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Effect of cold storage on the pupal development of two pollinators, *Eristalinus aeneus* and *Eristalis tenax*

Andrés Campoy*, Olga Egea-Casas, Celeste Pérez-Bañón & Santos Rojo

Department of Environmental Sciences & Natural Resources, University of Alicante, PO Box 99, Alicante E-03080, Spain

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Abstract

Eristalinus aeneus (Scopoli) and *Eristalis tenax* (L.) (Diptera: Syrphidae, Eristalini) are important pollinator species that can be artificially reared and commercialised. To achieve better control over the pupal development time and adult emergence, cold storage techniques are suitable tools. Insects were reared under controlled conditions: 25 ± 1 °C, 50% r.h., and L12:D12 photoperiod. Pupae of both species were stored at 5 °C at the beginning (early treatments) or at the end (late treatments) of their development for various periods of time (5, 10, 15, 20, or 30 days). Development stopped completely at 5 °C in both treatments, but in general, pupae stored at the beginning of the pupal stage provided better results in terms of survival (adult emergence) and quality of the adults (general morphology). The cold tolerance of *E. tenax* was lower than that of *E. aeneus*, with their pupal developmental time successfully extended up to 18 and 23 days, respectively, without compromising survival and morphology. The number and types of morphological alterations due to cold storage were recorded.

Introduction

The important role played by syrphids (Diptera) as pollinators is widely accepted (Larson et al., 2001; Ssymank et al., 2008). The subfamily Eristalinae, tribe Eristalini, comprises some of the most efficient hoverfly pollinators (Jarlan et al., 1997; Pérez-Bañón et al., 2007; Sajjad et al., 2008; Rader et al., 2009; Howlett & Gee, 2019). This fact has awoken interest in developing artificial rearing systems of some of these species (Gladis, 1994a; Rosso et al., 1994). Most protocols have focused on small-scale production for biological studies in the laboratory (Heal, 1979; Francuski et al., 2014; Nicholas et al., 2018). However, the increased interest in using these species as pollinators requires the development of protocols for medium- to large-scale rearing in order to conduct inundative releases of insects, especially in greenhouses or isolation cages.

Eristalinus aeneus (Scopoli) and *Eristalis tenax* (L.) are two eristalines that share a wide distribution around the world, saprophagous biology in their larval stage, and a remarkable pollination efficiency (Howlett & Gee, 2019; Latif et al., 2019; Speight, 2020), which can even be compared in some cases with commercial honey bees and bumble bees (Rader et al., 2009; Willmer, 2011). From such positive opinions have arisen interest in their artificial rearing.

A key aspect that insect producers need to sort out is the mass production of beneficial insects at an appropriate time. Independent of their application (e.g., biological control or pollination), cold storage is considered an essential tool in mass-rearing and commercialisation. This is commonly carried out under sub-optimal conditions above 0 °C (Leopold, 1998; Colinet & Boivin, 2011). The chosen temperature needs to be low enough to stop or decrease development and metabolic rate without reducing survival, adult fitness, and biological action (López & Botto, 2005; Rathee & Ram, 2018).

This technique presents multiple advantages: it allows the storage of sufficient numbers of beneficial insects for release in the field when required, improving the efficiency of the rearing process (Leopold et al., 1998; Prasad & Ansari, 2000; Colinet & Hance, 2010; Rathee & Ram, 2018). It permits synchronisation of the life cycles of multiple cohorts, facilitating the availability of beneficials during periods of high demand (Riddick & Wu, 2010; Costa

*Correspondence: Andrés Campoy, Department of Environmental Sciences & Natural Resources, University of Alicante, PO Box 99, ?E-03080, Alicante, Spain. E-mail: andrescp.bio@gmail.com

et al., 2016). It ensures sufficient stocks if any unexpected problem compromises production, such as disease, failure to keep the correct conditions, or genetic deterioration (Leopold, 2007). Cold storage also aids in transportation to where they are needed, regardless of distance (Li et al., 2014). Additionally, it is not just focused on commercial production, but also provides a viable stock of insects for research and other scientific purposes (Colinet & Boivin, 2011).

