcity. Future studies will address the possibility to combine the recovery of the eggs laid throughout the cage by captive females (Dindo et al. 2007) with their storage under suboptimal conditions.

2.6 Acknowledgments

2.7 Statement of Authorship

MB, EM and MLD conceived and designed the experiments. MB conducted the experiments. MB and MLD analysed data. MB wrote the Chapter, and all authors read and approved the Chapter.
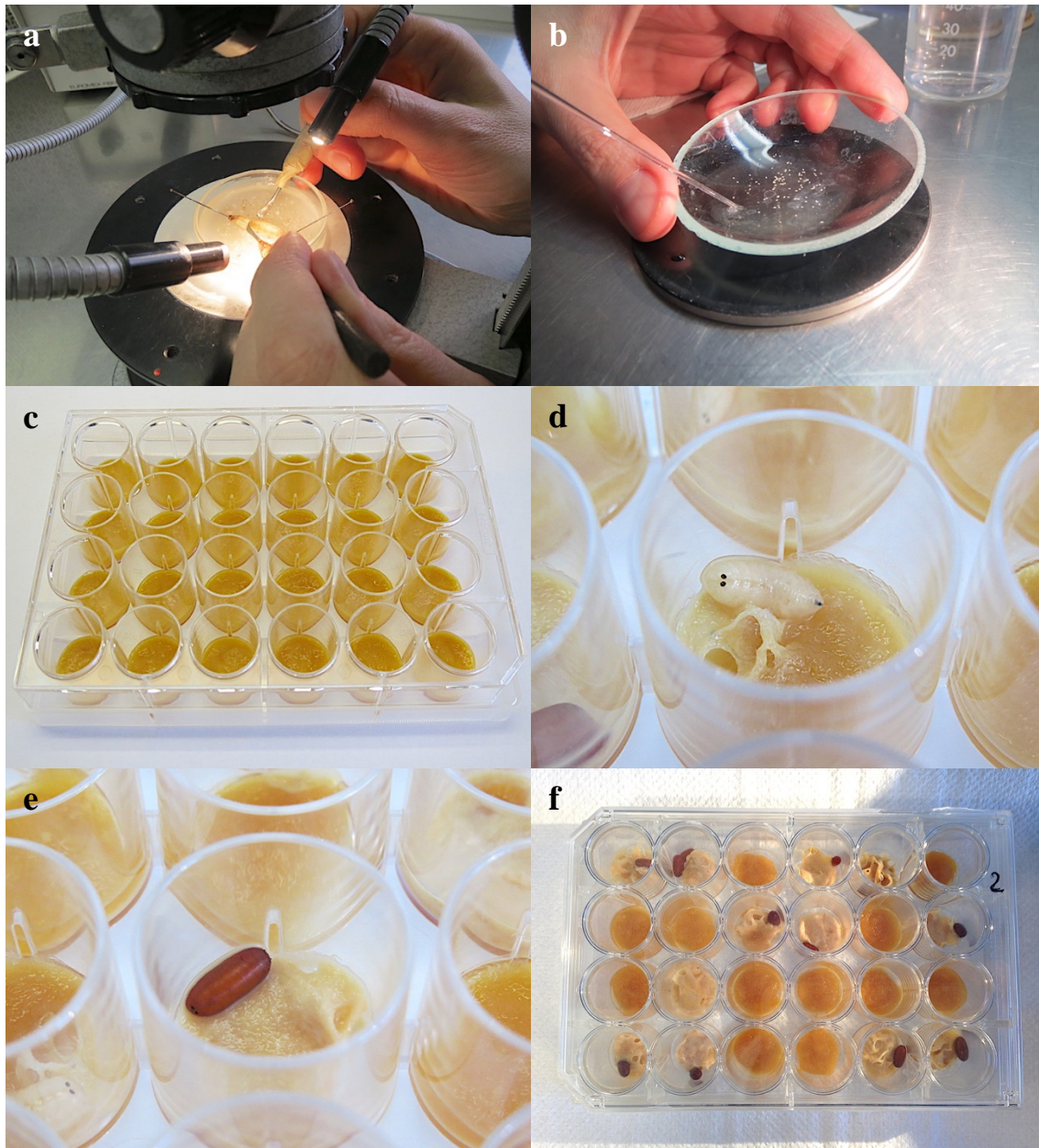
2.8 List of figures

Figure 2.1 – *Exorista larvarum* in vitro rearing procedure utilised in the experiments: (a) eggs are obtained from superparasitized *Galleria mellonella* larvae exposed to *E. larvarum* females and removed from their integument with the aid of spatulas under the stereomicroscope; (b) once placed on a glass lens, eggs are disinfected through alcohol washings using a glass pipette; (c) 24-well plastic rearing plate containing 4 ml of larval medium per well on cotton support, in which eggs are placed (one egg per well); (d) mature *E. larvarum* third instar larva developed in the artificial medium; (e) newly-formed *E. larvarum* puparium; (f) rearing plate at the end of the rearing period, the in vitro-obtained puparia are about to be removed, weighed and transferred in an adult rearing cage. All photos were taken by the author.

Figure 2.2 – Developmental period (mean ± SE) from egg placement on media to hatching for *Exorista larvarum* eggs stored continuously at 26 °C (control) or maintained at 20 °C for 5 days or until pupation ($F_{2,9} = 15.07$; $P = 0.0013^*$).

Figure 2.3 – Hatching rate (mean ± SE) calculated on the original egg number (= 24) for in vitro-reared *Exorista larvarum* stored continuously at 26 °C (control) or maintained at 20 °C for 5 days or until pupation ($F_{2,9} = 3.39$; $P = 0.0799$).
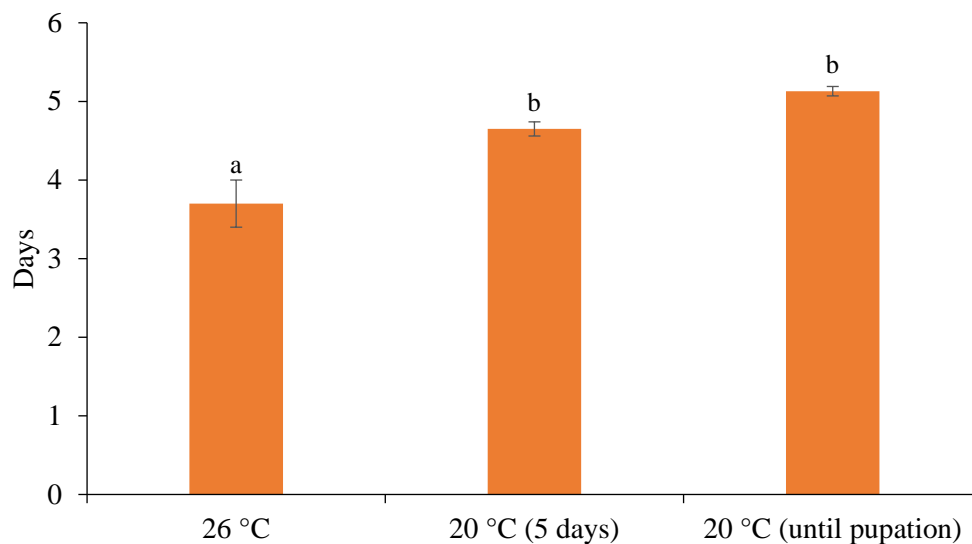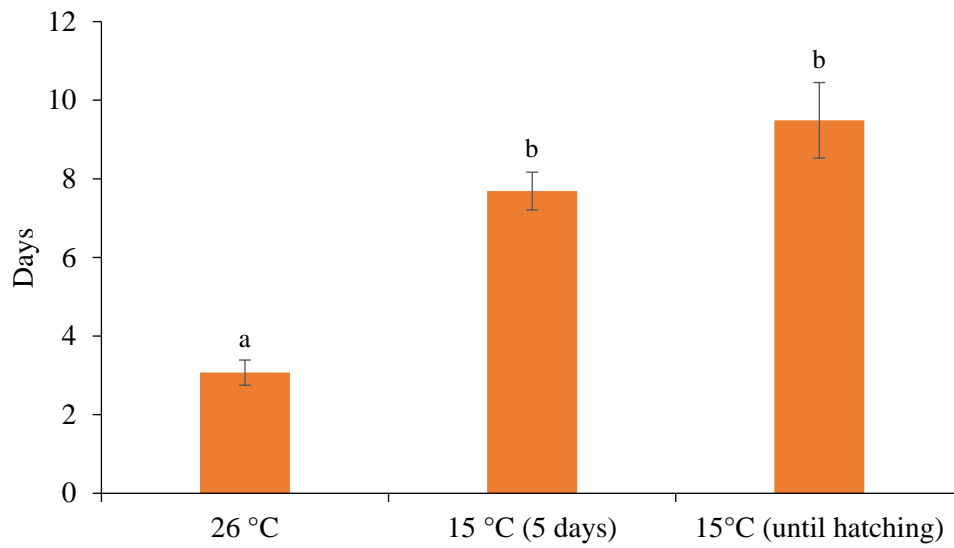
Figure 2.4 – Developmental period (mean ± SE) from egg placement on media to hatching for in vitro-reared *Exorista larvarum* stored continuously at 26 °C (control) or maintained at 15 °C for 5 days or until hatching (= when the first hatched egg of the plate was detected) ($H_{12} = 9.85$; $P = 0.0073*$).
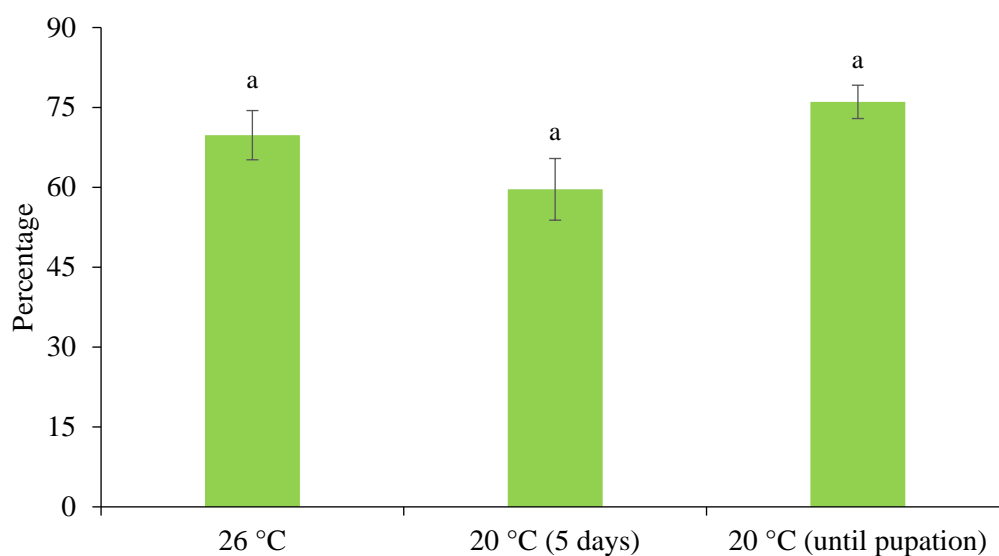

Figure 2.5 – Hatching rate (mean ± SE) calculated on the original egg number (= 24) for in vitro-reared *Exorista larvarum* stored continuously at 26 °C (control) or maintained at 15 °C for 5 days or until egg hatching ($H_{12} = 2.367$; $P = 0.3062$).
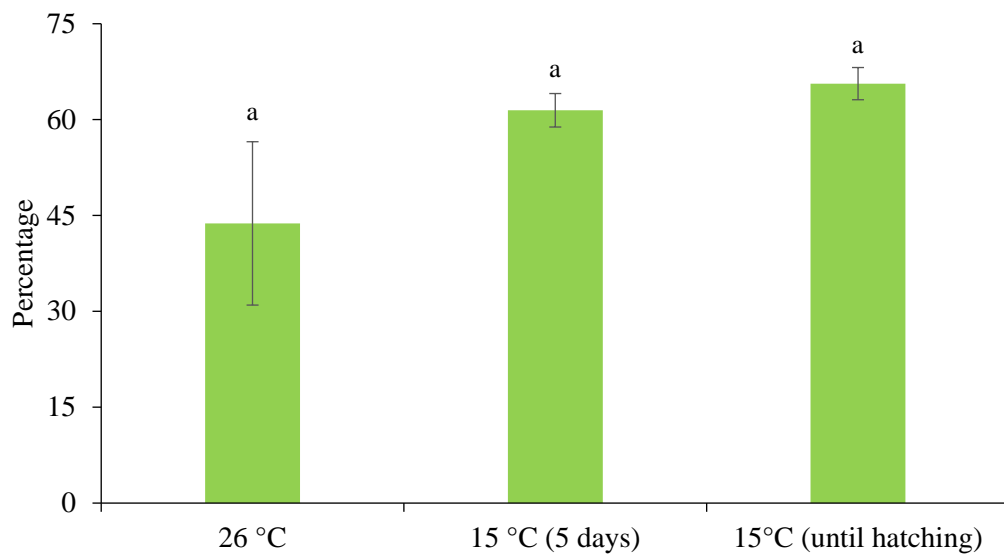
2.9 List of tables

Storage	Larval and pupal development period (days)		Puparium yield (%)[1]	Adult yield (%)[1]
	Females	Males		
26 °C	17.3±0.77[a]	16.65±0.86[a]	58.33±5.1[ab]	53.13±3.56[ab]
20 °C (5 d)	19.43±0.3[b]	18.4±0.52[b]	45.84±3.8[a]	39.58±3.99[a]
20 °C (until pupation)	24.63±1.09[c]	23.35±0.96[c]	67.7±1.9[b]	59.38±3.13[b]
F (df)	90.73 (2, 9)	74.95 (2, 9)	8.09 (2, 9)	8.006 (2, 9)
P	< 0.0001*	< 0.0001*	0.0097*	0.01*

Table 2.1 – Developmental parameters (mean±SE) for the in vitro reared *Exorista larvarum* stored continuously at 26 °C (control) or maintained at 20 °C for 5 days or until pupation. When the storage at 20 °C was completed, the rearing plates (or puparia) were transferred at 26 °C. For each treatment 4 replicates were carried out, each consisting of a rearing plate with 24 eggs. Values followed by different letters in a column are significantly different at $P < 0.05$ (one-way ANOVA followed by the Tukey test).
[1]Calculated on the egg number of the rearing plate (= 24).

Storage	Puparial weight (mg)		Sex ratio (% of females)	Female longevity (days)	Fecundity (F1 eggs/female)	F1 adult yield (%)[1]
	Females	Males				
26 °C	45.58±2.58[a]	49.9±5.74[a]	49.66±6.46[a]	13.2±1.72[a]	142.29±48.22[a]	22.14±2.65[ab]
20 °C (5 d)	47.3±2.02[a]	48.93±4.99[a]	52.08±5.51[a]	8.48±2.72[b]	64.93±42[ab]	28.49±5.81[a]
20 °C (until pupation)	43.98±2.92[a]	44.25±3.02[a]	47.08±3.62[a]	8.78±1.84[b]	37.35±19.01[b]	10.24±1.04[b]
F (df)	1.72 (2, 9)	1.63 (2, 9)	0.04 (2, 9)	6.08 (2, 9)	-----	7.51 (2, 9)
H (N)	-----	-----	-----	-----	6.27 (12)	-----
P	0.2323	0.2487	0.9575	0.0213*	0.0435*	0.012*

Table 2.2 – Puparial weight, sex ratio, female longevity, fecundity and F1 adult yield (mean±SE) for the in vitro reared *Exorista larvarum* stored continuously at 26 °C (control) or maintained at 20 °C for 5 days or until pupation. When the storage at 20 °C was completed, the rearing plates (or puparia) were transferred at 26 °C. Emerged adults were kept at 26 °C. For each treatment 4 replicates were carried out, each consisting of a rearing plate with 24 eggs. Values followed by different letters in a column are significantly different at $P < 0.05$ (one-way ANOVA followed by the Tukey test or Kruskal-Wallis H non-parametric test followed by the Mann-Whitney U test).
[1]Percentage calculated on F1 eggs laid on *Galleria mellonella* larvae.

Storage	Larval and pupal development period (days)		Puparium yield (%)[1]	Adult yield (%)[1]
	Females	Males		
26 °C	17.89±1.52[a]	16.88±1.98[a]	35.42±8.42[a]	30.21±5.98[a]
15 °C (5 d)	16±1.02[a]	14.67±0.6[a]	35.41±5.51[a]	31.25±4.96[a]
15 °C (until hatching)	17.63±1.36[a]	16.58±1.72[a]	33.33±4.50[a]	29.17±5.10[a]
F (df)	2.43 (2, 9)	2.4 (2, 9)	0.0298 (2, 9)	0.04 (2, 9)
P	0.143	0.1461	0.9707	0.9601

Table 2.3 – Developmental parameters (mean±SE) for the in vitro reared *Exorista larvarum* stored continuously at 26 °C (control) or maintained at 15 °C for 5 days or until egg hatching (when the first hatched egg of the plate was detected). When the storage at 15 °C was completed, the rearing plates were transferred at 26 °C. Newly-formed puparia were kept at 26 °C. For each treatment 4 replicates were carried out, each consisting of a rearing plate with 24 eggs. Values followed by different letters in a column are significantly different at $P < 0.05$ (one-way ANOVA followed by the Tukey test).
[1]Calculated on the egg number of the rearing plate (= 24).

Storage	Puparial weight (mg)		Sex ratio (% of females)	Female longevity (days)	Fecundity (F1 eggs/female)	F1 adult yield (%)[1]
	Females	Males				
26 °C	43.91±3.94[a]	46.26±4.98[a]	54.72±10.22[a]	16.35±4.14[a]	69.55±32.2[a]	27.22±3.29[a]
15 °C (5 d)	37.34±5.34[b]	42.34±3.79[a]	54.17±5.83[a]	13.87±2.94[a]	70.25±34.7[a]	37.16±1.99[a]
15 °C (until hatching)	35.66±2.74[b]	40.00±1.82[a]	47.22±1.61[a]	14.25±5.96[a]	52.92±21.02[a]	30.79±3.19[a]
F (df)	4.422 (2, 9)	2.863 (2, 9)	-----	0.349 (2, 9)	0.430 (2, 9)	3.17 (2, 9)
H (N)	-----	-----	0.422 (12)	-----	-----	-----
P	0.0459*	0.1091	0.8096	0.7141	0.6629	0.0907

Table 2.4 – Puparial weight, sex ratio, female longevity, fecundity and F1 adult yield (mean±SE) for the in vitro reared *Exorista larvarum* stored continuously at 26 °C (control) or maintained at 15 °C for 5 days or until egg hatching (when the first hatched egg of the plate was detected). For each treatment 4 replicates were carried out, each consisting of a rearing plate with 24 eggs. When the storage at 15 °C was completed, the rearing plates were transferred at 26 °C. Newly-formed puparia and emerged adults were kept at 26 °C. Values followed by different letters in a column are significantly different at $P < 0.05$ (one-way ANOVA followed by the Tukey test or Kruskal-Wallis H non-parametric test followed by the Mann-Whitney U test).
[1]Percentage calculated on F1 eggs laid on *Galleria mellonella* larvae.

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3. Storage at suboptimal temperature of *Exorista larvarum* (Diptera: Tachinidae) puparia for mass rearing programs

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3.1 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 increase their use in biological control programs. *Exorista larvarum* (L.) is a Palearctic tachinid, 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 have investigated the effects of storage of 1-day old puparia at 15 °C for 7, 14 or 21 days. Lower temperatures (5 and 10 °C) were excluded following the outcome of a preliminary test. Parasitoid emergence and quality control parameters of the female flies obtained from the stored puparia were recorded. A lipid extraction with Chloroform was also performed on adults of both sexes. 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, fecundity and

F1 puparium yield). Our findings may prove useful to increase the flexibility of *E. larvarum* colony management, not only for large-scale rearing of the parasitoid, but also for small-scale research laboratories.

Keywords: Mass rearing, Tachinidae, *Exorista larvarum*, suboptimal temperature, storage.

3.2 Introduction

Biological control programs based on augmentative releases depend upon releasing mass-reared natural enemies into the environment for controlling the population level 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.

Since biological control is a component of Integrated Pest Management (IPM) programs (Kogan 1998), insect mass rearing companies ("biofactories") are in competition with other companies offering alternative pest management solutions. Despite the incredible advances in insect mass rearing technologies made during the last 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 delivered on time in field or greenhouses, especially during the outbreak of their prey or hosts.

Except for species which can enter an induced diapause (Vallo et al. 1976; Quednau 1993), living insects have a fairly short shelf life, meaning that they have to be released in field shortly after their production. The storage at temperatures that are lower than the optimal rearing one 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 and Tommasini 2003), trying to attain a slowdown of 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 depends mostly on the pest population levels in field (Leopold 1998). Moreover, the continuous production chain of entomophagous natural enemies can also experience periods of low production, due to the difficulties raised when rearing their prey or hosts, but also because of equipment failure or temporary unavailability of trained personnel. Regardless of the storage period, a reduction in fitness of the stored insects is commonly observed and represents a serious drawback that must be taken into account (Posthuma-Doodeman et al. 1996; Colinet and Boivin 2011).

Despite their secondary importance in terms of number of species compared to hymenopterans (Feener and Brown 1997), dipteran parasitoids have shown potential for regulating phytophagous insect populations in applied biological control (Dindo and Grenier 2014). Fly parasitoids for use against insect pests, with application for mass rearing and release, belong to the families of Tachinidae, Sarcophagidae, Phoridae, Cryptochaetidae and Bombyliidae (Eggleton and Belshaw 1992). Tachinidae comprise 8,500 worldwide described species and represent the largest and most significant family of nonhymenopteran parasitoids (O'Hara 2013).

The Palearctic tachinid fly *Exorista larvarum* (L.) is a gregarious parasitoid recorded as natural enemy of several species of lepidopteran pests, including the gypsy moth *Lymantria dispar* (L.), the lackey moth *Malacosoma neustria* (L.), the green oak moth *Tortrix viridana* L., *Mamestra brassicae* (L.) and other defoliators of forest or agricultural interest (Herting 1960; Delrio et al. 1983; 1988; Cerretti and Tschornig 2010). The

availability of different mass rearing techniques for *E. larvarum* make it a good candidate to be produced at an industrial scale level and released against target pest species. Indeed, this tachinid showed good potential, in terms of yields and quality, when reared in vivo, in particular on the factitious host *Galleria mellonella* (L.) (Mellini et al. 1993; Depalo et al. 2010), or in vitro, using artificial media (with or without host components) in replacement of live hosts (Bratti and Coulibaly 1995; Mellini and Campadelli 1996; Dindo et al. 2006; 2016). In all techniques, the rearing was performed at 26 °C, which was selected as standard, based on previous experience with other tachinids (Baronio and Campadelli 1979; Bratti et al. 1992).

At present, the use of *E. larvarum* as natural enemy has been limited to sporadic releases in North America for the classical biological control of *L. dispar* in forestry ecosystems (Sabrosky and Reardon 1976). Finally, the parasitoid became established and contributed in lowering the population level of the defoliator (Kenis and Lopez Vaamonde 1998). The tachinid has indeed been considered the second most important antagonist of the gypsy moth (Herting 1960), a host on which up to 50% mortality was observed after attack by the parasitoid in cork oak forests in Sardinia (Luciano and Prota 1979).

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 the storage at suboptimal temperatures of the tachinid puparia may attain a prolonged pupal development while having the minimum impact on the fitness of the resulting adults (Easwaramoorthy et al. 2000). As outlined by van Lenteren and Tommasini (2003), the pupal stage is the most suitable stage for insect short-term storage. In the view of possible utilisation of low temperature storage protocols for mass rearing of tachinid flies, our study on *E. larvarum* is justified by the potential

usefulness of this species to be used against several insect pests (Simões et al. 2004; Depalo et al. 2010; 2012). Moreover, the tachinid production may also be useful in the view of laboratory studies evaluating the impact of insecticides on non-target species (Marchetti et al. 2009; Francati and Gualandi 2017). Since preliminary tests excluded the possible use of 5 and 10 °C for our purpose, we focused on the use of 15 °C as a storing temperature, investigating the effects of three different storage periods, namely 7, 14 or 21 days. Similar storage periods were tested by Gross and 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 to be one of the reasons of failure following field release of these parasitoids in biocontrol strategies (Grenier 1988; Dindo and Grenier 2014). Therefore, the development of an efficient rearing technique for *E. larvarum*, including protocols for its storage at suboptimal temperature, may contribute in making this species as an even more suitable candidate for future production and utilisation in applied biological control programs.