Exposure to low temperatures, however, may cause some detrimental effects on the physiology, morphology, and behaviour of treated insects (Leopold, 1998; López & Botto, 2005). Low temperatures can trigger various effects depending on exposure duration and severity of the cold (Lee & Denlinger, 2010). These reasons justify the importance of having a better understanding of the technique when applied to a particular species. The temperature, time of exposure, and effects caused by storage at low temperatures comprise essential information needed for correct implementation (López & Botto, 2005). In order to develop a cold storage protocol it is crucial to assess the lower and upper thresholds for each species so that development can be stopped without reducing fitness, biological action, or survival, all of which usually are lessened at lower temperatures (López & Botto, 2005; Bernardo et al., 2008). In some cases, a small difference of 0.5 °C can lead to totally different results in terms of survival (Leopold et al., 2004). The duration of cold exposure also has an effect on treated insects—usually, the longer the exposure, the higher the mortality (Colinet & Hance, 2010). This varies among species, as some can be stored for no more than a few days (López & Botto, 2005; Bernardo et al., 2008), whereas others can be stored for months (Mills & Nealis, 1992). The mortality is frequently associated with chilling injuries which can be immediate, accumulative, or latent (Rojas & Leopold, 1996; Leopold, 1998).

Traditionally, most studies related to insect storage at low temperature have been focused on biocontrol agents (parasitoids and predators), especially Hymenoptera (Jayanth & Nagarkatti, 1985; Jalali & Singh, 1992; Bernardo et al., 2008; Rathee & Ram, 2014) and, less frequently, Diptera (Easwaramoorthy et al., 2000; Benelli et al., 2017; Rathee & Ram, 2018). Few studies have paid attention to the cold storage of pollinators, most of them focused on the overwintering of Hymenoptera species (Undurraga & Stephen, 1980; Bennett et al., 2013; Rinehart et al., 2013). Additionally, the scarce studies that address the effect of cold storage on syrphids do not provide enough data on emergence rate, quality of the resultant adults, or cold thresholds, as their target is not the mass production of pollinators (Heal, 1989; Ottenheim et al., 1995; Nicholas et al., 2018).

Not all the developmental stages are equally resistant to stressful conditions. Some species are more cold tolerant during the immature stages, whereas others are more tolerant as adults. In this study, the pupa was the chosen stage for several reasons. This stage is more resistant against some adverse conditions (such as desiccation or extreme temperatures) commonly encountered during transport or in commercial greenhouses. Furthermore, this immobile stage allows better handling as well as more accurate control of the exact moment of adult emergence (van Lenteren & Tommasini, 2002; Colinet & Boivin, 2011).

This shortage of information and the growing interest in these species have encouraged this study. Its objectives were: (1) to find out whether *E. aeneus* and *E. tenax* respond favourably to cold storage to delay development; (2) to determine the maximum time that pupae can be stored at 5 °C without compromising survival and biological action; and (3) to characterise the effect of cold storage on the intra-puparial development from a morphological point of view.

Materials and methods

The pupae of *E. aeneus* and *E. tenax* used in this study were obtained from the third laboratory generation established at the rearing facilities of the University of Alicante (Spain), reared following the method described by Gladis (1994b) based on soaked oat grains (see also Campoy et al., 2020a).

Cold storage

Preliminary tests were conducted to determine the pupal development time for both species. In these tests, 200 pupae of *E. tenax* and *E. aeneus* were kept under controlled conditions (25 ± 1 °C, 50% r.h., and L12:D12 photoperiod) until adult emergence. In the case of *E. tenax*, adults emerged after eight (67.5%) and nine (32.5%) days, whereas in *E. aeneus*, the pupal stage lasted seven (6.4%), eight (68.2%), or nine (25.4%) days. This information was required to establish some of the treatments described hereinafter.