3.3 Materials and Methods

3.3.1 Insect colonies

A stock colony of *E. larvarum* was kept in the Entomology Laboratory of the Department of Agricultural and Food Sciences (DISTAL, University of Bologna) (Figure 3.1), using *G. mellonella* as factitious host for its continuous in vivo rearing. The colony was started in 2004 with adults that had emerged from *L. dispar* and *Hyphantria cunea* (Drury) (Lepidoptera: Erebidae) larvae collected in 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) (Emilia Romagna, northern Italy). Adult flies were kept in plexiglass cages (40 x 30 x 30 cm, with 50-70

individuals per cage) in a controlled environment room set at 26±1 °C, 65±5% relative humidity (RH), and a photoperiod of 16:8 (L:D) h ("standard conditions"). Adult diet consisted of sugar cubes, cotton balls soaked in a honey and water solution and water provided in drinking cups with soaked cotton.

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

3.3.2 Preliminary test

A preliminary test was carried out to assess the possibility of 1-day old *E. larvarum* puparia to complete their development and lead to adult emergence when short-term stored at 5 °C and 10 °C. For this purpose, 10 puparia were maintained at each of these suboptimal temperatures and 10 control puparia were kept at 26 °C. After 21 days of storage, the cold-treated puparia were brought back at 26 °C, but none of them completed the pupal development after additional 21 days, resulting in 100% mortality. Conversely, 100% of the control puparia emerged as adults. For these reasons, and also considering the studies carried out with *E. larvarum* eggs by Benelli et al. (2017), we selected 15 °C as a first lower-bound suboptimal temperature to be studied.

3.3.3 Experimental conditions

In our experiment, we tested the capacity of *E. larvarum* pupae to survive and complete development when stored at 15 °C for 7 (1), 14 (2) or 21 days (3) and give origin to high quality adults, in comparison with pupae kept at the standard temperature of 26 °C (4).

For the experiment, *E. larvarum* puparia were collected from the stock colony and weighed. Puparia to be stored at the experimental conditions (weight range 35-55 mg) were selected. For each of the four treatments, four replicates were carried out, each consisting of 5 puparia placed in a plastic cup (6-cm diameter) with small ventilation holes in the cover lid. The plastic cups were stored in an incubator set at 15 ± 1 °C, $65 \pm 5\%$ RH and complete darkness. Temperature and RH were monitored by data loggers (RHT10, Extech, USA). To allow the newly-formed puparia to complete their first crucial period of development (Tsiropoulos 1972), they were transferred to 15 °C after having spent the first 24 h at 26 °C, while the control puparia were kept continuously at 26 °C. For treatments (1), (2) and (3), once the storage period at 15 °C was completed, puparia were restored at 26 °C. Each plastic cup was inserted into a small plexiglass cage (20 x 20 x 20 cm) waiting for adult emergence to occur.

3.3.4 Adult quality control

The quality control assessment was performed for *E. larvarum* females. When an adult female emerged, it was individually placed in a small plexiglass cage of the same type described above and provided water, sugar cubes and a cotton ball soaked in honey/water solution (Figure 3.2). Immediately after, the 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 could be placed together, in the same cage, and paired with an equal number of males from the stock colony. As the preoviposition period lasts about 3 days (Mellini et al. 1994), the first host exposure to flies took place on the 3rd day from pairing. Adult food was removed and 3 mature *G. mellonella* larvae per female were inserted 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 in small plastic cups, similar to those used for storing

puparia, under the standard environmental conditions. Newly-formed F1 puparia and F1 adults were counted. Since *E. larvarum* females lay most eggs during their first 10 days from the beginning of oviposition (Dindo et al. 1999), the parasitization procedure was repeated daily for 10 days, or until death if female flies died before this timeframe.

3.3.5 Lipid analysis

Newly emerged adults, both males and females which were not utilised for the quality assessment test, were frozen at -20 °C within 24 h from their emergence for lipid content analysis. For this purpose, chloroform was used as a moderately polar solvent for the extraction (Raubenheimer et al. 2007; Ponton et. al 2011; 2015).

Flies were first dried in oven at 60 °C for 48 h, individually placed in 5-mL glass tubes. 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 were measured with a microscale balance (AT21, Mettler Toledo, USA). Chloroform (Sigma-Aldrich) was added to the samples (1 mL per fly) using a pipette and a plastic plug applied to the tubes. 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 lately expressed as a percentage of the initial dry weight.

3.3.6 Parameters

The parameters measured during the experiment were: pupal development period (both sexes, males and females) (days), pupal development from restoring at 26 °C (both sexes, males and females) (days), adult yield (calculated over the original puparium number) (%), sex ratio (% of females), female longevity (from emergence) (days). 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 throughout female life span (= E), as an estimate of fecundity (Dindo et al. 1999; 2006). Additional parameters were: lipid content (both sexes, males and females) (%), F1 puparium yield (calculated over the number of eggs laid throughout female life span) (%), F1 adult emergence (calculated over the number of F1 puparia) (%), F1 deformed adults (calculated over the total number of emerged adults) (%).

3.3.7 Statistical analysis

Data analysis was performed using the statistical program STATISTICA 10.0 (StatSoft 2010). The percentage values were transformed using an arcsine transformation prior to analysis (Zar 1984). Data (pupal development period, pupal development from restoring at 26 °C, adult emergence, sex ratio, female longevity, fecundity and lipid content) were analysed by a one-way ANOVA. A Levene's test was performed before the analysis to assess if the variances of means were homoscedastic. The Tukey's HSD or the Unequal N HSD (fecundity and lipid content) tests were used to separate the means. The independence of storage conditions for *E. larvarum* puparia and F1 puparium yield, F1 adult emergence and F1 deformed adults was tested using 2 by 2 contingency tables. The significance level was set at 0.05 for all statistical procedures.

3.4 Results

The exposure at 15 °C, regardless of sex, progressively and significantly extended the pupal development as the storage period increased (Table 3.1). In particular, compared to control puparia, the development was prolonged of about two weeks when puparia were stored at 15 °C for 21 days (Figure 3.3). Adult emergence did not occur while puparia were stored at the suboptimal temperature, but only when they were restored at 26 °C

(Table 3.1). Nonetheless, development progressed during storage and the longer the storage period, the shorter the time (days) required for emergence to occur once brought back at the control temperature (puparia stored at 15 °C for 7 days took about 6 days to emerge when restored at 26 °C, while puparia stored for 21 days took only 1.3-1.8 days) (Table 3.1). On the contrary, adult emergence and sex ratio were not significantly affected by storage conditions (Table 3.2).

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 emerged from the puparia stored at 15 °C for 7 days, compared with control flies (Table 3.2).

The average number of eggs laid by females throughout their life span (F1 eggs) was significantly and considerably reduced when *E. larvarum* puparia were stored at 15 °C for 14 days (61.5 vs 170.75 for control females). A reduction in fecundity was also observed when puparia were stored at 15 °C for 7 and 21 days (120.5 and 105.75, respectively), but this reduction was not significant in comparison with control females (Table 3.2).

The lipid content of the flies did not show any statistical difference among treatments when data from male and female flies were analysed together (Figure 3.4), or when the analysis was performed only on females (Table 3.3). Conversely, the lipid content of male flies was significantly reduced to 12.85% when puparia were stored at 15 °C for 14 days in comparison with the other treatments, including the control (Table 3.3).

Female flies emerged from control puparia laid eggs that gave origin to the lowest F1 puparium yield (18.89%). The highest yield was obtained for the females emerged from puparia stored at 15 °C for 21 days (30.02%) (Figure 3.5). There were significant differences between the control and the storage temperature of 15 °C applied for 14 or 21

days. The difference was also significant between the storage at 15 °C for 7 and 21 days ([Table 3.4](#)). F1 adult emergence and F1 deformed adults were not significantly influenced by storage conditions ([Tables 3.5, 3.6](#)).

3.5 Discussion

Tachinid parasitoids, to be mass-reared and released into the environment for controlling target insect pest populations, have commonly received less attention than hymenopteran parasitoids, on which more research has been performed so far (Dindo and Grenier 2014). Nonetheless, several examples can be found in literature on the use of tachinids in classical and augmentative biological control (Grenier 1998).

Lixophaga diatraeae Townsend is one of the best examples of a tachinid parasitoid mass-reared in facilities and released for the control of the sugarcane borer *Diatraea saccharalis* (F.) (King et al. 1981). The largest biocontrol program entailing augmentative releases of *L. diatraeae* has been established in Cuba, where the tachinid is considered endemic, for the effective control of lepidopterous sugarcane borers (Nicholls et al. 2002; Montes et al. 2008). The successful program reached the productivity of more than 100 million tachinid flies per year produced in a network of more than 50 mass rearing government-funded facilities throughout the island (Medina 2002; Massó Villalón 2007). Other examples of tachinid flies released for augmentation purposes, whether experimental or not, include *Blepharipa pratensis* Meigen and *Compsilura concinnata* Meigen against the gypsy moth (Blumenthal et al. 1979) and *A. marmoratus* against noctuid pests (Proshold et al. 1998). The development of efficient rearing techniques for these and other tachinid species, also including storage at low temperatures, could reduce the rate of failure in biocontrol programs already in place and encourage the development of new ones.

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The possibility to store insect immatures at suboptimal temperatures represents a powerful tool for increasing the flexibility of the production in mass rearing facilities and better adapt it according to the demand (Glenister and 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 on the requirements of the selected insect species.

Our model species, *E. larvarum*, was released in classical biocontrol programs carried out in the 20th century against the gypsy moth in north America (Sabrosky and Reardon 1976). This tachinid fly has, however, shown potential for the control of other forest (Delrio et al., 1983; 1988) or agricultural insect pests, including *Spodoptera littoralis* (Boisduval) (Depalo et al. 2010), *Mythimna unipuncta* (Haworth) (Depalo et al. 2012), *Xestia c-nigrum* L., *Pseudaletia unipuncta* (Haworth), and *Peridroma saucia* (Hübner) (Simões et al. 2004). Valid in vivo and in vitro rearing techniques for *E. larvarum* are already available (Dindo et al. 2006), but protocols for its storage at suboptimal temperatures are scant at present and deserve more attention (Benelli et al. 2017).

In our experiment, we proved that by storing 1-day old *E. larvarum* puparia at 15 °C for 21 days the pupal development can be prolonged of more than 14 days when compared with puparia kept continuosly at 26 °C, with no detrimental effects for all the tested quality parameters. Adult emergence was comparable for each of the tested treatments, meaning that the suboptimal temperature of 15 °C did not arrest the development during storage, but extended it without lethal consequences for the parasitoid. As hypothesised (following the findings of Benelli et al. 2017), insects have adapted to the low temperature maintaining their vital functions (Hance et al. 2007). This observation is also supported by the fact that the longer the storage period at 15 °C, the shorter the time required for

adults to emerge when puparia were restored at the control temperature. The result is consistent with the findings of Mills and Nealis (1992), who investigated cold storage procedures for the puparia of the tachinid *Aphantorhaphopsis* (= *Ceranthia*) *samarensis* (Villeneuve).

When insect parasitoids are stored at suboptimal temperature, the extension of their shelf life routinely results in a decrease in quality (van Baaren et al. 2006; Colinet and 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 and Johnson 2005). In our case, female longevity was significantly shortened by the storage at 15 °C only when the exposure lasted for 7 days (compared to control flies), but a milder reduction was observed for the other storage periods. Our observations are in line with those of Foerster and 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 brought back at the control temperature (25 °C). Fecundity of *E. larvarum* females was also negatively influenced by the suboptimal temperature storage, but only when puparia were exposed to 15 °C for 14 days the difference was significant compared to the control. As demonstrated by Levie et al. (2005), fecundity of female parasitoids that overcome storage at low temperature can be dramatically reduced. Since *E. larvarum* puparia exposed to 15 °C for 21 days achieved a comparable fecundity with the control, also for this parameter we observed a non-linear relationship with the duration of the storage period. A more expected proportional reduction was observed for other stored parasitoids, such as the scelionid *Telenomus busseolae* Gahan (Bayram et al. 2005).

Energetic reserves may be critical to overcome low temperatures exposure and lipid reserve depletion during storage has been found to be correlated with insect survival and longevity (Colinet et al. 2006). In our experiment, we found that *E. larvarum* males

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displayed a lipid content (fraction soluble in chloroform) decline when stored at 15 °C for 14 days during the pupal stage. However, when males were stored for an additional week, lipid decline was not observed. A detailed quality control investigation also on males in future experiments will help to verify a possible effect of fat depletion during cold exposure on parasitoid fitness (Renault et al. 2003).

Beneficial effects following storage at low temperatures are less widely observed than detrimental effects, however, they can be detected (Colinet and Boivin 2001). Considering the F1 puparium yields, we observed that the highest yields were obtained from 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 suboptimal temperature for 10 days gave origin to more progeny than unstored ones. Reasons for these phenomena have not been found in the literature. In our study, the F1 puparium yield may have been influenced by the number of eggs per host laid by parental control females. Excessive superparasitism has been previously found to be detrimental for successful *E. larvarum* development (Mellini and Campadelli 1997).

Furthermore, it has to be taken into account that consequences of storage at low temperatures may not be observed directly on the stored insects but may carry over the next generation (intergenerational effect) (Rako and Hoffmann 2006; Leopold and Chen 2007). However, in our experiment we have not found any negative effect for the parameter we have considered, i.e., the number of F1 deformed adults.

Overall, the outcomes of the present study exclude the possibility to store 1-day old *E. larvarum* puparia at 5 and 10 °C but suggest that storage at 15 °C is suitable for increasing the flexibility of the parasitoid rearing, both on a small or large-scale. As a consequence, this procedure may prove useful for better scheduling releases in field in

biological control programs. In terms of duration of attainable storage, all the tested exposures (7, 14 and 21 days) may be adopted if a certain degree of fitness reduction is tolerable, but this depends on the purposes of the rearing (Grenier 2009). Further research on suboptimal temperature storage of *E. larvarum* immatures is needed to better understand the mechanisms underpinning the physiological mechanisms taking place during storage. Additional factors to be considered may incude pupal age and the possibility to store puparia following a period of acclimation, as a mean to increase their cold hardiness (Hoffmann et al. 2003; Luczynski et al. 2007).

3.6 Acknowledgments

3.7 Statement of Authorship

MB and MLD conceived and designed the experiment. MB conducted the experiment. MB and MLD analysed data. MB wrote the Chapter, and all authors read and approved the Chapter.

3.8 List of figures

Figure 3.1 – *Exorista larvarum* laboratory colony at the Department of Agricultural and Food Sciences (University of Bologna). Newly-formed puparia are placed in plexiglass cages waiting for adult emergence. The provided food consists of sugar cubes, cotton balls soaked in a honey and water solution and a drinking plastic cup with cotton soaked in water (author's photo).
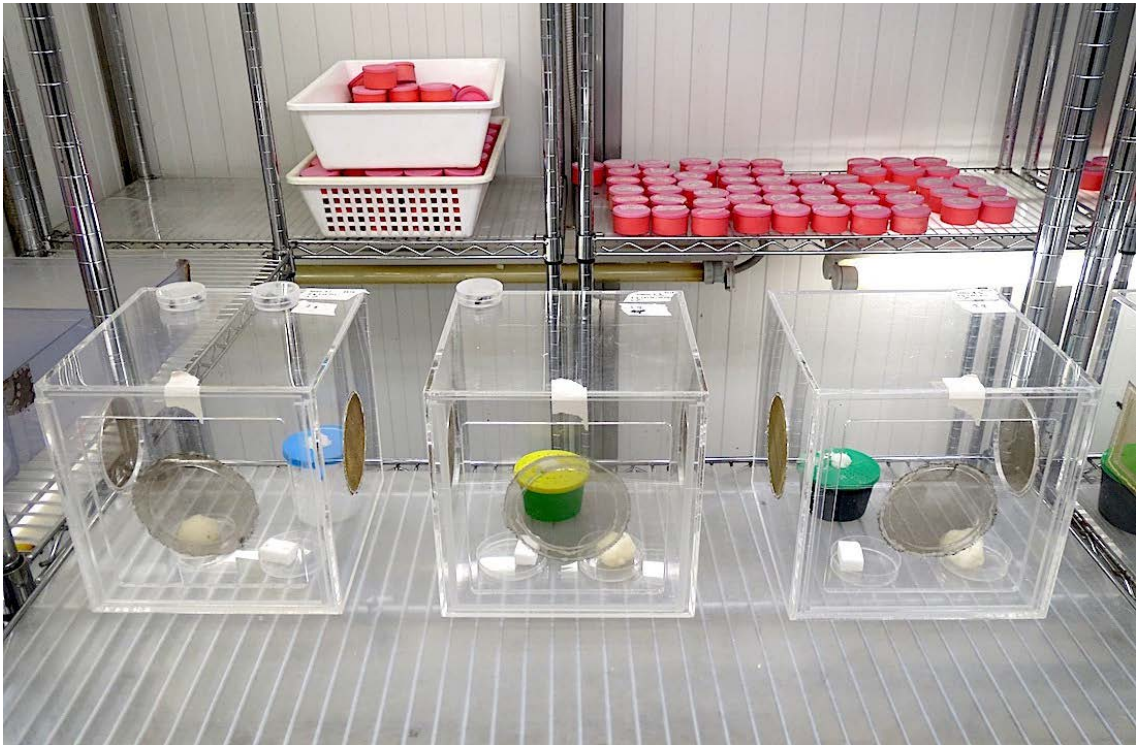

Figure 3.2 – Quality control assessment of *Exorista larvarum* female flies. Following the storage of puparia, each newly emerged female was placed in a small plexiglass cage (20 x 20 x 20 cm) and paired with a same-age male from the stock colony. Food was provided in each cage and removed only during the parasitization procedure, when 3 mature *Galleria mellonella* larvae per female were inserted into the cages (author's photo).
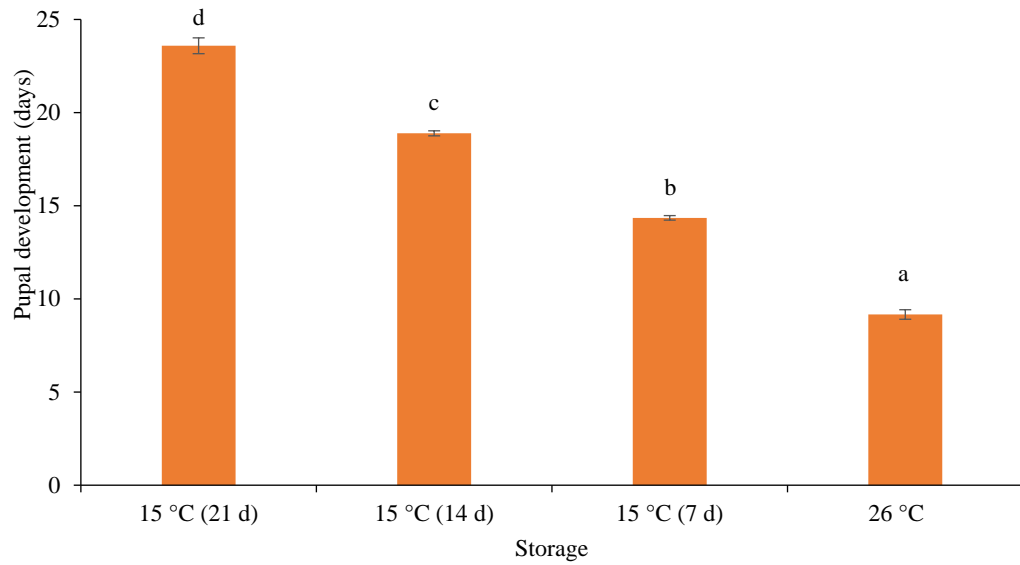
Figure 3.3 – Pupal development (both sexes; mean±SE) of *Exorista larvarum* 1-day old puparia stored at 15 °C for 21, 14 or 7 days, or kept continuously at 26 °C (control). When the storage at 15 °C was completed, the puparia were transferred at 26 °C. Different letters above the columns indicate significant difference at $P < 0.05$ ($F_{3, 12} = 2190.34$; $P < 0.0001*$).
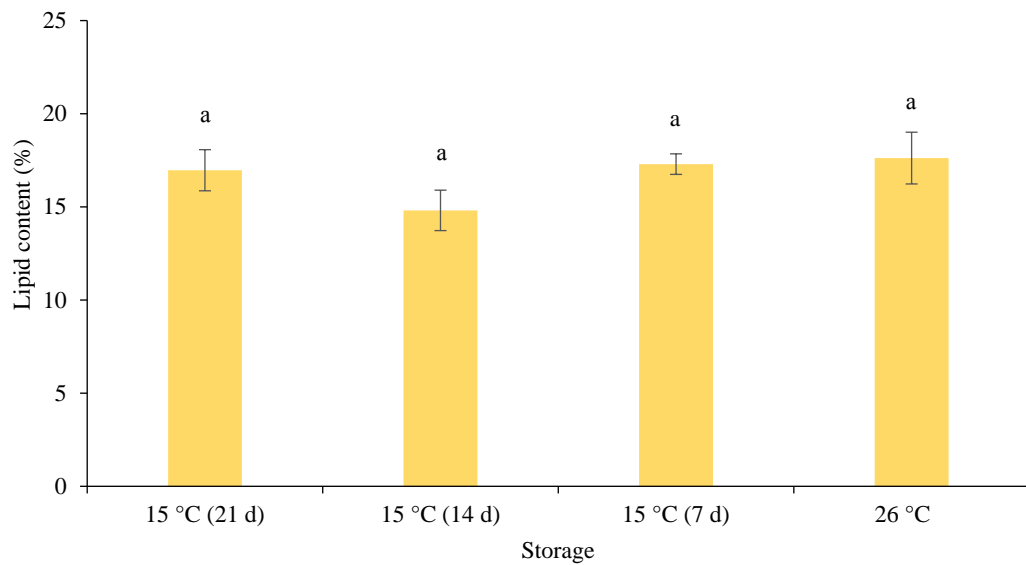

Figure 3.4 –Lipid content (both sexes; mean±SE) of *Exorista larvarum* adults emerged from puparia stored at 15 °C for 21, 14 or 7 days, or kept continuously at 26 °C (control). When the storage at 15 °C was completed, the puparia were transferred at 26 °C. Different letters above the columns indicate significant difference at $P < 0.05$ ($F_{3, 49} = 1.2$; $P = 0.3196$).

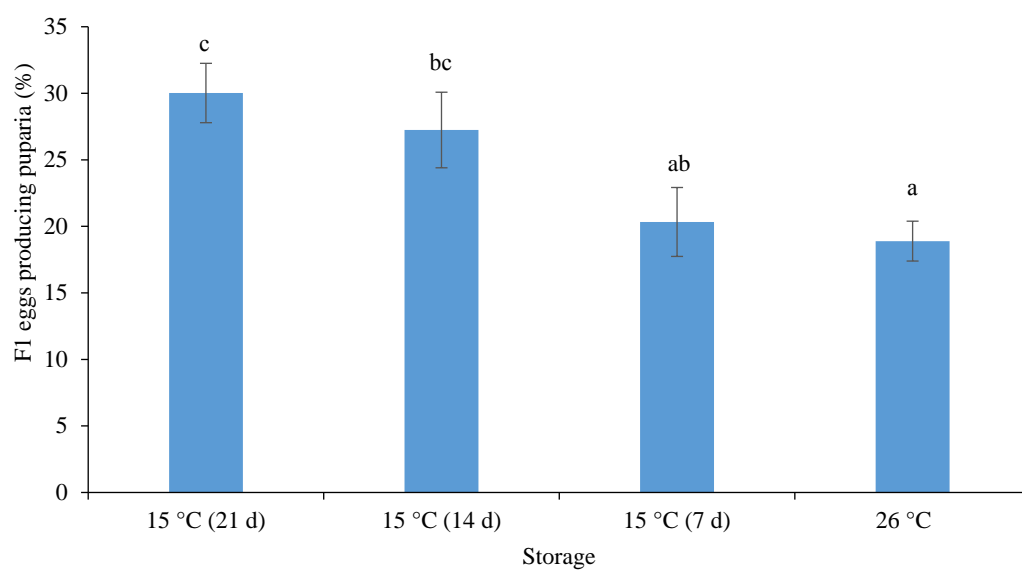**Figure 3.5** – F1 eggs that gave origin to puparia (mean±SE) laid by *Exorista larvarum* females emerged from puparia stored at 15 °C for 21, 14 or 7 days, or kept continuously at 26 °C (control). When the storage at 15 °C was completed, the puparia were transferred at 26 °C. Different letters above the columns indicate significant difference at $P < 0.05$ (as determined by 2 x 2 contingency tables. See Table 3.4 for statistics).