During the 1st day, all pupae from the rearing facilities were collected and discarded. Thus, pupae collected on the 2nd day at the prepupal phase (Fraenkel & Bhaskaran, 1973) were labelled as 1-day-old pupae and placed in a rearing chamber at 25 ± 1 °C, 50% r.h., and L12:D12 photoperiod. The randomness of the samples was ensured by collecting pupae from different trays (different mothers) but same age group. After 48 h, when the pupal spiracles were already present, pupae were individually separated into Petri dishes (3.5 cm diameter, 1 cm high). Pupae without pupal spiracles or malformed in any way

were directly discarded to keep a standard quality of insect.

Each of the different treatments were composed of three replicates of 100 pupae (a total of 300 pupae per treatment), placed together in plastic trays. Additional samples of 10 pupae were added to each cold storage treatment for dissection. These extra pupae were used for the morphological studies.

Treatments were divided into two main groups, one cold stored at the beginning of the pupal stage (early treatments), and the other at the end of it (late treatments). Storage was in a cold room at 5 °C, 60 ± 10% r.h., and total darkness. Early treatments were started just after the pupal spiracles protruded. Late treatments were started 1 day before adult emergence. Following the results from preliminary tests carried out before the main experiment, pupae of *E. aeneus* were stored at 6 days old and pupae of *E. tenax* at 7 days old, as the minimum pupal developmental time of these species was 7 and 8 days, respectively. For each early and late group, five cold storage periods (5, 10, 15, 20, or 30 days) were applied, plus two control treatments kept under controlled conditions (25 ± 1 °C, 50% r.h., and L12:D12 photoperiod). When the storage period was over, the various treatments were transferred again into the climatic chamber until adult emergence. In total, 12 treatments were carried out for each species. Pupal development time, adult survival (emergence), and the number and type of morphological alterations were recorded for both *E. aeneus* and *E. tenax*.

Morphological study

The extra samples of 10 pupae added to each treatment (100 pupae for each species in total) were dissected the day that their corresponding cold storage period was over. The rest of the pupae from the main treatments were moved back into the climatic chamber until adult emergence.

Pupae were carefully dissected from the anterior to the posterior end using micro-scissors, an anatomical tweezer, and hypodermic needles. Dissected pupae were preserved in 70% ethanol and photographed using an SZX16 stereo microscope (Olympus, Tokyo, Japan) with an SC100 integrated camera (Olympus) and Olympus cellSens Dimension v.1.18 software. The morphology was characterised and the intra-puparial phases were identified. The results were compared with the intra-puparial development of *E. aeneus* and *E. tenax* under optimal conditions (Campoy et al., 2020b), in order to describe the effect of cold storage on pupal development. The terminology for the different intra-puparial phases was determined following Fraenkel & Bhaskaran (1973), Cepeda-Palacios & Scholl (2000), and Martín-Vega et al. (2016).

Statistical analysis

To determine whether the cold storage could affect one sex more than the other, the developmental time of males and females within each treatment was compared. Additionally, in the same analysis, both sexes were compared separately between early and late treatments within the same cold storage duration. As data did not match the assumption of normality and homoscedasticity, non-parametric multifactorial analyses were performed. Fixed factors were 'treatment' (early and late) and 'sex' (male and female), the variable was 'developmental time'. Thus, the data were analysed using the Kruskal–Wallis test, and pairwise comparisons were made by the Wilcoxon rank-sum test. Post-hoc Bonferroni corrections were applied. Data analyses were carried out using R v.4.0.3 (R Core Team, 2020). Data used for the statistical analysis are publicly shared in Figshare repository (Campoy et al., 2021).