3.9 List of tables

Storage	Pupal development period[1] (days)			Pupal development from restoring[2] (days)		
	Both sexes	Males	Females	Both sexes	Males	Females
26 °C	9.163 ± 0.256^a	8.583 ± 0.288^a	9.708 ± 0.21^a	-----	-----	-----
15 °C (7 d)	14.35 ± 0.122^b	14.375 ± 0.25^b	14.333 ± 0.236^b	6.35 ± 0.122^a	6.375 ± 0.25^a	6.333 ± 0.236^a
15 °C (14 d)	18.888 ± 0.135^c	18.75 ± 0.288^c	19 ± 0.408^c	3.888 ± 0.132^b	3.75 ± 0.288^b	4 ± 0.408^b
15 °C (21 d)	23.588 ± 0.425^d	23.333 ± 0.562^d	23.875 ± 0.478^d	1.588 ± 0.425^c	1.333 ± 0.562^c	1.875 ± 0.478^c
F (df)	2190.34 (3, 12)	1163.93 (3, 12)	1197.78 (3,12)	319.705 (2,9)	165.633 (2,9)	132.2 (2,9)
P	< 0.0001*	< 0.0001*	< 0. 0001*	< 0. 0001*	< 0.0001*	< 0. 0001*

Table 3.1 – Development period (mean±SE) for *Exorista larvarum* puparia stored continuously at 26 °C (control) or maintained at 15 °C for 7, 14 or 21 days. When the storage at 15 °C was completed, puparia were restored at 26 °C. For every treatment 4 replicates were carried out, each consisting of 5 puparia. Values followed by different letters in a column are significantly different at *P* < 0.05 (one-way ANOVA followed by the Tukey HSD test).
[1]From puparium formation until adult emergence.
[2]From the moment of restoring at 26 °C until adult emergence.

Storage	Adult emergence[1] (%)	Sex ratio[2] (% of females)	Female longevity (days)	Fecundity (F1 eggs/female)
26 °C	(n=4) 95 ± 5^a	(n=4) 52.5 ± 4.787^a	(n=4) 23 ± 5.115^a	(n=4) 170.75 ± 17.941^a
15 °C (7 d)	(n=4) 85 ± 5^a	(n=4) 52.5 ± 2.5^a	(n=4) 6.75 ± 2.594^b	(n=2) 120.5 ± 43.5^{ab}
15 °C (14 d)	(n=4) 80 ± 8.165^a	(n=4) 50 ± 10.206^a	(n=4) 10.5 ± 2.723^{ab}	(n=4) 61.5 ± 19.765^b
15 °C (21 d)	(n=4) 90 ± 5.774^a	(n=4) 45 ± 2.887^a	(n=4) 15 ± 0.408^{ab}	(n=4) 105.75 ± 21.474^{ab}
F (df)	0.9937 (3, 12)	0.333 (3, 12)	4.831 (3, 12)	4.526 (2, 10)
P	0.429	0.802	0.0198*	0.0298*

Table 3.2 – Adult emergence, sex ratio, female longevity and fecundity (mean±SE) following storage of *Exorista larvarum* puparia at 26 °C (control) or at 15 °C for 7, 14 or 21 days. When the storage at 15 °C was completed, puparia were restored at 26 °C. The number of replicates is given in parentheses above the means. Each replicate consisted of 5 puparia (adult emergence and sex ratio), or 1 adult female (female longevity and fecundity). Values followed by different letters in a column are significantly different at *P* < 0.05 (one-way ANOVA followed by Tukey HSD test or Unequal HSD test).
[1]Calculated over the original puparium number (=5/replicate).
[2]Calculated over the number of emerged adults.

Storage	Lipid content (%)		
	Both sexes	**Males**	**Females**
26 °C	(n=14) 17.619 ± 1.389^a	(n=8) 20.293 ± 1.555^a	(n=6) 14.053 ± 1.654^a
15 °C (7 d)	(n=13) 17.295 ± 0.552^a	(n=8) 18.033 ± 0.595^a	(n=5) 16.115 ± 0.907^a
15 °C (14 d)	(n=12) 14.811 ± 1.085^a	(n=8) 12.853 ± 0.715^b	(n=4) 18.725 ± 1.702^a
15 °C (21 d)	(n=14) 16.967 ± 1.104^a	(n=10) 17.778 ± 0.625^a	(n=4) 14.939 ± 3.693^a
F (df)	1.2 (3, 49)	11.532 (3, 30)	0.9356 (3, 15)
P	0.3196	< 0.0001*	0.4479

Table 3.3 – Lipid content (mean±SE) for *Exorista larvarum* adults emerged from puparia kept continuously at 26 °C (control) or stored at 15 °C for 7, 14 or 21 days. When the storage at 15 °C was completed, puparia were restored at 26 °C. The number of replicates is given in parentheses above the means. Each replicate consisted of 1 adult fly. Values followed by different letters in a column are significantly different at $P < 0.05$ (one-way ANOVA followed by Unequal HSD test).

	Storage conditions	F1 eggs				χ^2(df=1)	*P*
		Producing puparia		Not producing puparia			
		N	%	N	%		
A)	26°C	129	18.89	554	81.11	0.24	0.625
	15 °C (7 d)	49	20.33	192	79.67		
B)	26°C	129	18.89	554	81.11	7.57	0.0059*
	15 °C (14 d)	67	27.24	179	72.76		
C)	26°C	129	18.89	554	81.11	18.21	< 0.0001*
	15 °C (21 d)	127	30.02	296	69.98		
D)	15 °C (7 d)	49	20.33	192	79.67	3.2	0.0738
	15 °C (14 d)	67	27.24	179	72.76		
E)	15 °C (7 d)	49	20.33	192	79.67	7.4	0.0065*
	15 °C (21 d)	127	30.02	296	69.98		
F)	15 °C (14 d)	67	27.24	179	72.76	0.59	0.4435
	15 °C (21 d)	127	30.02	296	69.98		

Table 3.4 – The 2 by 2 contingency tables, created to test any possible combination of treatments, for testing the independence of storage conditions of *Exorista larvarum* puparia and F1 puparium yields. The latter were calculated over the total number of F1 eggs laid throughout the first 10 days from the first host exposure; χ^2 values are presented.

Storage conditions		**F1 puparia**				χ^2(df=1)	*P*
		Producing adults		**Not producing adults**			
		N	%	N	%		
A)	26°C	122	94.57	7	5.43	0.03	0.857
	15 °C (7 d)	46	93.88	3	6.12		
B)	26°C	122	94.57	7	5.43	0.89	0.3464
	15 °C (14 d)	61	91.04	6	8.96		
C)	26°C	122	94.57	7	5.43	0.09	0.7662
	15 °C (21 d)	119	93.7	8	6.3		
D)	15 °C (7 d)	46	93.88	3	6.12	0.32	0.5732
	15 °C (14 d)	61	91.04	6	8.96		
E)	15 °C (7 d)	46	93.88	3	6.12	0.001	0.9654
	15 °C (21 d)	119	93.7	8	6.3		
F)	15 °C (14 d)	61	91.04	6	8.96	0.46	0.4966
	15 °C (21 d)	119	93.7	8	6.3		

Table 3.5 – The 2 by 2 contingency tables, created to test any possible combination of treatments, for testing the independence of storage conditions of *Exorista larvarum* puparia and F1 adult emergence. The latter was calculated over the total number of F1 puparia obtained; χ^2 values are presented.

Storage conditions		**F1 adults**				χ^2(df=1)	*P*
		Normal		**Deformed**			
		N	%	N	%		
A)	26°C	99	81.15	23	18.85	0.53	0.4666
	15 °C (7 d)	35	76.09	11	23.91		
B)	26°C	99	81.15	23	18.85	0.02	0.8931
	15 °C (14 d)	50	81.97	11	18.03		
C)	26°C	99	81.15	23	18.85	1.3	0.2545
	15 °C (21 d)	103	86.55	16	13.45		
D)	15 °C (7 d)	35	76.09	11	23.91	0.56	0.4562
	15 °C (14 d)	50	81.97	11	18.03		
E)	15 °C (7 d)	35	76.09	11	23.91	2.66	0.1032
	15 °C (21 d)	103	86.55	16	13.45		
F)	15 °C (14 d)	50	81.97	11	18.03	0.67	0.4146
	15 °C (21 d)	103	86.55	16	13.45		

Table 3.6 – The 2 by 2 contingency tables, created to test any possible combination of treatments, for testing the independence of storage conditions of *Exorista larvarum* puparia and F1 deformed adults, calculated over the total number of F1 adults; χ^2 values are presented.

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4. Effects of storage at suboptimal temperatures of Queensland fruit fly eggs

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4.1 Abstract

The Queensland fruit fly (Q-fly), *Bactrocera tryoni* (Froggatt), is a highly polyphagous tephritid fly considered the most economically damaging insect pest of fruit crops in Australia. The Sterile Insect Technique (SIT) is currently being developed as a mainstream approach to manage this pest and relies on mass rearing facilities for the supply of high quality male Q-flies. Storage at suboptimal temperature is a valuable technology for prolonging the developmental time of insects, and has never been explored for Q-fly. The possibility to create a stockpile of flies makes their mass rearing more flexible and ensures readiness for use during field outbreaks. In this study, we investigate the effect of four storage temperatures (10, 13, 16 and 19 °C) and three storage periods (3, 6 or 9 days) on 1-day old Q-fly eggs. Control eggs were maintained at 25 °C. Biological parameters, including hatching rate, egg-larval development, pupation rate, pupal weight and emergence rate were assessed using a recently developed gel-based diet for larval rearing. In general, storage of eggs proved to be effective in prolonging developmental time of Q-flies. However, the storage at 10 °C was unsuitable with a high mortality rate. The hatching rate was also compromised when eggs were exposed for 6 or 9 days at 13 °C, with a dramatic reduction in the number of pupae recovered. Storage at 16 °C resulted in being the most promising since it prolonged the preimaginal development of Q-flies up to 6.5 days without affecting egg quality parameters.

4.2 Introduction

Fruit flies (Diptera: Tephritidae) comprehend some of the world's most damaging agricultural pests (Christenson and Foote 1960; Aluja and Norrbom 1999; Sabater-Muñoz et al. 2016). *Bactrocera tryoni* (Froggatt)*, or Queensland fruit fly (Q-fly), is a highly polyphagous tephritid species considered the most economically damaging pest of fruit crops in Australia (Dominiak et al. 2003; Clarke et al. 2011). Originally from tropical and subtropical Queensland and northern New South Wales, *B. tryoni* is now established throughout eastern Australia and other South Pacific Regions (Drew et al. 1978; Sutherst and Yonow 1998; Dominiak and Daniels 2012). Adult Q-flies oviposit into healthy fruits and larvae feed within their tissues causing direct damages and early fruit drop, resulting of major economic importance (Sutherst et al. 2000).

Australia has relied for decades on organophosphate insecticides, such as dimethoate, for the control of fruit flies, but regulatory agencies have now restricted their use and alternative control strategies are needed (Dominiak and Ekman 2013). As part of an Area-Wide Integrated Pest Management (AW-IPM) programme (Apple and Smith 1976; Elliot et al. 1995; Vreysen et al. 2007), the Sterile Insect Technique (SIT) is likely to become a mainstream control strategy against the Q-fly (PBCRC 2015). SIT aims at the reproductive failure of insect pest wild populations (Knipling 1955). Very large number of mass-reared male insects are sterilised, commonly through irradiation, and released in the field. In case of fruit flies, when wild females mate with sterile males, their eggs fail to hatch and develop in fruit-infesting larvae. As a result, the pest abundance is reduced in the next generation. SIT has a number of advantages such as being environmentally benign, not impacting non-target organisms and it can be integrated with other control strategies in AW-IPM programs (Dyck et al. 2005). Australia has started to adopt SIT for

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managing Q-fly in the 1960s (Monro and Osborn 1967) and over the past 20 years has relied on it to control outbreaks in the now abolished Fruit Fly Exclusion Zone (FFEZ) (Jessup et al. 2007; Fanson et al. 2014). More research is however needed to implement Q-fly SIT and optimise the production in the new national Q-fly mass rearing facility in Port Augusta (South Australia).

Efficient mass rearing methods are essential for the success of SIT (Steiner and Shizuko 1966; Vargas 1989; Parker 2005). Mass rearing facilities have not only to produce an adequate number of flies, but also optimise fly quality and delivery time, and minimise functioning costs (Tanaka 1978). While fly production is continuous, more releases are expected during field outbreaks. Providing the factory with ways to increase supply of sterile males periodically is important. As part of the rearing toolkit for insect colony management, cold storage represents a valuable technology for this purpose (Leopold 1998).

Insect exposure to temperatures below their optimal level has been investigated for a number of different reasons, including to increase our understanding of insects' physiology and behavior, and to develop control strategies for disinfesting fruits or even for establishing cryopreservation protocols to preserve genetic resources (Armstrong et al. 1995; López and Botto 2005; Leopold 2007). In our case, we will refer to the use of suboptimal temperatures as a mass rearing aid for prolonging insects' development. This procedure allows to establish insect stockpiles while waiting for shipment and release in field at seasonal infestation peaks, but also to overcome a series of possible drawbacks in the production chain (e.g., mechanical failures, absence of technical personnel, diseases) which can benefit from having an extended life cycle of the reared insect species (Parker 2005). To induce a prolonged development, insects are usually stored at temperatures above 0 °C, commonly in the range of 4-15 °C (van Lenteren and Tommasini 2003) and,

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anyway, below their optimal rearing temperature. Referring to the arbitrary calissification of Leopold (1998), storage can be classified as "short-storage" when lasts less than a month, or as "long-storage" for anything over this period of time. In any case, a reduction in the fitness-related traits of the stored insect species is expected and may be observed directly, immediately or later during development, or even in the next generation (Colinet and Boivin 2011).

Studies on Q-fly cold tolerance mainly focus on survival (O'Loughlin et al. 1984), distribution and overwintering (Bateman 1967; Fletcher 1975; Meats 2006), thermal conditioning before release (Fay and Meats 1987) or disinfestation of fruits (Jessup et al. 1998; De Lima et al. 2011). So far, cold storage protocols have not been implemented for Q-fly rearing and may prove particularly advantageous for the SIT programme.

The aim of this study is to develop baseline knowledge on the effect of suboptimal temperature storage on Q-fly immatures and outline a protocol for prolonging their developmental time, with potential for both small- and large-scale rearing. Specifically, we have investigated the possibility to expose 1-day old Q-fly eggs to a range of suboptimal temperatures (10, 13, 16 and 19 °C) for a short-term (3, 6 or 9 days). Control eggs were maintained at 25 °C. Hatching rate, developmental times, pupal productivity and other parameters measured on the emerged adults were evaluated using a recently developed gel-based diet for larval rearing (Moadeli et al. 2017).

4.3 Materials and Methods

4.3.1 Fly colonies

Queensland fruit flies were obtained as pupae from the Fruit Fly Production Facility at Elizabeth Macarthur Agricultural Institute (Menangle, NSW, Australia) from a population mass-reared on a lucerne chaff-based larval diet (Fanson et al. 2014). Upon

arrival at Macquarie University, ca. 2000 pupae were transferred into open 9-cm Petri dishes and placed in a $47.5 \times 47.5 \times 47.5$ cm mesh cage (Megaview Bugdorm 44545, Taiwan). A diet of water, available through drinking cups with soaked sponges, sucrose and yeast hydrolysate was provided. A new colony was established every 3 weeks, to ensure adult flies had the same age when the eggs were collected for the experiment. Fly colonies were kept in a controlled environment laboratory at 25 ± 0.5 °C, $65\pm5\%$ RH and a photoperiod of 13L:11D (standard rearing conditions) in which the light phase embraced a simulated daily dawn and dusk during which light level increased and decreased gradually, respectively, over 1 h.

4.3.2 Egg collection

Egg collection was performed using a 250-mL plastic bottle that contained ca. 10 mL of water and had numerous perforation holes for the females to oviposit through (oviposition device). A slice of organic apple was used as attractant, inserted in the oviposition device before closing its lid and placing it inside the fly colony cage. After allowing females to oviposit for 2 h, 0 to 2-h old eggs were collected at 10:30 am (AEST) and rinsed through washings with tap water from the oviposition device into a beaker. The excess water was removed by filtering the beaker content with a piece of black nylon and a funnel, while the eggs were put in an open Petri dish with a thin layer of water to avoid dehydration. The first egg collection of a colony was performed when flies were sexually mature and ca. 14-days old, but the eggs were discarded. The day next, when flies were ca. 15-days old, their eggs were collected and utilised for the experiment.

4.3.3 Storage conditions and experimental protocol

Egg viability and egg to adult survival was compared when Q-fly 1-day old eggs were stored at the suboptimal temperatures of 10, 13, 16 or 19 °C for 3, 6 or 9 days (exposure time). Control eggs were maintained at the standard rearing temperature of 25 °C. When

the storage period was completed, the eggs (or the resulting larvae) were all restored at 25 °C.

Egg storage was performed in 9-cm plastic Petri dishes. Considering the possibility that egg hatching could occur before the completion of the scheduled exposure time, 35 mL of larval medium was provided in each Petri dish. The medium consisted of a gel-based diet (1% agar) recently and effectively developed by Moadeli et al. (2017) to achieve high productivity in Q-fly rearing.

To prevent egg dehydration and facilitate egg counting, a double-layer of moistened filter paper was placed on the medium surface: half a disk of 5-cm white cellulose filter paper with a triangular piece of black filter paper on top. With the aid of a thin paintbrush, 100 eggs were transferred on top of the black filter paper in each of the Petri dishes. Subsequently, the Petri dish lid was applied and the dishes sealed with Parafilm (Pechiney Plastic Packaging Company, Chicago, Illinois) to maintain the saturation level of humidity and avoid eggs to dehydrate ([Figure 4.1](#)). Six replicates were prepared for each of the tested temperatures and exposure times (6 x 4 x 3 = 72 replicates), each consisting of a Petri dish with 100 eggs. Since one suboptimal temperature was tested at a time, 6 control replicates were prepared every time and kept at 25 °C (6 x 4 = 24 control replicates).

Similarly to what has been previously done on cold storage of tephritids (Tsiropoulos 1972), replicates containing the eggs were kept for 24 h in a controlled environment room under standard rearing conditons before being stored at low temperature. This incubation period allowed eggs to complete a crucial phase of their development and prevent hatching failure. When the incubation at 25 °C was completed, the Petri dishes were placed inside a plastic box (13 x 36 x 24 cm) covered by a transparent plastic wrap and transferred inside an incubator set at one of the four tested storing temperatures (10, 13,

16 or 19 °C) and complete darkness. The dishes were kept under the storing conditions for 3, 6 or 9 days, except when they were daily inspected for ca. 5 minutes. If egg hatching occurred before the completion of the exposure time, the parafilm that sealed the dishes was removed when eggs finished to hatch.

At the end of the exposure time, the dishes were restored at the standard rearing conditions (where they spent their first 24 h) and placed individually into clear plastic trays (17.5 x 12 x 4 cm), covered with plastic lids and daily inspected. If egg hatching occurred, during or after the storage at low temperature, and larvae reached L3 instar, the Petri dish lid and the filter paper were removed. Sterilised fine vermiculite (Ausperl, Orica Australia Pty. Ltd, Banksmeadow, NSW, Australia) was added in the plastic trays to form a 0.5-cm deep layer of substrate for pupation. Pupae were daily recovered by sifting the vermiculite and transferred in a separate 55-mm plastic Petri dish for each day of collection. Seven days from the beginning of pupation, pupae coming from the same treatment were put together into 15-cm plastic Petri dishes. Newly emerged adulted were recovered every day until emergence ended.

Control dishes were kept under standard rearing conditions at all time and transferred into the clear plastic trays after 2 days from egg seeding. Then, they were manipulated as the low temperature stored ones.

Throughout the experiment, both in the controlled environment room and the incubator, temperature variability was ±0.5 °C.

4.3.4 Quality control parameters

Flies reared under the 13 storage conditions (4 suboptimal temperatures, 3 exposure times + control temperature) were evaluated in terms of the following parameters:

Egg hatching

- *Hatching rate:* percentage of hatched eggs. The number of hatched eggs was divided by the sum of hatched and unhatched eggs and then multiplied by 100. Eggs were observed daily under a stereomicroscope. For the eggs stored at low temperature, if egg hatching did not occur after 5 days from being restored at 25 °C, eggs were discarded.

Developmental times

- *Egg-larval development:* time (in days) from the day on which the eggs were collected and deposited on the filter paper until the day on which pupation was first observed.

- *Egg-pupal development:* time (in days) from egg seeding until the day on which adult emergence was first observed.

- *Pupal development:* time (in days) from the day on which pupation was first observed until the day on which emergence was first observed.

- *Pupation period:* time (in days) necessary to complete pupation from the day on which pupation was first observed.

- *Peak day of pupation:* time (in days) from day of egg seeding to the day with highest production of pupae.

- *Emergence period:* time (in days) necessary to complete adult emergence from the day on which emergence was first observed.

- *Peak day of emergence:* time (in days) from day of egg seeding to the day with highest emergence of adults.

Pupal productivity

- *Pupal number:* the total number of pupae formed during the pupation period.

- *Pupation rate:* percentage of pupation rate was calculated as the total number of pupae obtained on the number of eggs that hatched.

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- *Pupal weight*: mean pupal weight (mg) when pupae were 0-24 h old. This parameter was estimated by measuring the weight of the pupae obtained in each day of the pupation period and dividing the total weight by the total number of pupae, at the end of the pupation period.

Adult parameters

Adult flies were categorized as: (1) fully emerged (completely outside the puparium); (2) not emerged (within the unopened pupal case); (3) partially emerged (adult stuck in puparium, but partially outside); (4) deformed (fully emerged but with curly wings).

- *Adult number:* the total number of fully emerged adults obtained.

- *Emergence rate:* percentage of fully emerged adults calculated on the total number of pupae obtained.

- *Partial emergence rate*: percentage of partially emerged adults calculated on the total number of pupae obtained.

- *Deformation rate*: percentage of deformed adults calculated on the number of fully emerged flies obtained.

- *Sex ratio:* percentage of females calculated on the total number of fully emerged flies obtained.

4.3.5 Statistical analysis

Data were analysed using Generalized Linear Models (GLM) with binomial distribution for data in percentages using SPSS (2012). Because we did not have access to five incubators simultaneously (i.e., five temperatures), experiments were not run all at the same time. Control temperature (i.e., 25 °C) samples were however run for each experiment and each dataset was normalized by mean of its respective control. Post-hoc comparisons (Tukey's HSD) were run to explore differences between all treatments.

4.4 Results

Descriptive statistics are shown in the form of histograms (Figures 4.3-4.18). The dotted lines in the graphs represent the average of the controls (eggs stored at 25 °C every time a lower temperature was tested). Statistical test values are given in Tables 4.1-4.2.

4.4.1 Egg hatching

The hatching rate was significantly influenced by the interaction between exposure and temperature (Table 4.1). When eggs were stored at 10 °C, regardless of the duration of the exposure, the hatching rate dropped dramatically. Less than 12% of eggs hatched following the 3-day storage and approximately zero after a longer exposure (Pairwise Tukey's HSD comparisons $P<0.05$ for the three exposure times at 10 °C compared with the other treatments; Figure 4.2a). Conversely, the hatching rate was negatively affected by the storage temperature of 13 °C only when egg exposure lasted 6 or 9 days (Pairwise Tukey's HSD comparisons $P<0.05$). No difference in egg hatching was detected when eggs were stored at 13°C (but only for 3 days), 16 °C and 19 °C, regardless of the exposure time, with levels of hatching all above 80 % (Pairwise Tukey's HSD comparisons $P>0.05$; Figures 4.2b, 4.3).

4.4.2 Developmental times

All developmental parameters were significantly influenced by the interaction between exposure and temperature with the exception of the pupal development (Table 4.1).

Egg-larval development displayed a linear relationship with the storing temperature and exposure time, meaning that the lower the temperature (and the longer the exposure), the longer the time required for eggs to hatch and for larvae to pupate. This parameter was about 7 days for control flies and was the longest (16 days) for eggs stored at 13 °C for 9 days (because of high mortality of flies after exposure to 10 °C for more than 3 days) (Pairwise Tukey's HSD comparisons $P<0.05$ between exposure 13 °C for 9 days and all

other treatments; Figure 4.4). No larvae began pupation during storage within the incubator, but only when brought back at 25 °C.

Egg-pupal development followed a similar trend of egg-larval development, reaching the peak of almost 28 days of duration after 9-day storage at 13 °C (Figure 4.5). Pupal development was comparable for all treatments (Figure 4.6).

Pupation period was similar among eggs stored at 16 °C and 19 °C (Pairwise Tukey's HSD comparisons $P>0.05$), however it was reduced when eggs were stored at 13 °C for 6 or 9 days (Pairwise Tukey's HSD comparisons $P<0.05$ only with 16 °C results, no significant difference was detected with 19 °C; Figure 4.7). The peak day of pupation was significantly influenced by temperature and exposure, and followed the already-observed pattern of egg-larval development. The largest delay for this parameter was observed after the 9-day storage at 13 °C (16.33 days from egg seeding) (Figure 4.8).

Emergence period, similarly to pupation period, was shortened only following storage at 13 °C for 6 or 9 days (Figure 4.9). However, the numerical differences were too small to detect any significant post-hoc comparisons (i.e, Pairwise Tukey's HSD comparisons $P>0.05$). The peak day of emergence was comprised between 21 days (storage at 19 °C for 3 days) and 28.27 days (storage at 13 °C for 9 days) from egg seeding (Figure 4.10).

4.4.3 Pupal productivity

Pupal number and pupal weight were significantly affected by the interaction between exposure and temperature; pupation rate was significantly affected by the temperature (Table 4.2).

Pupal number was not different between 16 °C and 19 °C, regardless of the exposure time (on average higher than 70 pupae per treatment, Pairwise Tukey's HSD comparisons $P>0.05$). This parameter was dramatically reduced at 13 °C and 10 °C compared to 16 °C

and 19 °C (Pairwise Tukey's HSD comparisons $P<0.05$; Figure 4.11). Pupation rate was negatively influenced only when the storage temperature was 13 °C or lower (Pairwise Tukey's HSD comparisons $P<0.05$; Figure 4.12).

Pupal weight was slightly reduced when eggs were stored at 10 °C for 3 days, however no significant post-hoc comparisons were detected (Pairwise Tukey's HSD comparisons $P>0.05$, Figure 4.13).

4.4.4 Adult parameters

Adult number was significantly influenced by the interaction between exposure and temperature (Table 4.2). Adult number was negatively affected by the storage at suboptimal temperatures with the number of adults at 10 °C and 13 °C being lower than at 16 °C and 19 °C, regardless of the exposure time (Pairwise Tukey's HSD comparisons $P<0.05$; Figure 4.14). No adults were obtained following storage for 6 days or more at 10 °C. Emergence rate was not significantly influenced by temperature or exposure (Figure 4.15).

Partial emergence and deformation rate were not significantly influenced by temperature, or exposure (Table 4.2). The greatest percentage of partially emerged adults was, however, found when eggs were stored at 10 °C for 3 days (10.71%), also considering that no adults were obtained following a longer storage at this temperature (Figure 4.16). Similarly, the same treatment gave the highest percentage of deformed adults (6.67%) (Figure 4.17). Sex ratio was not significantly influenced by all treatments (Figure 4.18).

4.5 Discussion

Suboptimal temperature storage is a useful technique for assisting insect colony management and would be advantageous for Q-fly industrial mass rearing at the core of SIT programs in Australia (Fanson et al. 2014; Moadeli et al. 2017).

Attemps to develop protocols for storage at suboptimal temperatures of tephritid eggs to assist mass rearing (not considering cryopreservation techniques) are scarce. Tsiropoulos (1972) investigated the effects of storage of *Bactrocera oleae* (Rossi) 1-day old eggs at four temperatures (8, 10, 15 and 20 °C) below the optimum of 25 °C, substantially prolonging their development. In the same study, *B. oleae* pupae of different age were successfully stored at temperatures between 5 and 25 °C, achieving a maximum of 34.5 days of extension for the pupal development. By investigating the same tephritid species, Tzanakakis and Stamopoulos (1978) evaluated the effect of cold storage of pupae and adults on survival and egg-laying ability. With the aim of developing an effective method for egg shipment in SIT programs for the Mediterranean fruit fly (Medfly), *Ceratitis capitata* (Wiedemann), Cáceres et al. (2007) studied the survival of Medfly embryos after storage at 5, 10, 15, 20 and 25 °C for different exposure times. A subsequent study of Mamán and Cáceres (2007) regarded the effect on egg, pupal and adult survival following storage of *C. capitata* eggs at two temperatures, for different periods and in two different substrates. Moreover, the effect of low temperature storage on Medfly was also evaluated on the oxygen levels inside plastic bags in which pupae are packed before shipping in SIT programs (Nestel et al. 2005). Protocols for long-term storage of tephritid flies which display an obligate diapause, such as the cherry fruit fly *Rhagoletis cerasi* L., were exploited and offer the possibility to attain a stockpile of viable pupae for several months (Vallo et al. 1976; Köppler et al. 2009).

Despite the numerous studies on cold treated insects and other arthropods, as remarked by Leopold (1998), taxonomic generalizations related to low temperature tolerance and survival on the basis of orders, families and also genera are very difficult to make, considering the tremendous variation displayed. In this study, we showed that by storing *B. tryoni* eggs at temperatures below the standard of 25 °C, it is possible to prolong preimaginal development and make the Q-fly rearing schedule more flexible. As a general observation, we found that the lower the storage temperature, the higher the mortality of exposed Q-flies. This expected finding is in line with those made following storage at low temperatures of different insect species (Jalali and Singh 1992; Lacey et al. 1999; Bernardo et al. 2008). The duration of exposure to suboptimal temperatures was also a key factor in determinig survival of Q-flies during storage. In particular, when eggs were exposed to 13 °C, we observed a linear mortality pattern, reflected by the hatching rate, and a subsequent linear reduction in the pupal and adult numbers (= yields of pupae and adults) suggesting that chilling injuries may have gradually accumulated during storage (Renault et al. 2004) and eventually became irreversible. Commonly in literature, when different exposure times were tested during similar experiments, survival decreased when the exposure time increased, making our findings consistent (Chen et al. 2008; El-Gawad et al. 2010; Colinet and Hance 2010).

Among our assessed storing temperatures, 10 °C proved to be unsuitable for the purpose of our experiment. Despite being able to delay the overall preimaginal development of the very few Q-flies that managed to survive, the yields of pupae and adults were extremely low, and dropped to zero when the exposure time lasted more than 3 days. The lethal consequences displayed after storage at 10 °C underline the inability of Q-flies to overcome negative effects of low temperature on insect metabolism (Denlinger and Lee 2010). For this reason, we consider this temperature as a lower-bound

limit for further studies on Q-fly immature storage. Similarly, the temperature of 13 °C cannot be recommended for storing Q-fly immatures, especially considering the negative effects on yields of pupae and adults caused by the 6- and 9-days exposures. On the contrary, eggs stored at 16 and 19 °C attained good results in terms of productivity and with no negative impacts on pupal weight. On the basis of our findings, we suggest the use of 16 °C as the most appropriate storage temperature for 1-day old Q-fly eggs, at least up to 9 days. Clearly, this storage temperature provided the most promising results in terms of longest attainable storage, by delaying the egg-pupal development of about 6.5 days compared with control flies, without having a detrimental impact on fly production and quality for the tested parameters.

Overall, it appears that storage at suboptimal temperatures of eggs, and the resulting larvae, does provide a useful tool for prolonging development of Q-flies and to constitute stockpiles for use in the short-run. In this study, we provided baseline information on how *B. tryoni* immatures respond to suboptimal temperatures and the consequences of storage on the following pupal and adult stages. We have shown that low temperature storage is a convenient tool to be added to the suite of rearing techniques that are currently available for *B. tryoni*, but this tool definitely needs to be further explored. Leopold (1998) gave examples of how low temperature exposure can be applied at any insect stage, from egg to adult. Our experiment focused on the consequences of cold storage on egg viability and egg to adult survival, however, it is part of a broader investigation. Later studies will focus on the effect of suboptimal temperatures applied during the pupal stage of Q-flies, considering both viability and quality of the resulting adults using standard international parameters (FAO/IAEA/USDA 2014).

4.6 Acknowledgments

4.7 Statement of Authorship

MB, FP and PWT conceived and designed the experiment. MB conducted the experiment. FP and MB analysed data. MB wrote the Chapter, and all authors read and approved the Chapter.

4.8 List of figures

Figure 4.1 – Suboptimal temperature storage of Queensland fruit fly eggs. Each replicate consisted of a 9-cm Petri dish containing 35 mL of gel-based larval medium and two layers of moistened filter paper on which 100 eggs were placed using a fine brush. Dishes were initially kept sealed with Parafilm (author's photo).
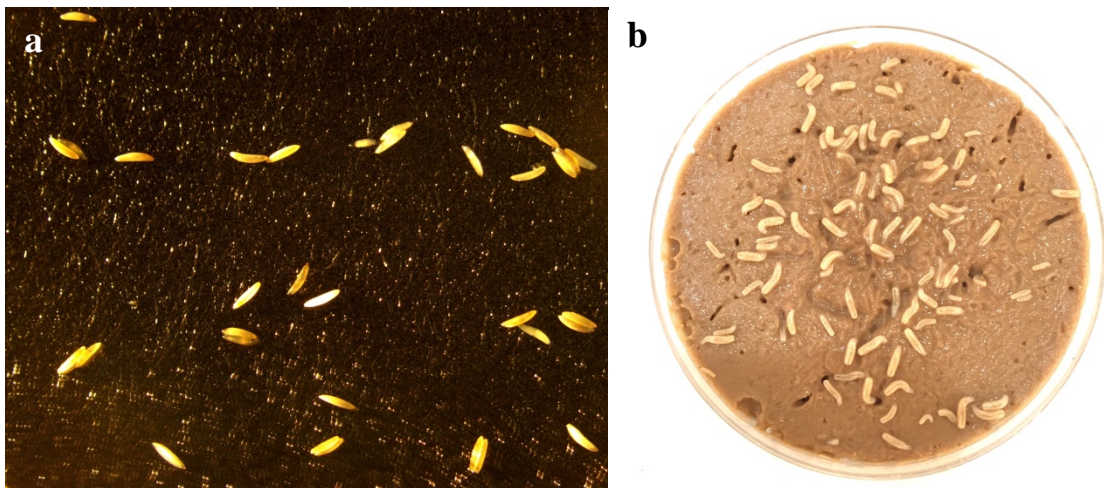
Figure 4.2 – Queensland fruit fly immatures after being stored at suboptimal temperatures: (a) following storage at 10 °C for 9 days eggs appeared of a darker colour and resulted to be non-viable; (b) following storage at 16 °C or 19 °C, regardless of the exposure time, the hatching rate was comparable to control eggs and larval development continued normally once the Petri dishes were restored at 25 °C. All photos were taken by the author.
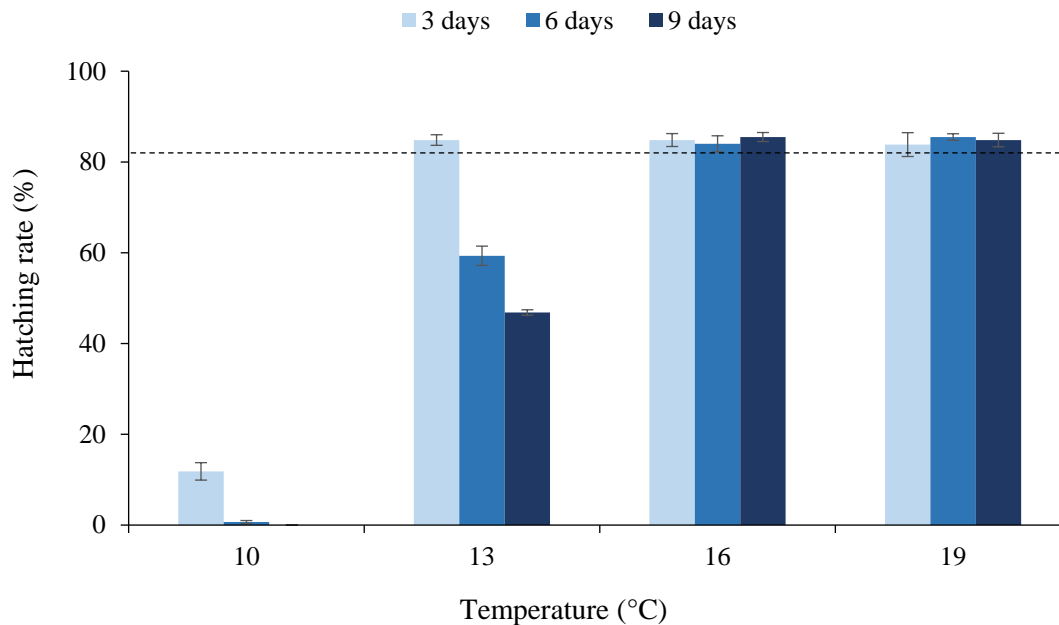
Figure 4.3 – Hatching rate (mean±SE) of 1-day old Q-fly eggs stored at 10, 13, 16 or 19 °C for 3, 6 or 9 days (exposure time). When the exposure time was completed, eggs were restored at 25 °C. Control eggs were maintained continuously at 25 °C (1 control was performed for each tested temperature = 4 controls). The dotted line represents the mean value of the 4 controls.
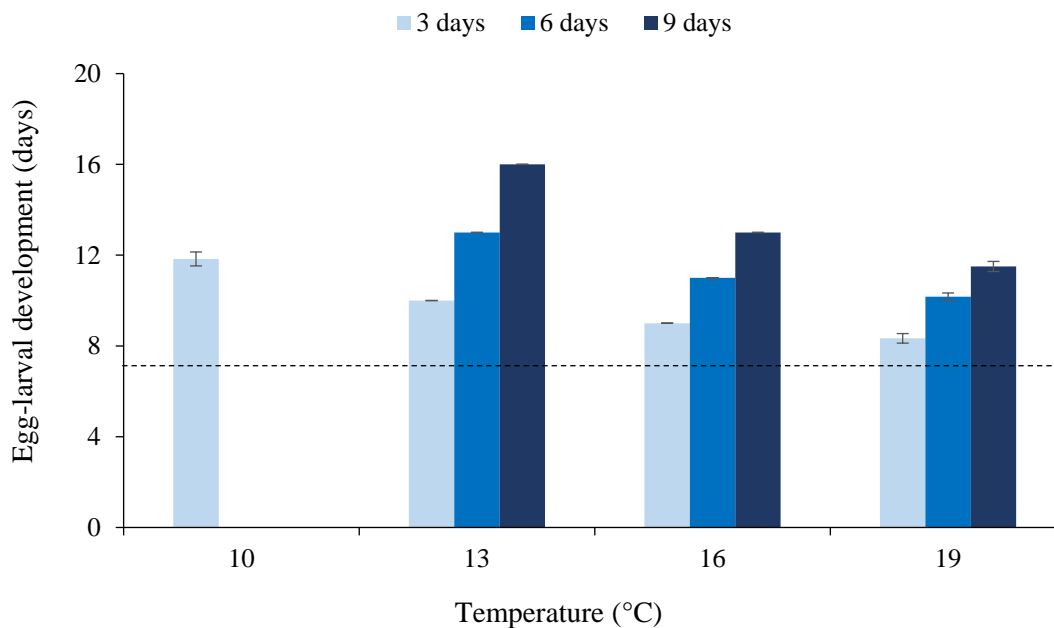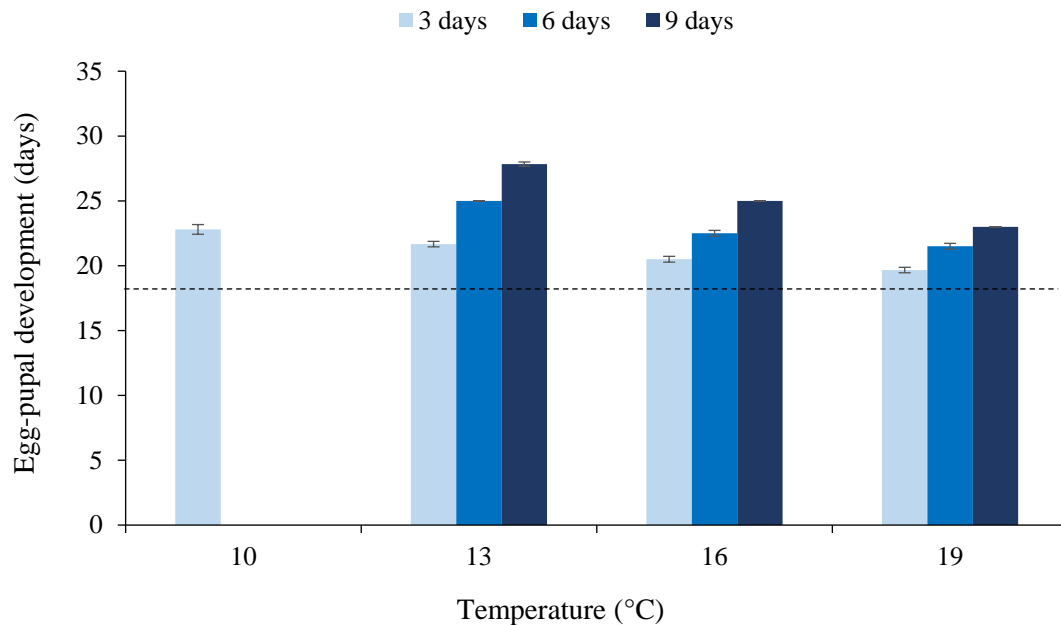

Figure 4.4 – Egg-larval development (mean±SE) obtained by storing 1-day old Q-fly eggs at 10, 13, 16 or 19 °C for 3, 6 or 9 days (exposure time). When the exposure time was completed, eggs were restored at 25 °C. Control eggs were maintained continuously at 25 °C (1 control was performed for each tested temperature = 4 controls). The dotted line represents the mean value of the 4 controls.
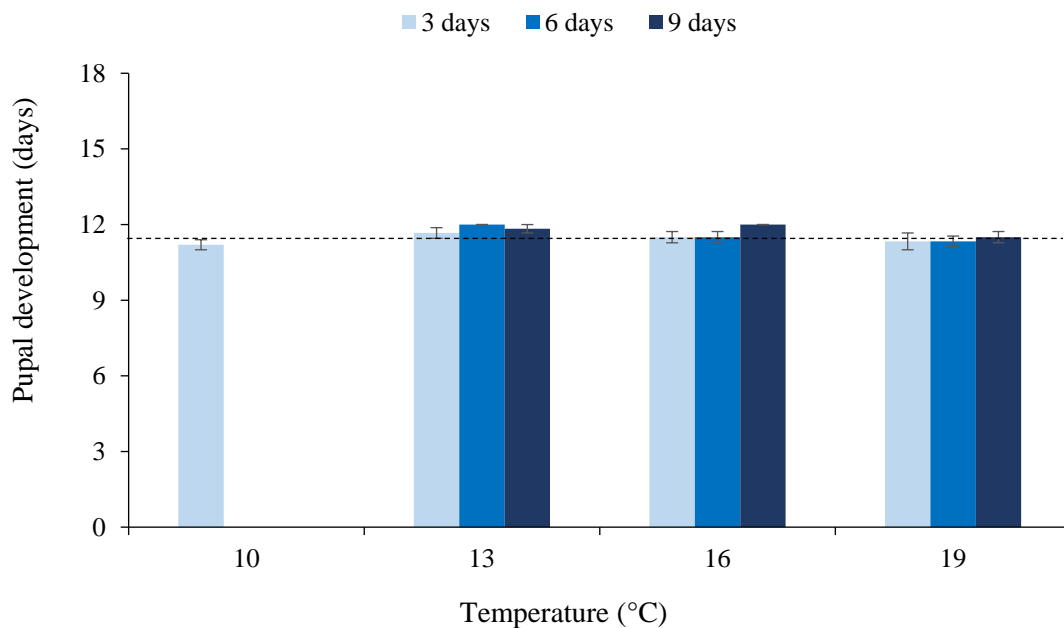
Figure 4.5 – Egg-pupal development (mean±SE) obtained by storing 1-day old Q-fly eggs at 10, 13, 16 or 19 °C for 3, 6 or 9 days (exposure time). When the exposure time was completed, eggs were restored at 25 °C. Control eggs were maintained continuously at 25 °C (1 control was performed for each tested temperature = 4 controls). The dotted line represents the mean value of the 4 controls.
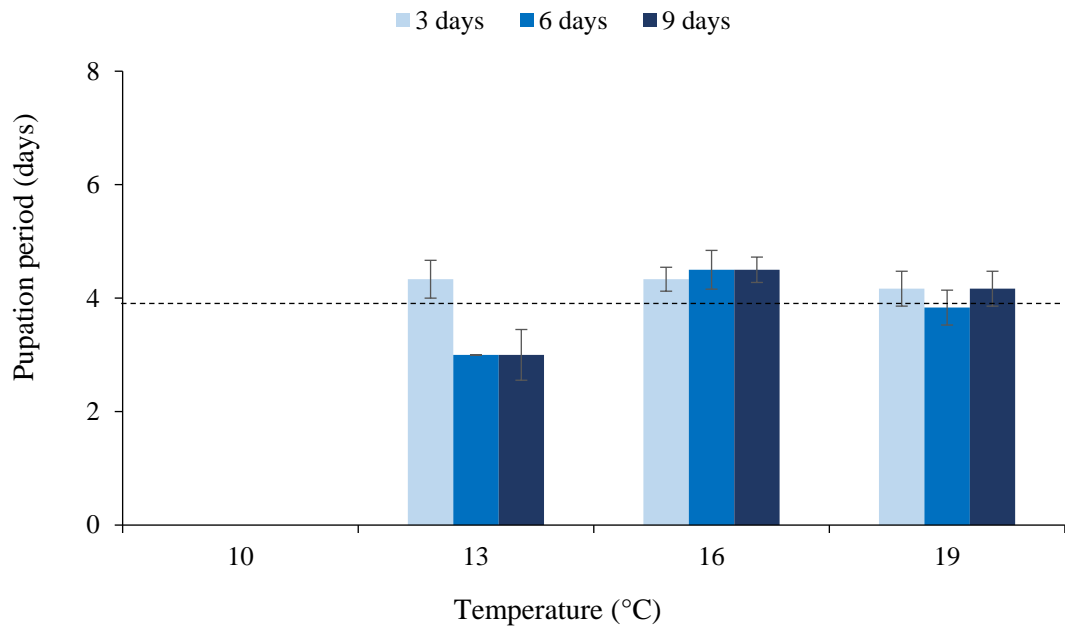

Figure 4.6 – Pupal development (mean±SE) of Q-fly pupae originated from eggs stored at 10, 13, 16 or 19 °C for 3, 6 or 9 days (exposure time). When the exposure time was completed, eggs were restored at 25 °C. Control eggs were maintained continuously at 25 °C (1 control was performed for each tested temperature = 4 controls). The dotted line represents the mean value of the 4 controls.
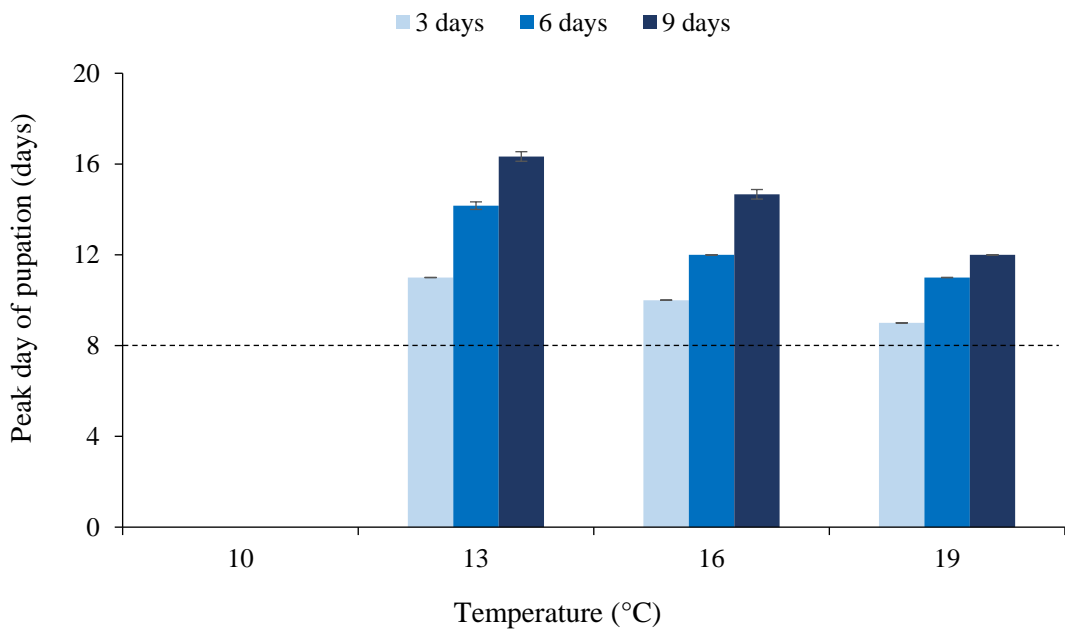
Figure 4.7 – Pupation period (mean±SE) observed following storage of 1-day old Q-fly eggs at 10, 13, 16 or 19 °C for 3, 6 or 9 days (exposure time). When the exposure time was completed, eggs were restored at 25 °C. Control eggs were maintained continuously at 25 °C (1 control was performed for each tested temperature = 4 controls). The dotted line represents the mean value of the 4 controls.

Figure 4.8 – Peak day of pupation (mean±SE) observed following storage of 1-day old Q-fly eggs at 10, 13, 16 or 19 °C for 3, 6 or 9 days (exposure time). When the exposure time was completed, eggs were restored at 25 °C. Control eggs were maintained continuously at 25 °C (1 control was performed for each tested temperature = 4 controls). The dotted line represents the mean value of the 4 controls.
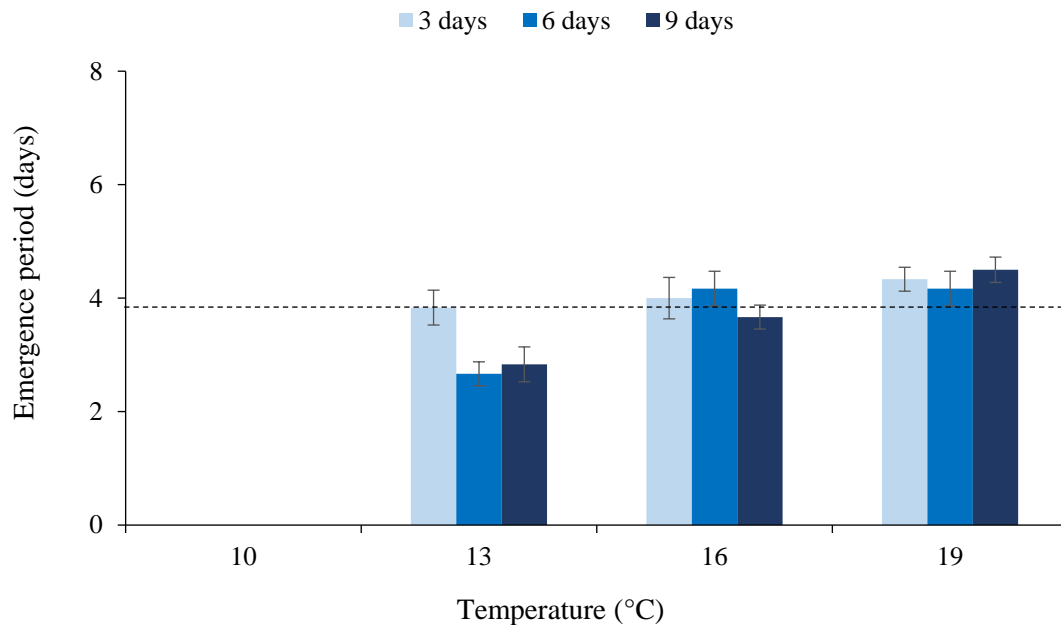
Figure 4.9 – Emergence period (mean±SE) observed following storage of 1-day old Q-fly eggs at 10, 13, 16 or 19 °C for 3, 6 or 9 days (exposure time). When the exposure time was completed, eggs were restored at 25 °C. Control eggs were maintained continuously at 25 °C (1 control was performed for each tested temperature = 4 controls). The dotted line represents the mean value of the 4 controls.
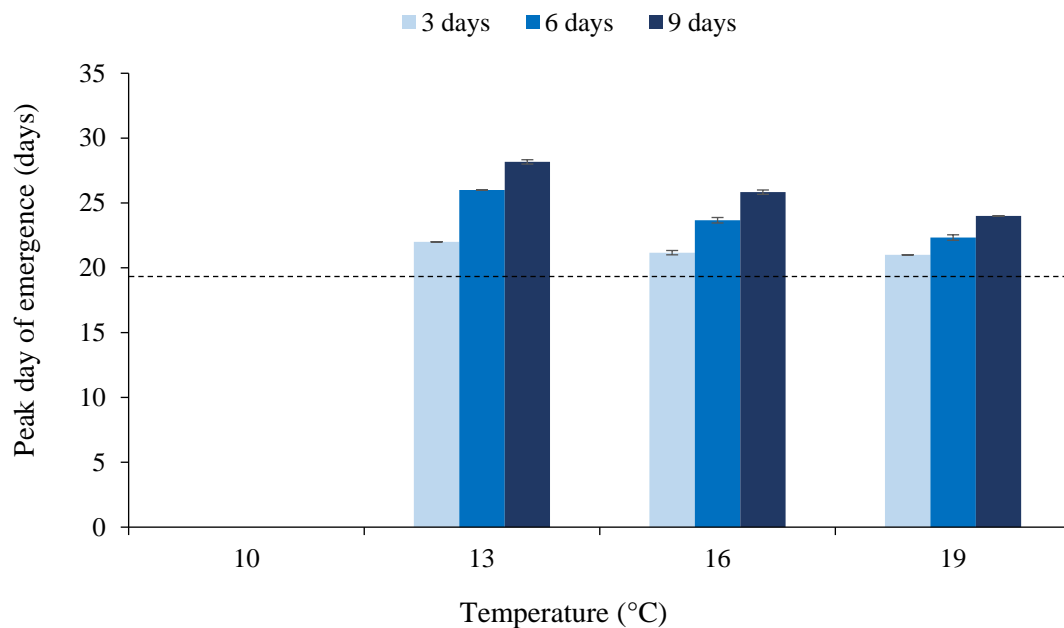

Figure 4.10 – Peak day of emergence (mean±SE) observed following storage of 1-day old Q-fly eggs at 10, 13, 16 or 19 °C for 3, 6 or 9 days (exposure time). When the exposure time was completed, eggs were restored at 25 °C. Control eggs were maintained continuously at 25 °C (1 control was performed for each tested temperature = 4 controls). The dotted line represents the mean value of the 4 controls.

Figure 4.11 – Pupal number (mean±SE) obtained following storage of 1-day old Q-fly eggs at 10, 13, 16 or 19 °C for 3, 6 or 9 days (exposure time). When the exposure time was completed, eggs were restored at 25 °C. Control eggs were maintained continuously at 25 °C (1 control was performed for each tested temperature = 4 controls). The dotted line represents the mean value of the 4 controls.
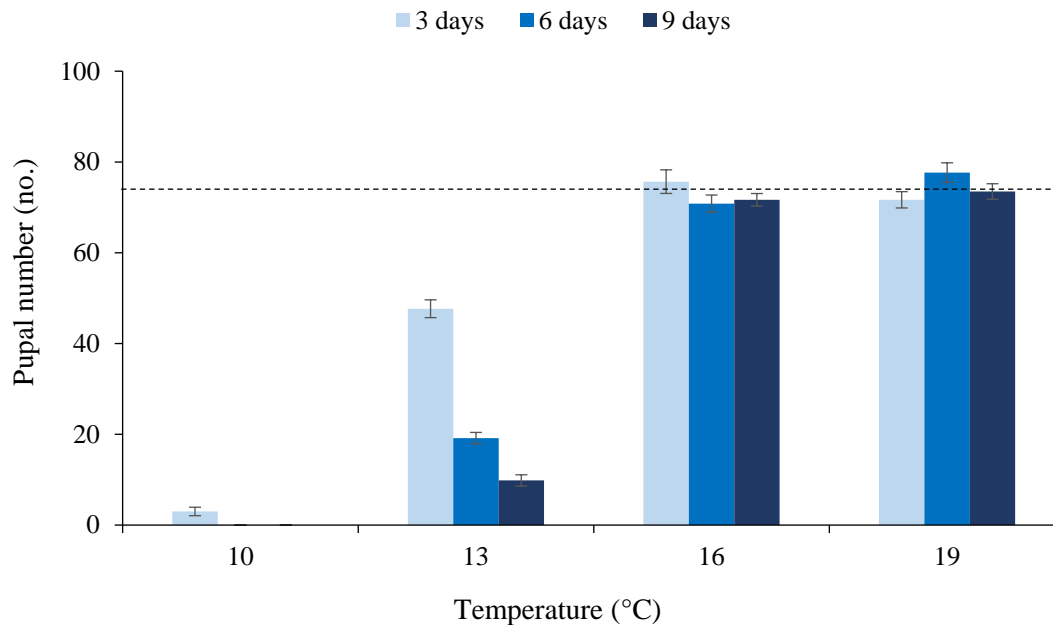

Figure 4.12 – Pupation rate (mean±SE) observed following storage of 1-day old Q-fly eggs at 10, 13, 16 or 19 °C for 3, 6 or 9 days (exposure time). When the exposure time was completed, eggs were restored at 25 °C. Control eggs were maintained continuously at 25 °C (1 control was performed for each tested temperature = 4 controls). The dotted line represents the mean value of the 4 controls.
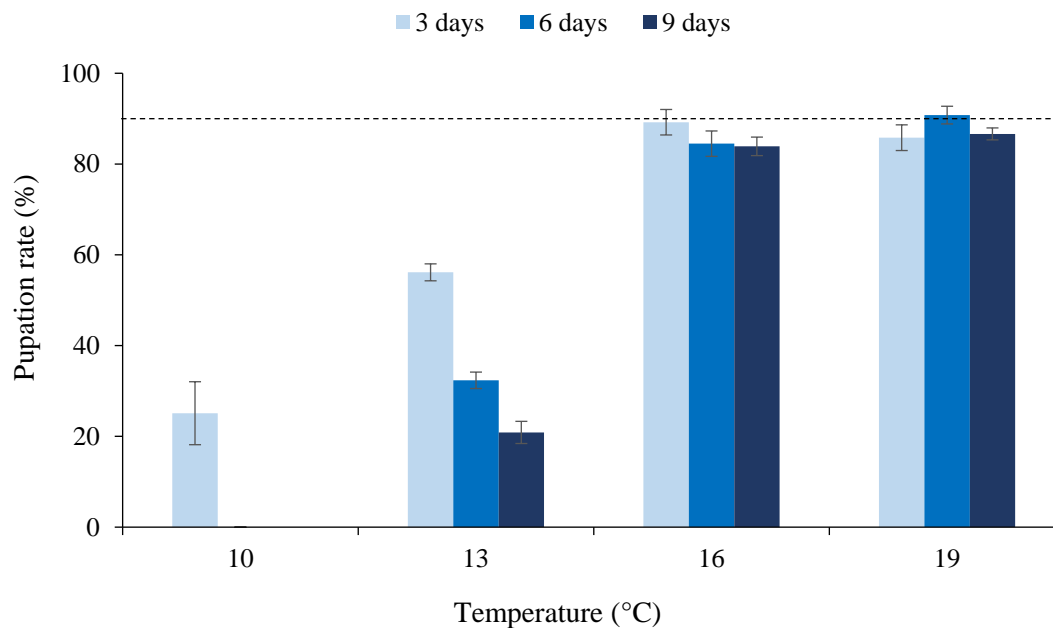
Figure 4.13 – Pupal weight (mean±SE) of Q-fly pupae obtained from 1-day old eggs stored at 10, 13, 16 or 19 °C for 3, 6 or 9 days (exposure time). When the exposure time was completed, eggs were restored at 25 °C. Control eggs were maintained continuously at 25 °C (1 control was performed for each tested temperature = 4 controls). The dotted line represents the mean value of the 4 controls.
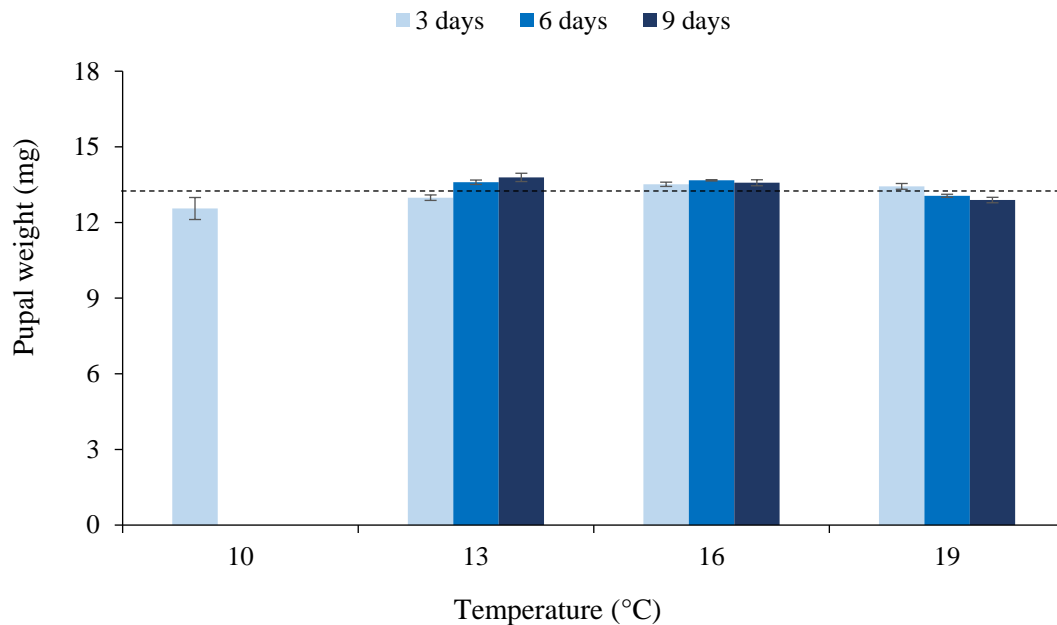

Figure 4.14 – Adult number (mean±SE) of Q-fly adults obtained from 1-day old eggs stored at 10, 13, 16 or 19 °C for 3, 6 or 9 days (exposure time). When the exposure time was completed, eggs were restored at 25 °C. Control eggs were maintained continuously at 25 °C (1 control was performed for each tested temperature = 4 controls). The dotted line represents the mean value of the 4 controls.

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Figure 4.15 – Emergence rate (mean±SE) observed following storage of 1-day old Q-fly eggs at 10, 13, 16 or 19 °C for 3, 6 or 9 days (exposure time). When the exposure time was completed, eggs were restored at 25 °C. Control eggs were maintained continuously at 25 °C (1 control was performed for each tested temperature = 4 controls). The dotted line represents the mean value of the 4 controls.
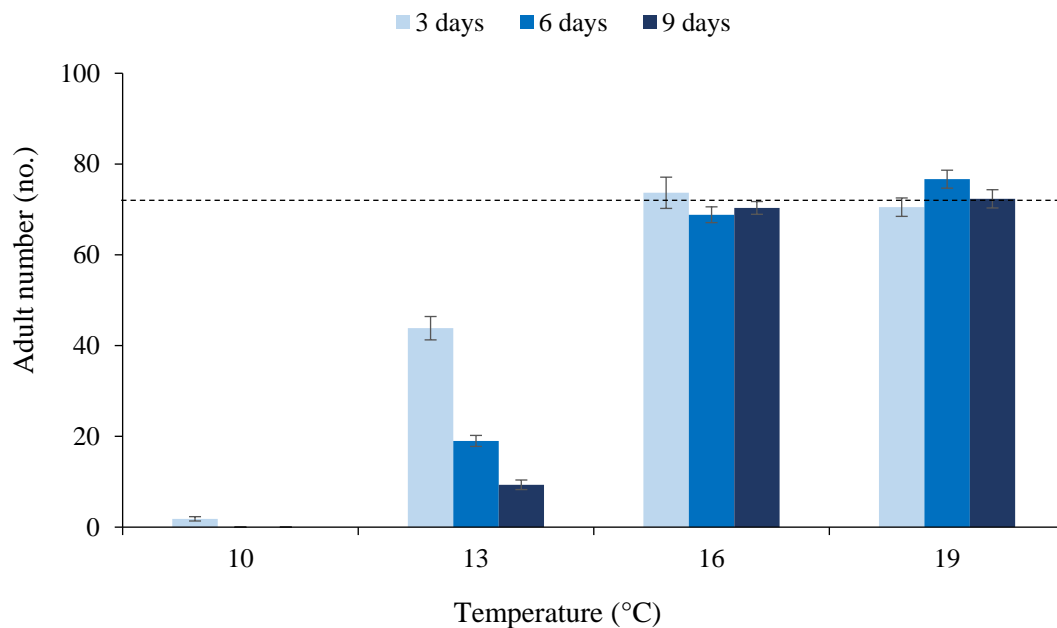

Figure 4.16 – Partial emergence rate (mean±SE) observed following storage of 1-day old Q-fly eggs at 10, 13, 16 or 19 °C for 3, 6 or 9 days (exposure time). When the exposure time was completed, eggs were restored at 25 °C. Control eggs were maintained continuously at 25 °C (1 control was performed for each tested temperature = 4 controls). The dotted line represents the mean value of the 4 controls.
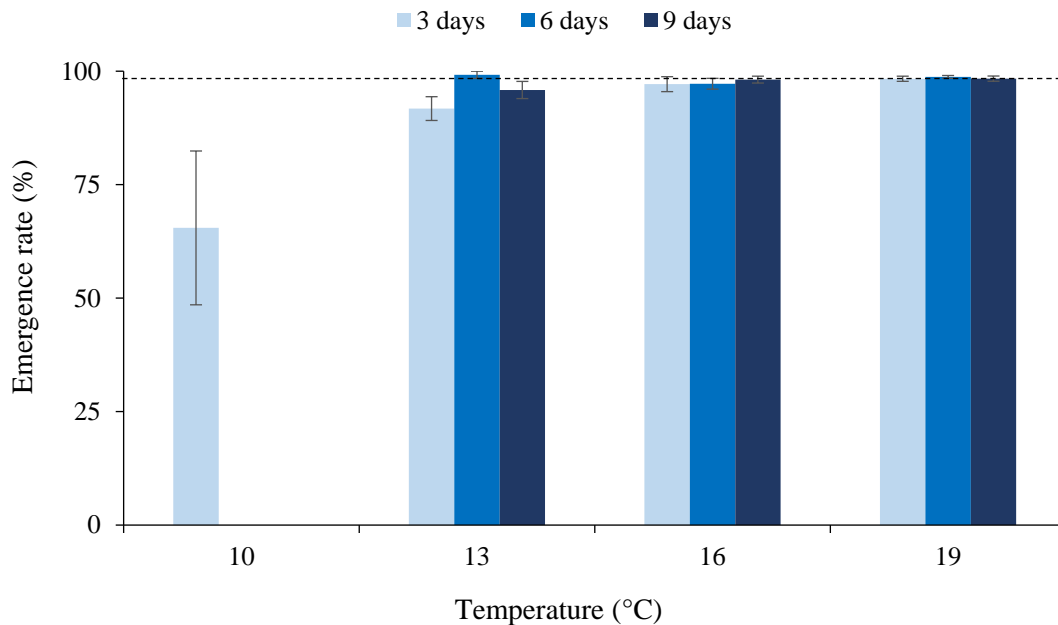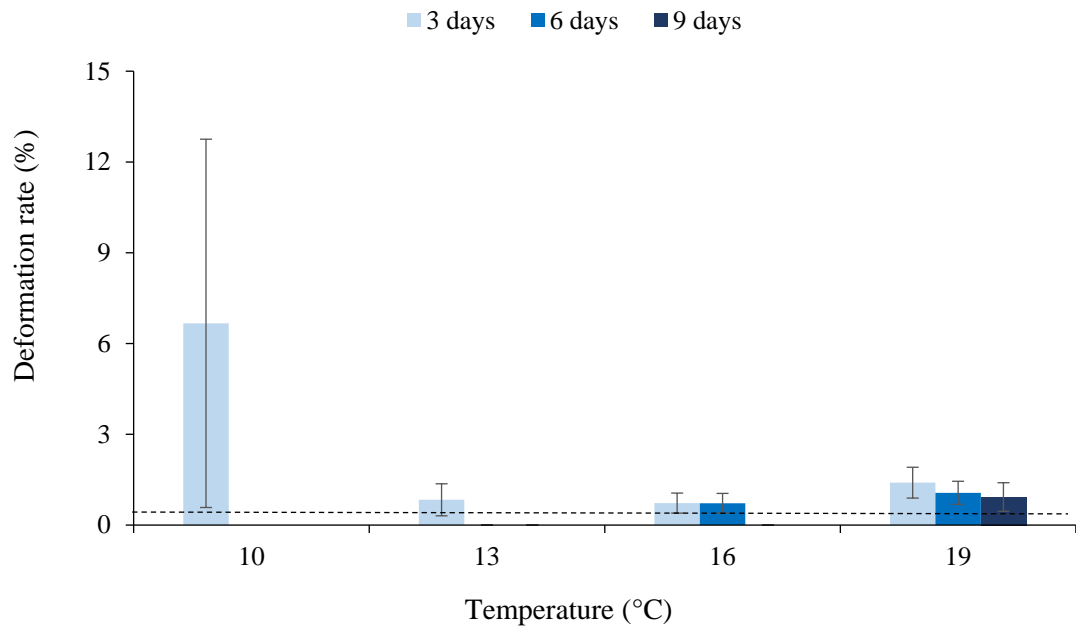
Figure 4.17 – Deformation rate (mean±SE) observed following storage of 1-day old Q-fly eggs at 10, 13, 16 or 19 °C for 3, 6 or 9 days (exposure time). When the exposure time was completed, eggs were restored at 25 °C. Control eggs were maintained continuously at 25 °C (1 control was performed for each tested temperature = 4 controls). The dotted line represents the mean value of the 4 controls.
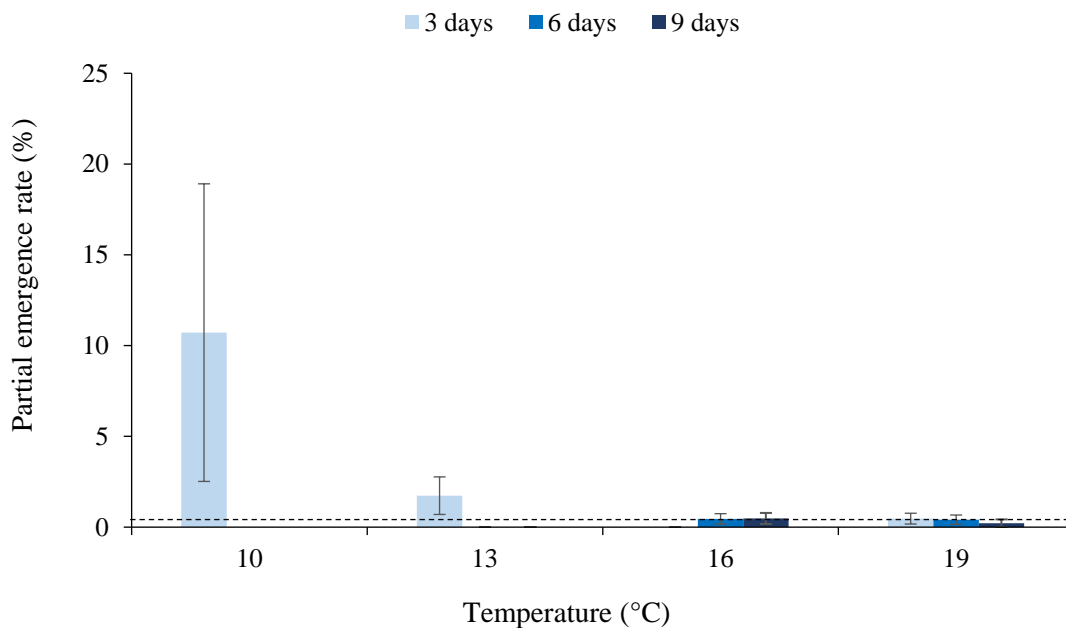

Figure 4.18 – Sex ratio (mean±SE) observed following storage of 1-day old Q-fly eggs at 10, 13, 16 or 19 °C for 3, 6 or 9 days (exposure time). When the exposure time was completed, eggs were restored at 25 °C. Control eggs were maintained continuously at 25 °C (1 control was performed for each tested temperature = 4 controls). The dotted line represents the mean value of the 4 controls.
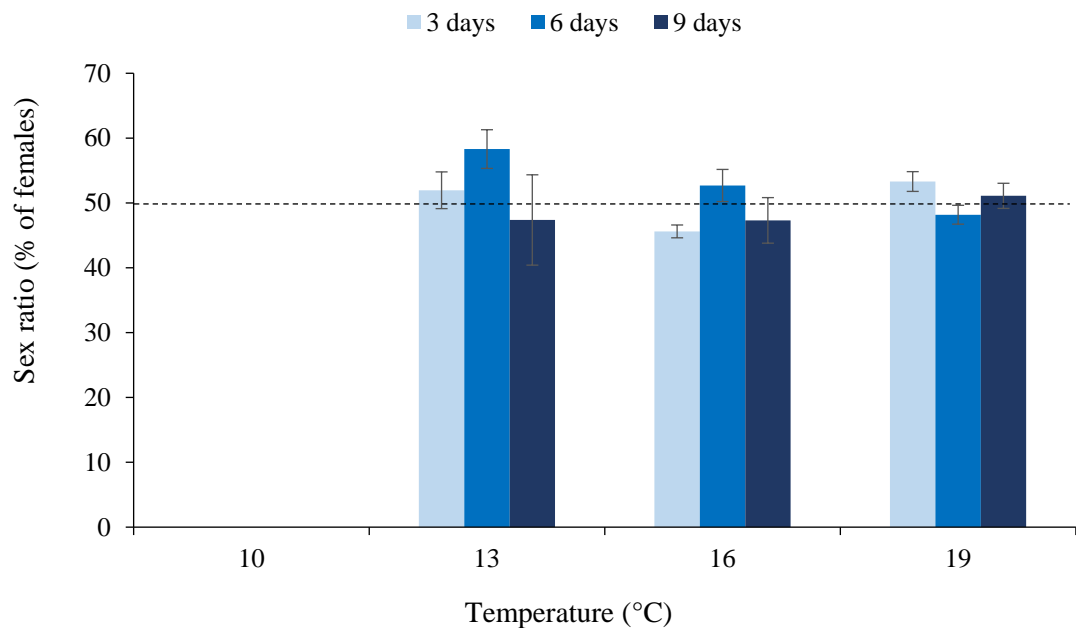
4.9 List of tables

	Hatching rate	Egg-larval development	Egg-pupal development	Pupal development	Pupation period	Peak day of pupation	Emergence period	Peak day of emergence
Exposure	df = 2 χ^2 = 6.668 P = 0.036*	df = 2 χ^2 = 1601.026 $P < 0.001$*	df = 2 χ^2 = 1051.299 $P < 0.001$*	df = 2 χ^2 = 3.233 P = 0.199	df = 2 χ^2 = 5.15 P = 0.076	df = 2 χ^2 = 2616.571 $P < 0.001$*	df = 2 χ^2 = 5.08 P = 0.079	df = 2 χ^2 = 2005.938 $P < 0.001$*
Temperature	df = 3 χ^2 = 64.271 $P < 0.001$*	df = 3 χ^2 = 1083.205 $P < 0.001$*	df = 3 χ^2 = 757.275 $P < 0.001$*	df = 3 χ^2 = 12.597 P = 0.006*	df = 2 χ^2 = 38.971 $P < 0.001$*	df = 2 χ^2 = 1392.571 $P < 0.001$*	df = 2 χ^2 = 19.934 $P < 0.001$*	df = 2 χ^2 = 1038.473 $P < 0.001$*
Exposure x Temperature	df = 5 χ^2 = 11.464 P = 0.043*	df = 4 χ^2 = 118.974 $P < 0.001$*	df = 4 χ^2 = 67.763 $P < 0.001$*	df = 4 χ^2 = 3.625 P = 0.459	df = 4 χ^2 = 11.177 P = 0.025*	df = 4 χ^2 = 162.286 $P < 0.001$*	df = 4 χ^2 = 11.161 P = 0.025*	df = 4 χ^2 = 188.103 $P < 0.001$*

Table 4.1 – GLM statistics of hatching rate and developmental times of Q-fly exposed for 3, 6 or 9 days to different suboptimal temperatures (10, 13, 16 and 19 °C).

	Pupal number	Pupation rate	Pupal weight	Adult number	Emergence rate	Partial emergence rate	Deformation rate	Sex ratio
Exposure	df = 2 χ^2 = 119.652 $P < 0.001$*	df = 2 χ^2 = 1.087 P = 0.581	df = 2 χ^2 = 1.089 P = 0.58	df = 2 χ^2 = 75.337 $P < 0.001$*	df = 2 χ^2 = 0.007 P = 0.996	df = 2 χ^2 = 1.031 P = 0.597	df = 2 χ^2 = 1.271 P = 0.53	df = 2 χ^2 = 0.67 P = 0.967
Temperature	df = 3 χ^2 = 5309.68 $P < 0.001$*	df = 3 χ^2 = 14.99 P = 0.002*	df = 3 χ^2 = 8.308 P = 0.04*	df = 3 χ^2 = 4199.67 $P < 0.001$*	df = 2 χ^2 = 0.002 P = 0.999	df = 2 χ^2 = 1.239 P = 0.538	df = 3 χ^2 = 5.864 P = 0.118	df = 2 χ^2 = 0.6 P = 0.97
Exposure x Temperature	df = 4 χ^2 = 271.154 $P < 0.001$*	df = 4 χ^2 = 1.892 P = 0.756	df = 4 χ^2 = 19.832 P = 0.001*	df = 4 χ^2 = 190.319 $P < 0.001$*	df = 4 χ^2 = 0.12 P = 1	df = 1 χ^2 = 0.418 P = 0.518	df = 4 χ^2 = 0.485 P = 0.975	df = 4 χ^2 = 0.162 P = 0.997

Table 4.2 – GLM statistics of pupal productivity and adult parameters of Q-fly exposed for 3, 6 or 9 days to different suboptimal temperatures (10, 13, 16 and 19 °C).

4.10 References

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5. Suboptimal temperature storage of Queensland fruit fly pupae for mass rearing programs

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5.1 Abstract

Bactrocera tryoni (Froggatt) (Queensland fruit fly, aka Q-fly) is a polyphagous tephritid that represents a serious biosecurity threat to the Australian horticultural industry. The Sterile Insect Technique (SIT) has attracted particular interest as an environmentally benign approach for Q-fly management. For SIT to be effective, millions of high-quality flies need to be produced continuously in mass rearing facilities. Mass-reared insects usually have a short shelf life, needing to be used almost immediately after production. Considering the complexity of synchronizing field releases with production, an increased flexibility in the timing of delivery with minimal loss of fly quality is desirable, however, protocols to establish stockpiles of Q-flies are limited. In this study, we developed experiments for cold storage of 1-day old Q-fly pupae at suboptimal temperatures and evaluated the effects on survival, development and quality of the resulting adults. In a first experiment, Q-fly pupae were stored at a wide range of temperatures, 13, 15, 17 and 19 °C, and effect of cold storage was assessed on adult emergence. In a second experiment, effects of a narrower range of storing temperatures (17, 19, 21 and 23 °C) were measured on adult flight ability, chill-coma recovery time and lipid content. Control pupae were maintained at 25 °C. Slight temperature reductions provided a modest delay of development without, or with minimum, negative impact on quality. However, larger

temperature reductions showed greater development extensions but were associated with progressively severe deleterious effects on flies' quality.

5.2 Introduction

Tephritid fruit flies are a significant threat to agriculture, causing direct loss of production and substantial costs for their control (Aluja and Norrbom 1999; Shelly et al. 2014). From an economic perspective, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) is, by far, the most significant insect pest attacking fruit crops in Australia (Sutherst et al. 2000). Also known as Queensland fruit fly or Q-fly, *B. tryoni* is an endemic species distributed along the east coast of Australia, from tropical Queensland to temperate eastern Victoria, and also established in New Caledonia, French Polynesia and Pitcairn Island (Dominiak and Daniels 2012). Recent findings demonstrated that Q-fly distribution in Australia is likely to be influenced by climate change (Sultana et al. 2017).

The Queensland fruit fly is highly polyphagous and capable of attacking hundreds of different species of fruit and vegetable crops (Hancock et al. 2000; Oliver 2007), thus posing a biosecurity risk to the Australian horticultural industry (Plant Health Australia 2016). In-field control is necessary in areas where Q-fly is present to ensure adequate protection to vulnerable fruits and guarantee market access, both domestic and international, for producers. Control strategies include cover spray, protein bait spray, coloured traps, Male Annihilation Technique (MAT), Sterile Insect Technique (SIT) and post-harvest treatments (Clarke et al. 2011; see Dominiak and Ekman 2013 for review).

For decades Australia has relied on dimethoate and fenthion, two broad-spectrum organophosphates insecticides, as an effective and economically sustainable method for protecting fruit crops, but their use has now been restricted by regulatory agencies (Reynolds et al. 2017). For this reason, alternative control strategies are now being

implemented, with particular emphasis given to environmentally benign approaches. In this frame, SIT, which is part of a broader Area-Wide Integrated Pest Management (AW-IPM) programme, is undergoing a substantial implementation in control strategies against Q-fly (PBCRC 2015). In SIT programmes, overwhelming numbers of sterile insects are repeatedly released in the field, with the aim of corrupting the reproductive system of wild popupations (Knipling 1955; Dyck et al. 2005). During the 1960s, the possibility to mass-rear, sterilise and release Q-flies in field for controlling natural pest populations was evaluated by Monro and Osborn (1967). Following further developments over the past decades, SIT provided a method for Q-fly control in areas particularly suitable to agriculture with periodic pest outbreaks (Meats et al. 2003; Jessup et al. 2007; Fanson et al. 2014).

Undoubtly, an efficient and cost-effective rearing technique of the target insect species is one of the fundamental pillars for the success of SIT (Parker 2005). For this control strategy, insect rearing takes place in large facilities equipped with controlled environment rooms, set at the optimal rearing conditions, where the production assumes the characteristics of an industrial process (Cáceres et al. 2012). Production in facilities is continuous over time, however, during SIT procedures there are situations that require field releases to be delayed or accelerated to release sterile insects at the right time, i.e., after a period of adverse weather or right before peaks of infestation in field (Enkerlin 2017). Moreover, non-routine situations during the rearing process, such as equipment failures, disease contaminations and breakdown in the release operations may also take advantage from a more flexible rearing schedule (Leopold 2007). In this view, the possibility to delay pupal development of fruit fly pupae during SIT mass rearing, by exposing them at suboptimal temperatures, is desirable (Resilva and Pereira 2014).

Cold storage is a valuable tool for increasing the shelf life of insects and proves particularly useful for pest control strategies entailing mass rearing and release, including SIT (Leopold 1998). The exposure at suboptimal temperatures indeed provides a slowdown of the insect metabolic activities during rearing, and thus increases the flexibility of the overall rearing process (Benelli et al. 2017). On the other hand, sub-lethal perturbations may accumulate during storage and eventually can lead the stored species to death (Lavy and Verhoef 1998; López and Botto 2005). Quality control, which is routinely performed on tephritid fruit flies destined to SIT (FAO/IAEA/USDA 2014), has therefore to be emphasized following any storage at low temperatures.

Protocols to establish stockpiles of Q-flies for assisting mass rearing and release in SIT programs have not yet being fully developed. In a previous study (Benelli et al. 2016, unpublished), suboptimal temperature storage for increasing *B. tryoni* developmental time following eggs cold storage was evaluated. Results show that Q-fly eggs can be stored at 16 °C for at least 9 days. With the aim of investigating the effect of exposure at 4 °C on recovery, longevity and flight ability of sterile adult Q-flies, Reynolds and Orchard (2011) have discussed the effect of this storage treatment in terms of the potential use of chilled adult Q-flies for release in SIT programs.

In this study, we tested for the effect of storage at different suboptimal temperatures of Q-fly pupae on survival, development and quality of the resulting adults. The pupal stage is considered as the most suitable stage for short-term storage at low temperatures in insects, since adult stage storage can lead to a higher and faster fitness reduction compared with storage of immatures (van Lenteren and Tommasini 2003). In a first experiment, effects of a wide range of temperatures were assessed on pupae survival and development. In a second experiment, the quality of adults emerged from the pupae stored under a new range of suboptimal temperatures, following results of experiment 1, was

evaluated performing a standard flight ability test, a chill-coma recovery test and a measurement of lipid body reserves. We discuss the results in terms of the possible use of suboptimal temperature storage for assisting Q-fly mass rearing in SIT programs.

5.3 Materials and Methods

5.3.1 Experiment 1

We ran a first experiment in order to test for a wide suboptimal temperatures range on pupae survival and development. One-day old Q-fly pupae were stored at 13, 15, 17 and 19 °C with 65% RH in complete darkness until adult emergence was completed. In a second experiment, the quality of adults emerged from the pupae stored under a refined range of suboptimal temperatures (17, 19, 21 and 23 °C) was evaluated. Control pupae were stored continuously at 25 °C.

Fly source

Queensland fruit fly eggs were sourced from New South Wales Department of Primary Industries (Ourimbah, NSW, Australia) from a Q-fly culture reared on a carrot-based larval diet. The eggs were collected at 8.30 am (AEST) after allowing flies to oviposit for 24 h. Egg collection was performed using an oviposition device made from a 1-L plastic jar that contained ~40 mL of water and had plentiful puncture holes for the females to oviposit through. Zero to twenty-four hours old eggs were rinsed from the inner surface of the oviposition device, where they adhere, into a beaker and then poured into a plastic vial. They were received at Macquarie University after being transported for approximately 1 h by an air-conditioned vehicle. To reduce the risk of damage by holding eggs in water, they were transferred onto larval medium within 1 h from their arrival. The medium consisted of a gel-based diet, containing 1% agar, developed by Moadeli et al. (2017) to achieve high productivity and performances in Q-fly rearing. Larval rearing

was performed into 500-mL clear plastic rearing trays (17.5 x 12 x 4 cm) into which 150 mL of diet was poured. After waiting for the diet to cool down and set at room temperature, 250 µL of eggs were transferred onto the diet surface of each tray using a 1000-µl pipette (~3500 eggs per tray). The rearing trays were then closed with plastic lids and placed in a controlled environment room set at 25±0.5 °C, 65±5% RH and 13L:11D photoperiod where lights stepped on and off to simulate dawn and dusk over the course of 1 h during the first and the last hour of the light phase, respectively (standard rearing conditions). When larvae reached the third instar (L3), lids were removed and the trays placed inside a 100-L clear plastic container (67.5 x 50 x 43 cm) were a 0.5-cm deep layer of fine vermiculite (Ausperl, Orica Australia Pty. Ltd, Banksmeadow, NSW, Australia) was added as pupation substrate. Newly-formed pupae were daily collected by sifting the vermiculite (Figure 5.1-a). To ensure the availability of an adequate number of same-age pupae for the experiment, only pupae from the second day of pupation (peak day) were utilized. Before commencing storage at suboptimal temperatures, pupae were maintained for 24 h under the standard rearing conditions mentioned above.

Experimental protocol

Six sets of 100 pupae per treatment were placed individually in 9-cm open Petri dishes. Each dish was then inserted in a 1-L clear plastic jar that had a 5 x 8 cm mesh-covered opening for ventilation. The jar was placed upside-down and the dish containing the pupae accommodated into its screw-top lid (Figure 5.1-b). Pupae were stored at 13, 15, 17, 19 or 25 °C (control) ±0.5 °C with 65±5% RH into incubators (A1000, Conviron, Melbourne, VIC, Australia; Figure 5.2) (6 replicates per treatment, 30 replicates in total). There was no illumination within the incubators, but pupae were exposed to light for ~5 min daily when they were inspected by opening the door of the incubators. A slight temperature fluctuation could also have occurred during this short period of time.

Newly emerged adult flies were daily collected, counted and sexed. Flies were categorized as: (1) fully emerged (completely outside the puparium); (2) not emerged (within the unopened pupal case); (3) partially emerged (adult stuck in puparium, but partially outside). Results were evaluated in terms of the following parameters:

- *Pupal development:* time (in days) from the day on which pupae were obtained until the day on which emergence was first observed.

- *Emergence period:* time (in days) necessary to complete adult emergence.

- *Peak day of emergence:* time (in days) from the day on which pupae were obtained to the day with the highest number of emerged adults.

- *Emergence rate:* percentage of fully emerged adults obtained from the pupae.

- *Partial emergence rate:* percentage of partially emerged adults calculated on the number of the pupae.

- *Sex ratio:* percentage of females calculated on the total number of fully emerged flies.

On the basis of the outcome of this experiment, a new norrowed range of storing temperatures was selected. Effects of suboptimal temperatures storage of Q-fly pupae were investigated on the quality of the resulting adults. For this purpose, a standard quality control test flight ability, a non-standard test chill-coma recovery and a measurement of lipid body reserves were performed.

5.3.2 Experiment 2: Flight ability

The aim of this assay is to evaluate the capacity of adult Q-flies to emerge following storage at suboptimal temperatures and, most importantly, their ability to fly.

Fly source

Eggs were obtained from a Q-fly culture reared on gel-based larval diet at Macquarie University. Adult flies (Generation 12) were kept in a metal frame cage (40 x 40 x 110

cm) in a controlled environment room under standard rearing conditions and provided a diet of sucrose, yeast hydrolysate and tap water available through soaked sponges. The eggs were collected by inserting two 250-mL perforated plastic bottles that contained ~10 mL of water and a slice of apple as attractant (oviposition devices) into the adult cage. After allowing females to oviposit for 6 h, eggs were rinsed with tap water into a beaker and then transferred onto larval rearing medium. The procedures for larval rearing and the following obtainment of pupae were identical as the ones described in experiment 1.

Experimental protocol

Six sets of 100 Q-fly pupae per treatment were prepared as in the experiment 1 and stored at 17, 19, 21, 23 or 25 °C (control) ±0.5 °C with 65±5 % RH and complete darkness into incubators (30 replicates in total). The standard flight ability test was performed following the guidelines of FAO/IAEA/USDA (2014). One day before the expected beginning of adult emergence for each storing temperature, pupae were transferred from the incubators to the flight ability room and each set was placed in a separate 5.5-cm plastic Petri dish lid. The lids were then centered on 9-cm plastic Petri dishes with the bottom overlaid with a disk of black filter paper. Simultaneously, 10-cm tall acrylic tubes (outside diameter 8.9 cm with 3-mm thick walls), painted black on the outside, were gently coated with unscented talcum powder on their inner surface to prevent flies from escaping. After tapping the tubes on a firm surface to remove the excess powder, a resting space for the newly emerged flies was created by wiping off the first 1-cm coated surface at the bottom of the tubes with a cloth. Each tube was then inserted onto the 9-cm Petri dish lid hosting the pupae, and transferred in a mesh cage (32.5 x 32.5 x 32.5 cm, Megaview BugDorm-43030F). Cages were positioned beneath always-on 20-W fluorescent tubes, located 5 cm above them, that generated ~1250 lx at the top and ~900 lx at the base of the flight tubes (Figure 5.3-a). Room temperature was set at the standard level of 25±0.5 °C with 65±5%

RH. To estimate the incidence of flies that escape the tube, but by chance return and die inside it (fly-back), a second black tube (coated with talcum powder, but empty), was inserted in each cage ~5 cm away from the first tube containing the pupae. Every second day, flies that managed to escape the tube were collected from the mesh cage with 50-mL plastic vials. The remaining content of the tubes was collected when adult emergence ceased, generally 6 days after detecting the first emerged fly.

Flies were categorized as: (1) fully emerged (adults completely outside the pupal cases); (2) not emerged (adults within the unopened pupal cases); (3) partially emerged (adults stuck in puparia, but partially outside; Figure 5.4); (4) fliers (the number of flies that escaped the tube and were collected from the mesh cage plus fly-back); (5) fly-back (the number of flies inside the second tube plus the same number of morphologically normal flies inside the first tube); (6) non-fliers (morphologically normal flies that were found inside the first tube minus the number of flies inside the second tube); (7) deformed (fully emerged flies with morphological alterations, such as curly wings). The following parameters were calculated:

- *Pupal weight:* mean pupal weight (mg) when the storage period was completed and the pupae were transferred into the flight ability room.

- *Emergence rate:* percentage of fully emerged adults calculated, as (N pupae – [N not emerged + N partially emerged] / N pupae) x 100.

- *Partial emergence rate:* percentage of partially emerged adults, calculated as (N pupae – [N fully emerged + N not emerged] / N pupae) x 100.

- *Deformation rate:* percentage of deformed adults, calculated as (N deformed / N pupae) x 100.

- *Fliers:* percentage of flies that are able to fly, calculated as (N pupae – [N not emerged + N partially emerged + N deformed + N non-fliers] / N pupae) x 100.

- *Rate of fliers:* percentage of fully emerged flies that are able to fly, calculated as (*N* pupae – [*N* not emerged + *N* partially emerged + *N* deformed + *N* non-fliers] / *N* fully emerged) x 100.

- *Sex ratio:* percentage of females calculated as (N females / N fully emerged) x 100.

5.3.3 Experiment 2: Chill-coma recovery time

The aim of this assay is to evaluate the capacity of adult Q-flies, emerged from pupae stored at different suboptimal temperatures, to overcome a chill-induced coma by regaining the standing position.

Fly source

Flies for this assay were sourced as for the flight ability assay.

Experimental protocol

Chill-coma recovery time (CCRT; Weldon et al. 2011) for Q-fly adults emerged from stored pupae were assessed when flies were 3- or 10-days old. Four sets of 100 Q-fly pupae per treatment were prepared as in the flight ability assay and stored under the same conditions (20 replicates in total). One day before the expected beginning of adult emergence for each storing temperature, pupae were transferred from the incubators to a controlled environment room set with standard rearing conditions. Each set, consisting of a 9-cm Petri dish containing 100 pupae, was placed singly in 12.5-L clear plastic cages that had a mesh-covered opening on top for ventilation (Cage 1, 2, 3 and 4). Food was provided inside each cage by adding a drinking cup with tap water, sucrose and yeast hydrolysate. To avoid changing in fly density during the course of the assay, Cage 1 and 2 were used to source 3-days old flies (~100 flies, ~50 from each cage), while Cage 3 and 4 were used to source 10-days old flies (~100 flies, ~50 from each cage). Flies were transferred to individual numbered 7-mL screw-cap plastic vials and submerged inside a

zip-lock bag into ice-water slurry (~0 °C) for 2 h. The plastic vials were then brought in a laboratory room set at 25±0.5 °C and placed on their side on a counter. The CCRT (i.e., time taken for flies to exhibit the righting response and thus return to a standing position) following return to room temperature was recorded (Figure 5.3-b). Flies were sexed immediately before the assay and discarded at the end of it.

5.3.4 Experiment 2: Lipid body reserves

The aim of this assay is to evaluate the lipid body reserves of adult Q-flies emerged from pupae stored at different suboptimal temperatures.

Fly source

Flies for this assay were sourced as for the flight ability assay.

Experimental protocol

Two sets of 100 Q-fly pupae per treatment were prepared as in the flight ability assay and stored under the same conditions (10 replicates in total), until adult emergence was completed, generally 6 days after detecting the first emerged fly. Newly emerged adults were daily collected in 50-mL plastic vials and frozen at -20 °C. Twenty adult flies were randomly selected from the 2 replicates of each treatment for lipid body reserve measurement. Flies were placed individually in 5-mL glass tubes and dried in oven at 60 °C for 48 h. Moderately polar solvent, such as chloroform, have been widely used in mixture with methanol for lipid extraction (Folch et al. 1957; Bligh and Dyer 1959). Since chloroform alone is a good solvent for lipids, we decided to simplify the extraction procedure and use chloroform on its own for our test, similarly to other protocols found in literature (Raubenheimer et al. 2007; Ponton et. al 2011; 2015). Immediately after removing the flies from the oven, the glass vials were put into a desiccator flask containing dry silica gel granules. The dry weight of the samples was measured with a microscale balance and the samples transferred under a fume hood. With the use of a

pipette, 1 mL of chloroform (Sigma-Aldrich) was inserted into each tube and a rubber plug applied. The chloroform was changed twice, every 24 h, so that flies stayed into the solvent for 72 h in total. When the extraction was completed, the solvent was discarded and the flies dried a second time at 60 °C for 48 h and weighed. The lipid content (mg) extracted by the solvent was calculated by the difference between the initial and the final dry weight of the flies and lately expressed as a percentage of the initial dry weight.

5.3.5 Statistical analysis

Data from experiment 1, flight ability assay and lipid body reserves assay were analysed using the software STATISTICA 10.0 (StatSoft 2010). Prior to analysis, the percentage values were transformed using an arcsine transformation (Zar 1984). A Levene's test was first performed to assess if the variances of means were homoscedastic or not. A one-way ANOVA was performed on data when found to be normally distributed, as the effect of only one factor (storing temperature) had to be evaluated (Kutner et al. 2005). The Tukey's HSD or the Unequal N HSD tests were used to separate the means. If data were not normally distributed, a Kruskal-Wallis H test (Siegel and Castellan 1988) was performed followed by non-parametric multiple comparisons. Analysis for chill-coma recovery time assay was performed in R 2.10.1 (R Development Core Team, 2010). Data were first normalised using a $\log(n+1)$ transformation and then analysed with a Generalised Linear Model (GLM), examining the effect of temperature, age and sex. The very small number of flies that did not recover from chill-coma within 1 h (n = 4 in total among all treatments) were excluded from the analysis. The significance level was set at 0.05 for all statistical procedures.

5.4 Results

5.4.1 Experiment 1

Results of experiment 1 are shown in Table 5.1 and Figures 5.5-5.7. No adult Q-fly emerged from the pupae stored at 13 °C and only 2 adults (from different replicates) out of 600 pupae emerged from the pupae stored at 15 °C. Their pupal development was around 41 days, the longest attained value for this parameter. Data for pupae stored at 13 and 15 °C were excluded from the analysis, except for the percentage of partially emerged adults obtained from the pupae stored at 15 °C (Table 5.1).

Pupal development was significantly influenced by the storage temperature and progressively increased the lower the temperatures were. Development was significantly longer for pupae stored at 17 °C compared to control pupae maintained at 25 °C (Table 5.1; Figure 5.5). Results for emergence period and peak day of pupation mirrored those for pupal development, with substantial prolongment when Q-fly pupae were stored at 17 °C (Table 5.1). Emergence rate was progressively and significantly reduced as the storage temperature decreased (Table 5.1; Figure 5.6). Partial emergence rate was significally higher following storage at 17 and 15 °C compared to control (Table 5.1; Figure 5.7). Sex ratio did not vary significantly among treatments (Table 5.1).

5.4.2 Experiment 2: Flight ability

Results from the flight ability assay are shown in Tables 5.2-5.3 and Figures 5.8-5.10. First adults emerged after 25 days of pupal development when pupae were stored at 17 °C, after 19 days when stored at 19 °C, 15 days when stored at 21 °C, 13 days when stored at 23 °C and 11 days when pupae were stored at the control temperature of 25 °C. We here remind that storage of these pupae was not continuous, but was terminated 1 day before the expected day of emergence (on the basis of the experiment 1 results), when they were all restored at 25 °C in the flight ability room.

Queensland fruit fly pupae were significantly lighter following storage at 17 °C than those stored at the other suboptimal temperatures. Moreover, pupae were lighter following storage at 21 °C compared to control pupae maintained at 25 °C (Table 5.2). Emergence rate was lowered by the storage temperatures and was significantly reduced when pupae were stored at 21 and 19 °C, and even more when stored at 17 °C compared to control flies (Table 5.2; Figure 5.8). Partial emergence closely resembled emergence rate linear pattern, but increased instead that decreasing for the lower tested temperatures. This parameter was significantly higher following storage at 21 and 19 °C, and this increment was even more accentuated following storage at 17 °C (Table 5.2; Figure 5.9). Deformation rate of Q-fly adults was significantly increased when they were stored at 21 and 17 °C during the pupal stage compared to control flies (Table 5.2).

The percentage of fliers was significantly affected by storage at suboptimal temperatures. It was reduced by storage at 19 °C and dramatically lowered by storage at 17 °C compared to control flies (Table 5.3; Figure 5.10). Similarly, rate of fliers was significantly affected by the storage temperature and decreased with decreasing temperatures. The decrease became, however, significant compared to control only following storage at 17 °C (Table 5.3). No significant effect of the storage temperature was detected on the sex ratio (Table 5.3).

5.4.3 Experiment 2: Chill-coma recovery time

Results of the chill-coma recovery assay are shown in Tables 5.4-5.5 and Figure 5.11. Chill-coma recovery was significantly influenced by fly's age and by the interaction between the storage temperature and fly's age (Table 5.4). Following storage at 17 °C of the pupal stage, adult Q-flies displayed a similar CCRT value, regardless of age and sex (Figure 5.11). Converseley, when adult Q-flies emerged from pupae stored at 19 °C, 3-days old flies recovered quicker from chill-coma than 10-days old flies, regardless of sex

(Figure 5.11). The shortest CCRT was displayed by 3-days old males emerged from pupae stored at 19 °C, while the longest one was displayed by 10-days old males emerged from pupae stored under the same temperature regime (Table 5.5).

5.4.4 Experiment 2: Lipid body reserves

Results are shown in Table 5.6 and Figure 5.12. Storage at suboptimal temperatures of Q-fly pupae significantly reduced lipid body reserves of the resulting adults (Table 5.6). A considerable reduction of the lipid content was observed for those adults stored at 23 °C during the pupal stage compared to control flies. The storing temperatures of 21, 19 and 17 °C led to an even stronger reduction of the lipid content. When data for both sexes were analysed together, not differences were found between flies stored at 21, 19 and 17 °C. When the analysis was performed only on males, or only on females, lipid body reserves from flies stored at 17 °C became comparable to lipid body reserves of flies stored at 23 °C (Table 5.6; Figure 5.12).

5.5 Discussion

Numerous studies have evaluated the effects of insect storage at low temperatures with the final aim of developing protocols to assist rearing procedures and utilisation in pest control strategies (Hanna 1935; Krishnamoorthy 1989; Prasad and Ansari 2000; Bayram et al. 2005; Yan et al. 2017).

The present study investigated the potential usefulness of insect low-temperature storage to aim for more flexible Q-fly rearing, in the view of improving the overall release schedules in SIT programs already in place in Australia (PBCRC 2015). Since many variables play an important role in insect cold tolerance (Colinet and Boivin 2011) and studies of this kind are limited for *B. tryoni*, we decided to provide baseline knowledge

on the effects of suboptimal temperature storage by targeting the pupal stage and selecting only 1 pupal age (1-day old pupae).

In experiment 1, storage of Q-fly pupae at temperatures ranging from 13 to 19 °C revealed an extreme sensitivity of the pupae to the lowest tested temperatures (13 and 15 °C). As expected, storage proved effective in prolonging Q-fly developmental time, similarly to what has been found by storing eggs at suboptimal temperatures (Benelli et al. 2016, unpublished). When pupae were stored at 17 °C, pupal development was more than doubled in compare with pupae maintained at 25 °C. However, the emergence rate was almost halved by this treatment. Generally, a reduction of the emergence rate of low-temperature stored insects is a rule and depends primarily on the temperature regime and the exposure time (Anwar et al. 2016). In the case of Q-fly pupae stored at 17 °C, the main reason for the substantial decrease in the emergence rate is related to the inability of Q-fly adults to fully emerge from the opened pupal case when their development was completed, resulting in a considerably high partial emergence rate. The occurrence of developed adults dying during the eclosion process following low-temperature storage has been also reported in hymenopteran parasitoids (Levie et al. 2005; Luczynski et al. 2007). On the basis of the outcome given by experiment 1, 17 °C was set as the lower-bound limit for the following quality control assays.

In SIT programs, sterile flies must be able to disperse from the release site to locate water, food and wild female partners for mating. Hence, flies that are not able to fly are useless to SIT (Dyck et al. 2005). In the flight ability assay, we estimated the percentage of adult Q-flies that were able to emerge from pupae stored at suboptimal temperatures and their ability to fly. Once storage was completed, and before the flight ability set up, the measurement of pupal weight highlighed that pupae stored at the lowest tested temperature (17 °C) were also the lighest (7.75 mg). The fact that these pupae also

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displayed the longest pupal development (25 days) might suggest that the lower weight is a consequence of water loss derived from aging (Vargas et al. 1987; Eskafi and Fernandez 1990; Hulthen and Clarke 2006). Similarly to experiment 1 outcome, Q-flies demonstrated a high sensitivity to the lowest tested temperatures by significantly reducing their emergence rate for the regimes equal to or below 21 °C in experiment 2. In addition, the lower the storage temperature was, the higher the partial emergence rate was. This linear trend was not observed in experiment 1 because pupae exposed to temperatures below 17 °C displayed a high mortality rate, reflecting the impossibility to overcome the low-temperature induced damages on development (Koštál 2010). Deformation rate was negatively affected by storage of Q-fly pupae. Morphological anomalies can be the consequence of the impact that low temperatures have on tissue differentiation and hormonal balance of insects (Sehnal 1991; Sibly and Atkinson 1994). Deformation rate of adult insects has also been found to be correlated with cold storage duration in *Trichogramma* eventually reaching 100% in some cases (Schread and Garman 1934; Tezze and Botto 2004). Propensity for flight ability was also negatively affected by cold storage treatments. The Q-fly low-temperature sensitivity suggests that storage of pupae should be performed within temperatures that are only few degrees below the standard of 25 °C, at least when pupae are 1-day old. Our results confirm the findings of previous studies investigating the effect of low-temperature treatments on tephritid flight ability. Reynolds and Orchard (2011) studied the effect of chilling on adult *B. tryoni* and found that flies fed on a protein and sucrose diet had a reduced tendency for flight as the chilling time increased. Similarly, Salvato et al. (2003) showed that chilled adult *Ceratitis capitata* (Wiedemann) displayed a significant decrease of their ability to fly.

In the chill-coma recovery time assay, we estimated the time taken for adult *B. tryoni*, emerged from pupae stored at suboptimal temperatures, to regain standing position after

a period of chill-coma (i.e., a loss of locomotory capacity due to impaired neuromuscular function) (Denlinger and Lee 2010). Storage at suboptimal temperatures may result in an acclimation response of the flies, which is defined as a rapid and reversible phenotypical change in response to the exposure to a new environmental condition (Woods and Harrison 2002). This change is commonly advantageous for insect survival in changing environments (Fay and Meats 1987a; Terblanche et al. 2006). Since CCRT is a measure of cold tolerance, it may prove useful for testing the quality of low-temperature stored flies for use in SIT programs. Indeed, under factory-like conditions flies are continuously kept at the optimal rearing temperature, but when they are released in field they may face different environmental conditions, including temperatures considerably below the optimum (Fay and Meats 1987b). Under this scenario, having an increased cold tolerance will be advantageous for the flies released in field. Storage of Q-fly pupae at 19 °C enhanced the ability of 3-days old adults to recover from chill-coma compared to control flies, thus displaying an acclimation response (= an increase of their cold tolerance). However, 10-days old flies previously stored at 19 °C recovered slower from chill-coma compared to control flies. Thus, acclimation response was not only lost with ageing (reversible change), but fly cold tolerance was even decreased. Further investigations on the physiological mechanisms taking place during storage and chill-coma are needed to clarify this phenomenon.

At last, we investigated the lipid body reserves of adult Q-flies emerged from the stored pupae. As discussed by Leather et al. (1995), during low temperature exposure, weight loss may be associated not only with water loss, but also with the depletion of energetic reserves, including carbohydrate and lipids. Our results interestingly showed that the suboptimal storage temperatures significantly reduced the Q-fly lipid content. This finding may be in line with results from other experiments where storage at low

temperatures induced consumption of lipid reserves and also decreased the quality of the stored insects (Colinet et al. 2006). Lipid content decline of Q-flies may be related to the reduction in the percentage of fliers observed in the flight ability assay. It has been suggested that insects display a trade-off, in terms of energy allocation, between survival and reproduction (Jervis et al. 2003; Colinet et al. 2007). Fat reserves are believed to play a major role in this process (Ellers and van Alphen 1997), and depletion of lipids during low-temperature exposure is expected to translate into a fitness cost (Renault et al. 2004).

Overall, *B. tryoni* suffered severe detrimental effects of suboptimal temperature storage under the procedures described in this study, even when stored only few degrees below the standard rearing temperature of 25 °C. While little reductions in storing temperature provided a modest delay of development without (or with minimum) negative impact on quality, larger reductions of storing temperature, that gave greater extension, were associated with severe deleterious effects on quality. The results of this study form a basis for the use of cold storage in Q-fly mass rearing programs, and open new avenues for further investigations of Q-fly cold tolerance and its related physiology. Since age of the stored tephritid immatures may play a central role in determining tolerance to low temperatures (Tsiropoulos 1972; Cáceres et al. 2007), in future studies pupal age will be considered with the aim of determining the most suitable storage conditions for *B. tryoni* pupae. Further research is also required to determine the effect of irradiation on the post release performances of low-temperature stored Q-flies destined to SIT.

5.6 Acknowledgments

5.7 Statement of Authorship

MB, FP and PWT conceived and designed the experiments. MB conducted the experiments with help from UL and KAM. MB and KAM analysed data. MB wrote the Chapter, and all authors read and approved the Chapter.
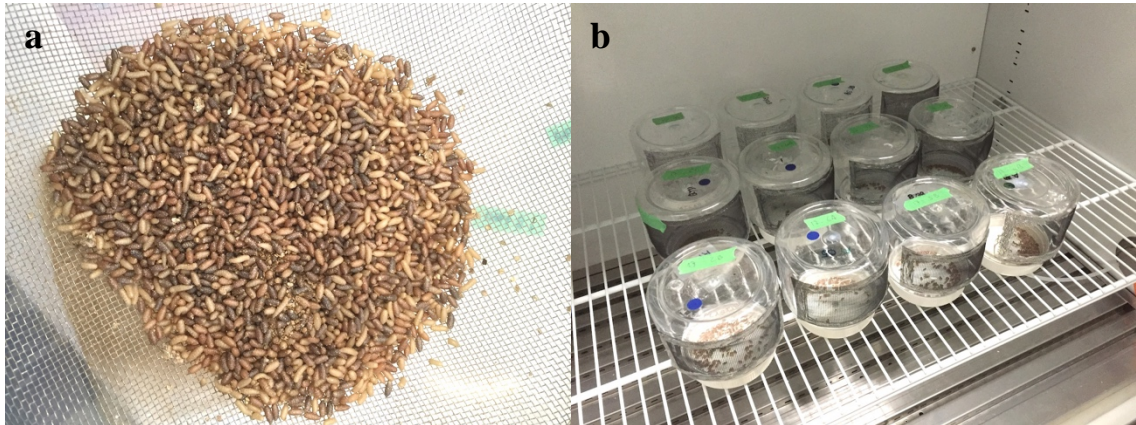
5.8 List of figures

Figure 5.1 – Suboptimal temperature storage of Queensland fruit fly pupae: (a) pupae were obtained by sifting the substrate in which L3 larvae pupated following rearing on a recently developed gel-based medium; (b) One-day old pupae were placed in 9-cm open Petri dishes (100 pupae per dish). Each dish was then inserted in a 1-L clear plastic jar that had a mesh-covered opening for ventilation. The jars were placed upside-down inside incubators and the dishes containing the pupae accommodated into their screw-top lid. All photos were taken by the author.

Figure 5.2 – Suboptimal temperature storage of Queensland fruit fly pupae: the incubators (Conviron A1000) used for the experiments were provided by the Department of Biological Sciences at Macquarie University. The incubators were set at different suboptimal temperatures with 65% RH and complete darkness. Storage conditions could be monitored by accessing remotely to the internal data logger system (author's photo).

Figure 5.3 – Quality control assessment of Queensland fruit fly adults emerged from pupae stored at 17, 19, 21, 23 or 25 °C (control): (a) standard flight ability test, fly cages are positioned beneath always-on 20-W fluorescent tubes; (b) chill-coma recovery test, plastic vials (each containing a fly) were brought back to the laboratory after having spent 2 h into an ice-water slurry. The time taken for flies to exhibit the righting response was measured. All photos were taken by the author.

Figure 5.4 – Queensland fruit fly partially emerged adults. Adults can remain stuck within the opened pupal case and die (author's photo).

Figure 5.5 – Experiment 1 results: pupal development (mean) for 1-day old Q-fly pupae stored continuously at 13, 15, 17, 19 or 25 °C (control). Different letters above the columns indicate significant difference at $P < 0.05$ ($H = 17$; $P = 0.002$*).

Figure 5.6 – Experiment 1 results: emergence rate (mean±SE) for 1-day old Q-fly pupae stored continuously at 13, 15, 17, 19 or 25 °C (control). Different letters above the columns indicate significant difference at $P < 0.05$ ($F_{2, 15} = 106.43$; $P < 0.0001$*).
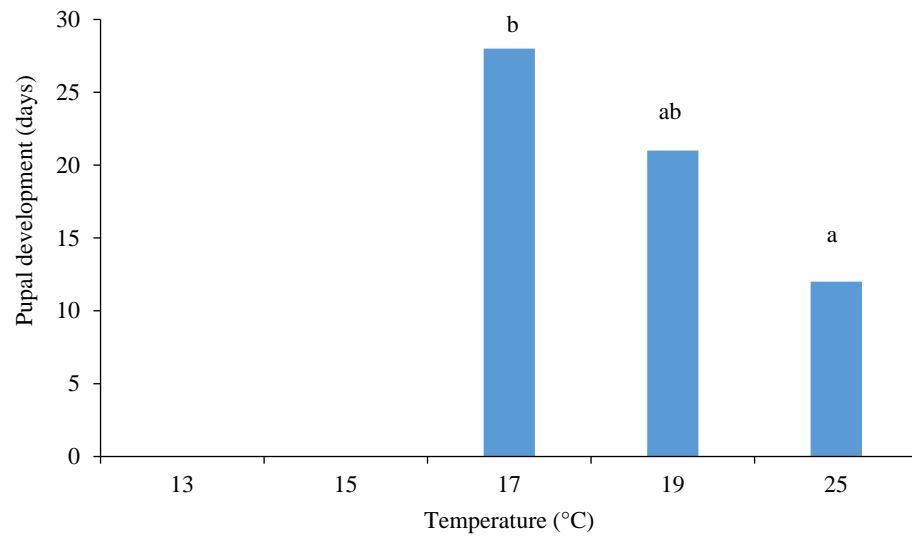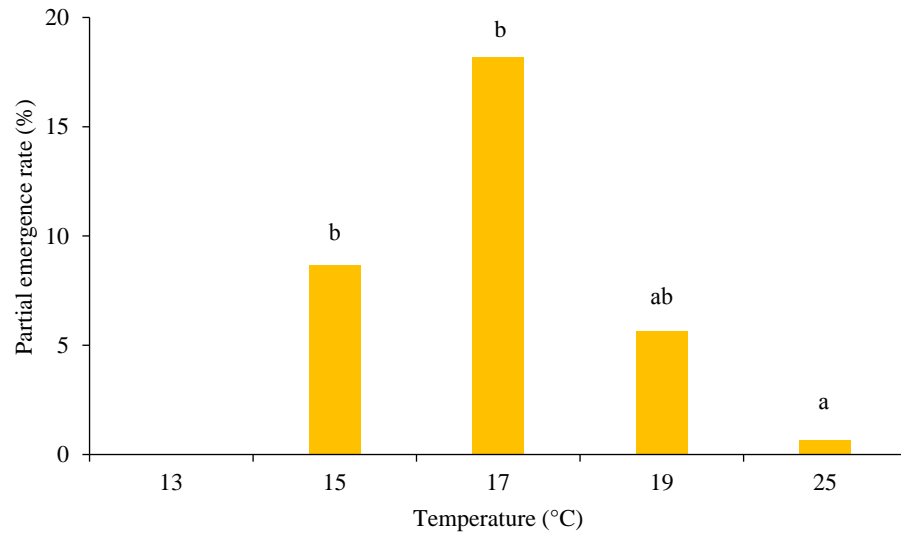
Figure 5.7 – Experiment 1 results: partial emergence rate (mean) for 1-day old Q-fly pupae stored continuously at 13, 15, 17, 19 or 25 °C (control). Different letters above the columns indicate significant difference at $P < 0.05$ ($H = 20.335$; $P = 0.0001*$).
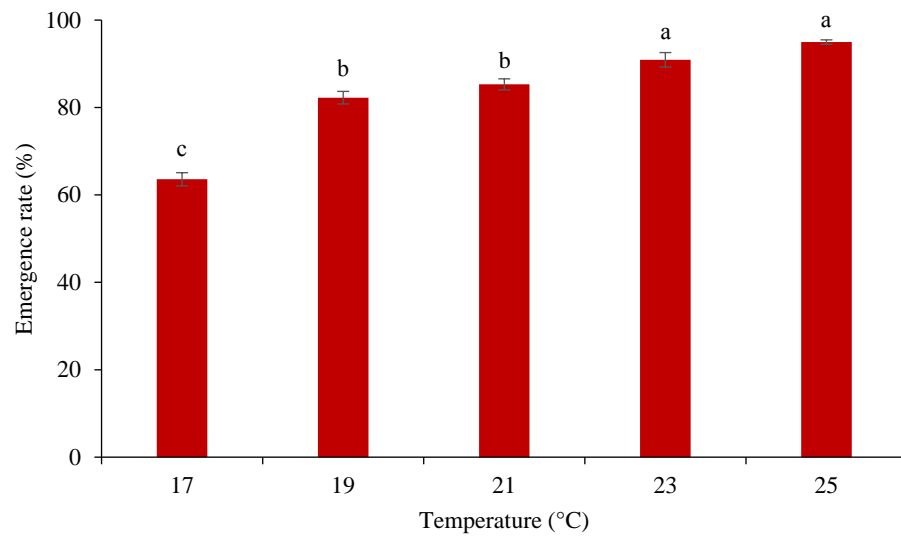
Figure 5.8 – Flight ability assay results: emergence rate (mean±SE) for 1-day old Q-fly pupae stored at 17, 19, 21, 23 or 25 °C (control). Different letters above the columns indicate significant difference at $P < 0.05$ ($F_{4, 25} = 57.41$; $P < 0.0001*$).
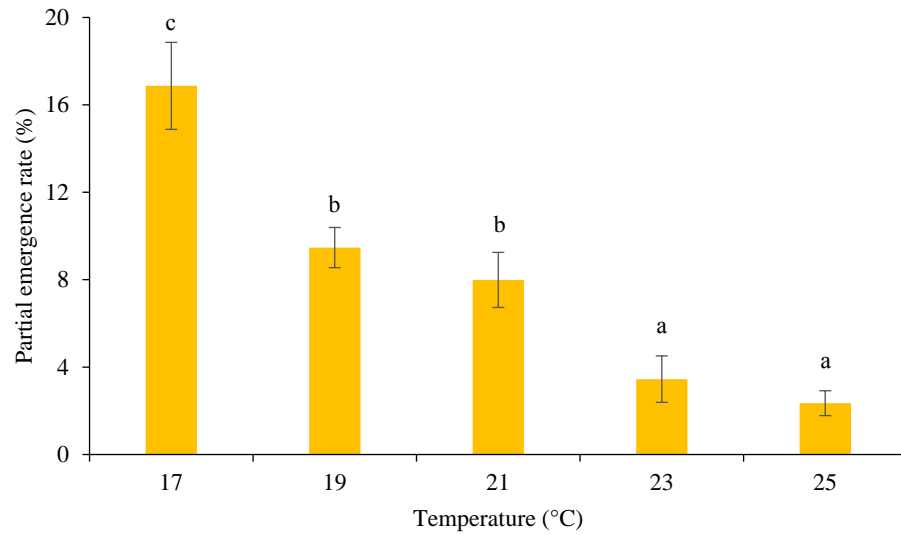
Figure 5.9 – Flight ability assay results: partial emergence rate (mean±SE) for 1-day old Q-fly pupae stored at 17, 19, 21, 23 or 25 °C (control). Different letters above the columns indicate significant difference at $P < 0.05$ ($F_{4, 25} = 18.511$; $P < 0.0001^*$).
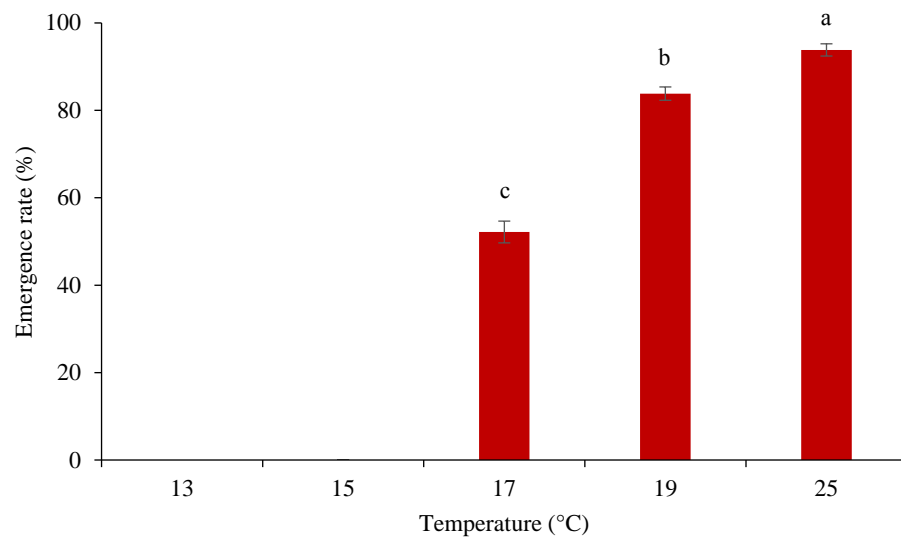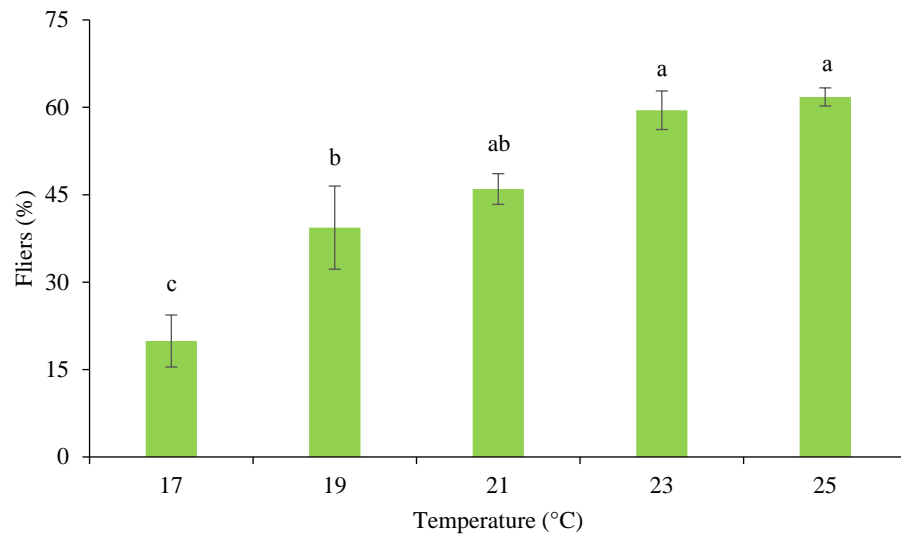

Figure 5.10 – Flight ability assay results: fliers (mean±SE) emerged from Q-fly pupae stored at 17, 19, 21, 23 or 25 °C (control). Different letters above the columns indicate significant difference at $P < 0.05$ ($F_{4, 25} = 14.462$; $P < 0.0001^*$).

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Figure 5.11 – Chill-coma recovery assay results: chill-coma recovery time (CCRT) (mean±SE) for 3- and 10-days old male and female Q-flies emerged from Q-fly pupae stored at 17, 19, 21, 23 or 25 °C (control).

Figure 5.12 – Lipid content (mean±SE) extracted by chloroform for both male and female Q-flies emerged from pupae stored at 17, 19, 21, 23 or 25 °C (control). Different letters above the columns indicate significant difference at $P < 0.05$ ($F_{4, 95} = 49.056$; $P < 0.0001^*$).
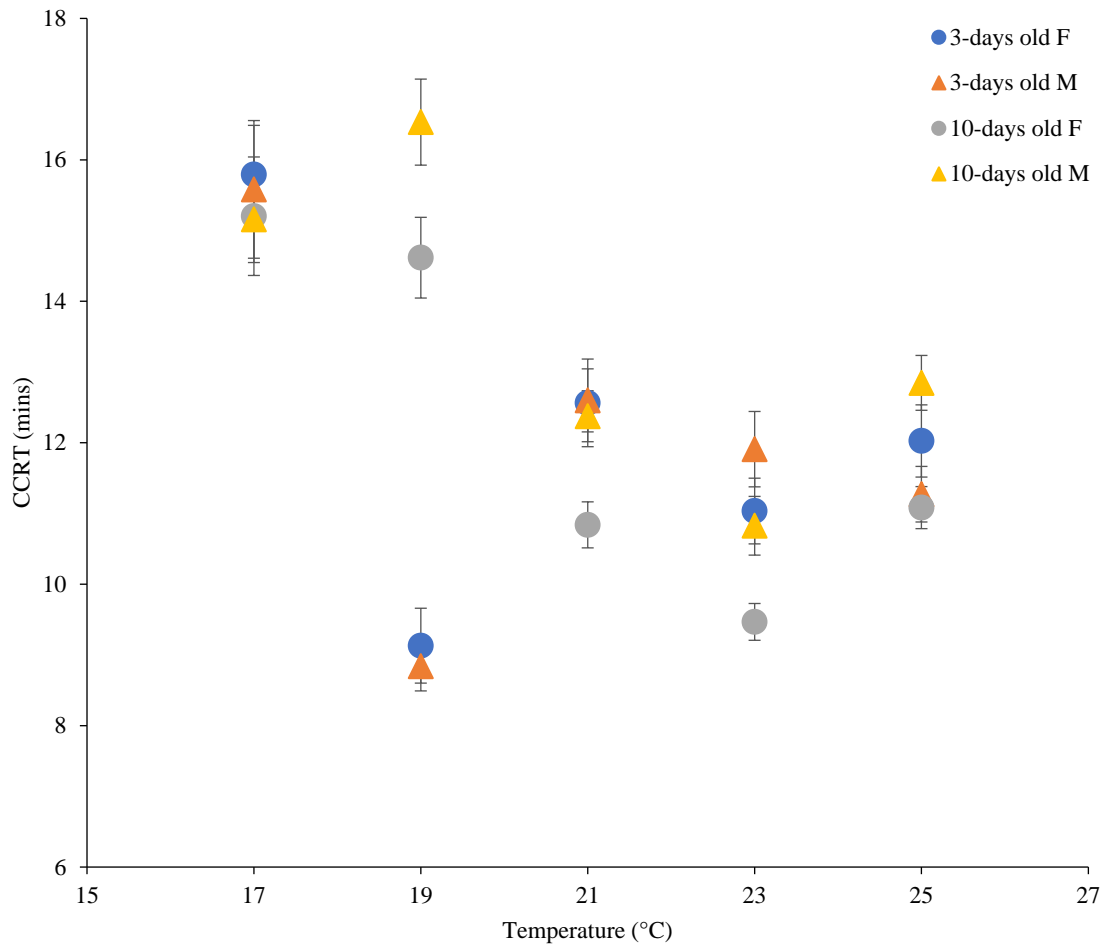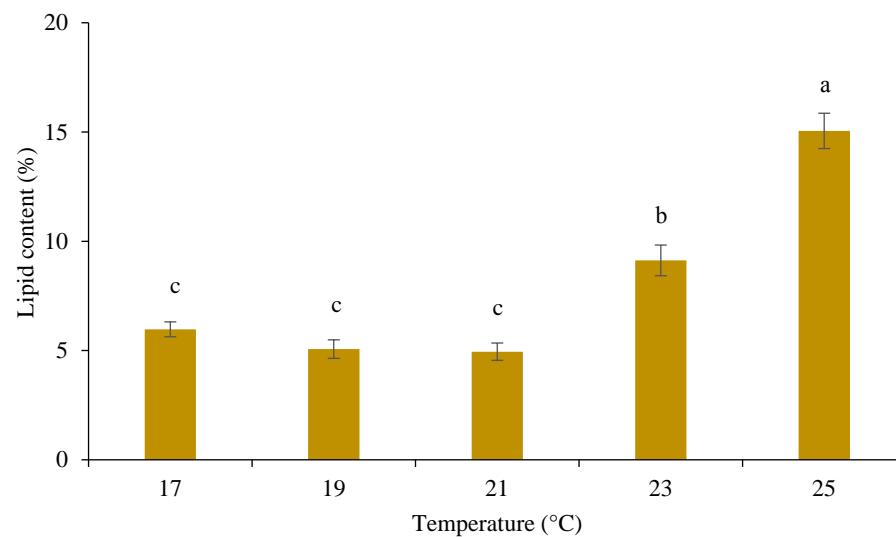
5.9 List of tables

Storage	Pupal development (days)	Emergence period (days)	Peak day of emergence (days)	Emergence rate (%)	Partial emergence rate (%)	Sex ratio (% of females)
25 °C	12[a]	3.167±0.167[a]	13[a]	93.833±1.4[a]	0.667±0.333[a]	49.78±0.753[a]
19 °C	21[ab]	3.333±0.211[ab]	22[ab]	83.833±1.537[b]	5.667±0.333[ab]	50.882±3.358[a]
17 °C	28[b]	4[b]	28.833±0.167[b]	52.167±2.496[c]	18.167±1.046[b]	50.683±2.837[a]
15 °C	-----	-----	-----	-----	8.667±1.358[b]	-----
13 °C	-----	-----	-----	-----	-----	-----
F (df)	-----	-----	-----	106.43 (2, 15)	-----	-----
H (N)	17 (18)	8.815 (18)	16.71 (18)	-----	20.335 (24)	0.245 (18)
P	0.002*	0.0122*	0.0002*	< 0.0001*	0.0001*	0.8844

Table 5.1 – Experiment 1 results: pupal development, emergence period, peak day of emergence, emergence rate, partial emergence rate and sex ratio (mean±SE) for 1-day old Q-fly pupae continuously stored at 13, 15, 17, 19 and 25 °C (control). For each treatment 6 replicates were carried out, each consisting of 100 pupae (n=6). Values followed by different letters in a column are significantly different at *P*<0.05 (one-way ANOVA followed by Tukey HSD test or Kruskal-Wallis H test followed by non-parametric multiple comparisons). No adult emergence was recorded when pupae were stored at 13 °C. Since emergence was approximately zero also for pupae stored at 15 °C, data for these storing temperatures were excluded from the analysis (except of partial emergence rate for pupae stored at 15 °C).

Storage	Pupal weight (mg)	Emergence rate (%)	Partial emergence rate (%)	Deformation rate (%)
25 °C	8.365±0.159[a]	94.972±0.508[a]	2.352±0.568[a]	1.012±0.261[a]
23 °C	8.166±0.14[ab]	90.913±1.662[a]	3.445±1.063[a]	1.968±0.346[ab]
21 °C	8.054±0.14[bc]	85.299±1.26[b]	7.989±1.162[b]	4.374±0.883[bcd]
19 °C	8.162±0.171[ac]	82.249±1.44[b]	9.47±0.92[b]	2.001±0.371[ac]
17 °C	7.745±0.139[d]	63.581±1.513[c]	16.869±1.991[c]	5.091±0.973[d]
F (df)	13.63 (4, 25)	57.41 (4, 25)	18.511 (4, 25)	8.137 (2, 15)
P	< 0.0001*	< 0.0001*	< 0.0001*	0.0002*

Table 5.2 – Flight ability assay results: pupal weight, emergence rate, partial emergence rate and deformation rate (mean±SE) for 1-day old Q-fly pupae stored at 17, 19, 21, 23 and 25 °C (control) until 1 day before the beginning of adult emergence, when they were transferred to the flight ability room under the standard rearing temperature (25 °C). For each treatment 6 replicates were carried out, each consisting of 100 pupae (n=6). Values followed by different letters in a column are significantly different at *P*<0.05 (one-way ANOVA followed by Tukey HSD test).

Storage	Fliers (%)	Rate of fliers (%)	Sex ratio (% of females)
25 °C	61.778±1.555[a]	65.045±1.561[a]	50.198±0.63[a]
23 °C	59.5±3.309[a]	65.476±3.557[a]	48.92±1.863[a]
21 °C	45.984±2.637[ab]	53.871±2.885[ab]	47.509±1.667[a]
19 °C	39.351±7.136[b]	47.776±8.361[ab]	47.984±2.46[a]
17 °C	19.9±4.463[c]	35.255±7.915[b]	47.778±1.152[a]
F (df)	14.462 (4, 25)	-----	-----
H (N)	-----	13.16 (30)	2.508 (30)
P	< 0.0001*	< 0.0001*	0.6432

Table 5.3 – Flight ability assay results: fliers, rate of fliers and sex ratio (mean±SE) for 1-day old Q-fly pupae stored at 17, 19, 21, 23 and 25 °C (control) until 1 day before the beginning of adult emergence, when they were transferred to the flight ability room under the standard rearing temperature (25 °C). For each treatment 6 replicates were carried out, each consisting of 100 pupae (n=6). Values followed by different letters in a column are significantly different at *P*<0.05 (one-way ANOVA followed by Tukey HSD test or Kruskal-Wallis H test followed by non-parametric multiple comparisons).

Factor	Wald's χ^2	Error	*P*
Temperature	-0.0051	0.0046	0.2671
Age	0.0341	0.0136	0.012*
Sex	-0.2367	0.1415	0.0948
Temperature x Age	-0.0016	0.0006	0.0105*
Temperature x Sex	0.0087	0.0067	0.1899
Age x Sex	0.0158	0.0191	0.4085
Temperature x Age x Sex	-0.0003	0.0009	0.7804

Table 5.4 – Chill-coma recovery assay results: data were first normalised with a log(n+1) transformation and the analysed with a Generalised Linear Model. The analysis examines the effect of temperature (storing temperature under which Q-fly pupae were kept before testing the resulting adults for the CCRT), age (3- or 10-days old) and sex and their interactions. The significance level was set at 0.05.

Storage	Age (days)	Sex	CCRT (mins)
25 °C	3	F	(n=49) 12.025±0.511
23 °C	3	F	(n=54) 11.034±0.465
21 °C	3	F	(n=46) 12.564±0.62
19 °C	3	F	(n=34) 9.13±0.529
17 °C	3	F	(n=56) 15.793±0.695
25 °C	3	M	(n=47) 11.272±0.393
23 °C	3	M	(n=45) 11.909±0.533
21 °C	3	M	(n=50) 12.599±0.446
19 °C	3	M	(n=57) 8.839±0.349
17 °C	3	M	(n=40) 15.583±0.973
25 °C	10	F	(n=47) 11.083±0.297
23 °C	10	F	(n=55) 9.467±0.26
21 °C	10	F	(n=51) 10.839±0.325
19 °C	10	F	(n=46) 14.617±0.571
17 °C	10	F	(n=42) 15.203±0.838
25 °C	10	M	(n=50) 12.847±0.388
23 °C	10	M	(n=43) 10.826±0.415
21 °C	10	M	(n=49) 12.375±0.361
19 °C	10	M	(n=54) 16.534±0.608
17 °C	10	M	(n=53) 15.159±0.611

Table 5.5 – Chill-coma recovery assay results: chill-coma recovery time (CCRT) (mean±SE) for adult Q-flies that were stored during their pupal stage at 17, 19, 21, 23 and 25 °C (control) until 1 day before the beginning of adult emergence, when they were transferred to a controlled environment room under the standard rearing temperature (25 °C). Flies were provided water, sucrose and yeast hydrolysate and tested when they were 3- or 10-days old. The number of individuals is given in parentheses above the means.

Storage	Lipid body reserves (%)		
	Both sexes	Males	Females
25 °C	(n=20) 15.051±0.808[a]	(n=10) 15.731±1.181[a]	(n=10) 14.37±1.122[a]
23 °C	(n=20) 9.125±0.702[b]	(n=10) 9.015±1.071[b]	(n=10) 9.236±0.965[b]
21 °C	(n=20) 4.947±0.397[c]	(n=10) 5.028±0.427[c]	(n=10) 4.866±0.693[c]
19 °C	(n=20) 5.066±0.425[c]	(n=9) 5.246±0.684[c]	(n=11) 4.918±0.559[c]
17 °C	(n=20) 5.971±0.341[c]	(n=8) 5.593±0.412[bc]	(n=12) 6.223±0.498[bc]
F (df)	49.056 (4, 95)	27.206 (4, 42)	20.916 (4, 48)
P	< 0.0001*	< 0.0001*	< 0.0001*

Table 5.6 – Lipid measurement results: lipid body reserves of both sexes, lipid body reserves of males and lipid body reserves of females (mean±SE) for adult Q-flies emerged from 1-day old Q-fly pupae continuously stored at 17, 19, 21, 23 and 25 °C (control). The number of individuals is given in parentheses above the means. Values followed by different letters in a column are significantly different at $P<0.05$ (one-way ANOVA followed by the Tukey HSD test or Unequal HSD test).

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6. General conclusions

Amongst all the reared insect species, dipterans represent the most frequently propagated ones and they are also reared in the highest numbers (Leopold 2000).

There are a variety of reasons for dipterans to be routinely reared. Besides fundamental research on physiology, genetics and molecular biology, where *Drosophila melanogaster* Meigen constitutes a widely adopted model organism (Jennings 2011), flies are reared in very high numbers for use in pest control strategies such as Sterile Insect Technique (SIT) (Knipling 1955). Officially born with the New World Screwworm (*Cochliomyia hominivorax* [Coquerel]) eradication program in the USA and Mexico (Vargas-Terán et al. 2005), SIT clearly marked a milestone for dipteran mass rearing. In addition, biological control programs releasing flies as natural enemies have also contributed to the establishment of dipteran mass rearing facilities, for the production of both predators (e.g., *Aphidoletes aphidimyza* [Rondani]) and parasitoids (e.g., *Lixophaga diatraeae* Townsend) (van Lenteren 2012).

Insect colony management presents several challenges and critical points, especially when it is performed at an industrial scale level (Morales-Ramos et al. 2014). This is related to the need for a production that has to be sufficient in terms of quantity of organisms produced at the right time. Specifically, it is necessary that the insect release in the field starts before the target pest population reaches the peak, thus being synchronized with the actual pest occurrence. In temperate areas, pest presence may be recorded only during a specific season (e.g., spring), meaning that to cut on costs, the production in mass rearing facilities should be maintained at the minimum level during the unfavourable season for the pest (e.g., winter). As a consequence, the efficiency of the overall production process may be lowered. Moreover, during the favourable season, quantities of needed insects may be higher than the amount produced weekly. A stockpile

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of insects, waiting for some more to be released all at once, is thus a valuable opportunity (Parker 2005). Short-term storage at low temperatures, sensu Leopold (1998), may allow to increase the flexibility of insect rearing, with particular advantage for the above-mentioned release programs.

Throughout the trials carried out in the present thesis, storage conditions (temperature and exposure time) were manipulated for different immature stages of two model dipteran species (*Exorista larvarum* [L.] and *Bactrocera tryoni* [Froggatt]) for use in pest control strategies entailing mass rearing and release. The main aim of this work was to develop protocols adopting suboptimal temperatures for facilitating the colony management of these two flies. In practical terms, the possibility to lengthen *E. larvarum* and *B. tryoni* developmental time was tested, ensuring also that their quality was not compromised by the storage treatments. The level of the tested suboptimal temperatures varied according to the fly species and also the stored stages. As stated by Sehnal (1991), any temperature below that to which a species is best adapted may be regarded as "cold".

Studies on *E. larvarum* developed methods for constituting a useful short-term reserve of immatures, for both small- or large-scale rearing. In Chapter 2, a method for stockpiling the tachinid eggs (and larvae) is provided. It has been demonstrated that once the eggs are collected and placed on artificial medium, flies can be stored at 15 or 20 °C. Storage at 15 °C was terminated when egg hatching was first observed, thus regarding only the egg stage of the parasitoid. Conversely, storage at 20 °C regarded also the larval stages and, probably for this reason, the detrimental effects were more severe than those observed storing eggs at 15 °C. The experiment was planned with the perspective to store the eggs that captive females usually oviposit on the cage surfaces. These eggs can be retrieved through the in vitro rearing, otherwise they would be lost (Dindo et al. 2007; Marchetti et al. 2008). In this scenario, a quality reduction of the adults derived from

those eggs may be acceptable, especially in the view of an urgent need of flies to overcome problems with the management of the main colony. Since we have tested eggs removed from previously parasitized *Galleria mellonella* (L.) larvae exposed to *E. larvarum* females, future experiments will investigate the storage of out-of-host laid eggs.

In Chapter 3, a method for stockpiling *E. larvarum* puparia was developed. Storage at 5 and 10 °C was unsuitable, but 1-day old puparia were stored successfully up to 21 days at 15 °C for this purpose. The possibility to maintain puparia at 15 °C and restore them at 26 °C when needed, as a way to delay development, may prove useful to provide an adequate reserve of parasitoids to be released in due time in augmentative biological control programs. At present, *E. larvarum*, has already been released in the field for controlling the gypsy moth *Lymantria dispar* (L.) in the USA (Sabrosky and Reardon 1976; Kenis and Lopez Vaamonde 1998). As shown in Chapter 3, several laboratory trials suggested that *E. larvarum* may be effective in controlling several pest species, besides the gypsy moth. Therefore, biological control programs involving this tachinid will be encouraged by this study, aimed at the improvement of the parasitoid rearing technology. As emphasised by Dindo and Grenier (2014), "mass rearing capability is one of the main factors that influence the selection of a parasitoid species for applied biological control". Since our investigation adopted 1-day old puparia, future experiments will consider puparium age as variable to better understand the role of ageing on *E. larvarum* cold tolerance. In addition, since dietary changes can play a role in determining insects' cold tolerance (Coudron 2007; Shreve et al. 2007), a comparison with in vivo and in vitro-obtained puparia could also prove useful, in the view of improving *E. larvarum* tolerance to low-temperature treatments through the use of different food sources. Artificial media supplemented with insect components may also be tested (Dindo et al. 2016).

Studies on *B. tryoni* provided baseline knowledge for the development of useful protocols for increasing its shelf life for a short period. In Chapter 4, a method for stockpiling the tephritid eggs (and larvae) was shown. It has been demonstrated that once the eggs are collected and placed on a gel-based larval medium, flies can be stored at 16 or 19 °C for 3, 6 or 9 days without any consequence for all the tested biological parameters and with no decrease in pupal and adult yields. Detrimental effects where severe when eggs were stored at 10 and 13 °C instead. Protocols of this kind have a potential for use on a laboratory and factory-like scale and are particularly convenient for insects targeted by SIT programs. For instance, in case of the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann), they have been explored for constituting methods for egg shipment from a central production facility to satellite centres that would mass-rear flies for release (Cáceres et al. 2007). Further investigations on the effects of suboptimal temperature storage of Q-fly eggs will explore different exposure times and, possibly, also the effects of diet composition on Q-fly cold tolerance.

In Chapter 5, a method for increasing the shelf life of 1-day old Q-fly pupae was shown. A first experiment outlined the necessity to narrow the range of suboptimal temperatures to use for storage, with 17 °C being the lower-bound limit. Pupal development was efficiently prolonged, although some severe detrimental effects were observed during a second experiment investigating adult quality following storage. Additional variables that may influence Q-fly cold tolerance (such as pupal age, nutrition, and oxygen concentration during storage [see Dominiak et al. 2011]) will be taken into account in future experiments to better assess the maximum attainable storage with the minimum impact on quality. The possibility to constitute a stockpile of Q-fly pupae, waiting for irradiation and release in SIT programs, may prove helpful in synchronizing

the operations with the occurrence of peaks of infestation in the field. Further experiments are also needed to evaluate the impact of irradiation on the low-temperature stored pupae.

Overall, the present thesis constitutes an additional contribution to the usefulness of short-term cold storage for assisting insect mass rearing (Leopold 2007). The baseline knowledge provided by the experiments performed adopting *E. larvarum* and *B. tryoni* as model species to be produced and release in pest control strategies needs to be further improved also considering the physiological mechanisms occurring during cold storage. Amongst the numerous factors that can impact insects' capacity to overcome storage at low temperature, acclimation and fluctuating thermal regimes deserve to be exploited for the refinement of the storage protocols for our model dipterans. The acclimation phenomenon refers to an increased cold tolerance following a short- or long-term pre-exposure to sub-lethal low temperatures (Hoffmann et al. 2003; Chown and Terblanche 2006). As a method for decreasing the detrimental effects observed in the stored *E. larvarum* e *B. tryoni* flies, a pre-exposure to suboptimal temperatures may prove effective, similarly to what has been found in other insect species (Luczynski et al. 2007; Lee and Denlinger 2010). Furthermore, short exposures to the optimal temperature during a longer storage at low temperature can reduce the amount of accumulated cold injuries and provide opportunities for physiological repair (Colinet et al. 2007; Koštál et al. 2007). Considering that injuries are cumulative during storage (Renault et al. 2004), fluctuating thermal regimes may prove useful compared to a constant storage at low temperature for increasing insects' shelf life while limiting the negative impacts on their survival and fitness (Colinet et al. 2006; Lalouette et al. 2007; Lee 2010).

In conclusion, storage at suboptimal temperatures proved effective for improving the rearing techniques of *E. larvarum* and *B. tryoni* and also for constituting useful stockpiles of immatures, but further research is required to better increase the longest attainable

storage and lessen the detrimental effects observed on their survival and quality of the

resulting adults.

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