Results

Morphological study

Regarding intra-puparial development, pupae from all the early treatments showed a similar morphology, even between species, as all were at the beginning of their development and the ultimate adult morphology was still undefined (Figures 1A and 2A). Pupae were characterised by yellowish-translucent eyes, clear differentiation of the tagmata, appendages (legs, wings, and mouthparts) that had almost reached their final length, and the whole body weakly sclerotised. Additionally, the scutellum was distinguishable, the spiracular sacs providing support and surrounding the air connections (the 'vestigial felt chamber' sensu Gäbler, 1930) were well defined and pigmented, and the 'pupal mask', described for the first time in these species by Campoy et al. (2020b), was fully developed over the head, with a couple of dark markings under the mask (Figures 1A and 2A).

In late treatments, all the dissected pupae belonging to the same species showed a similar morphology. These pupae were more differentiated as their development was closer to the final adult. Dissections were conducted at different ages, i.e., at 6 days old in the case of *E. aeneus* and at 7 days old in the case of *E. tenax* (Figures 1B and 2B).

In *E. aeneus*, the eyes were reddish, and the whole body had not yet reached the final level of sclerotisation, although it was already covered by setae and the abdominal sclerites were well defined. Legs and mouthparts were already sclerotised and pigmented. Wings were membranous, and the venation had started to be distinguishable. The genital structures were almost defined. The pupal cuticle looked looser, and the ultimate morphology of the adult was more evident (Figure 1B).

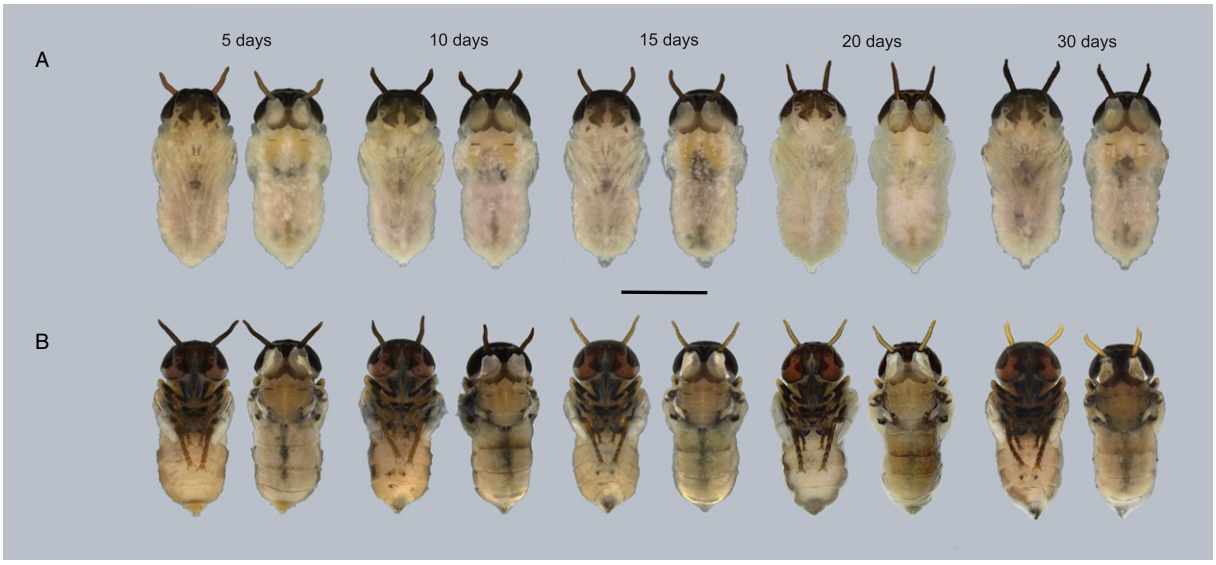

Figure 1 Intra-puparial development of *Eristalinus aeneus* when stored at 5 °C during increasing periods of time (0–30 days), either (A) shortly after pupariation (early) or (B) just before adult emergence (late). Storage time of 0 days implies under controlled conditions (constant 25 °C). Each treatment shows ventral (left) and dorsal (right) view. Scale bar: 5 mm.

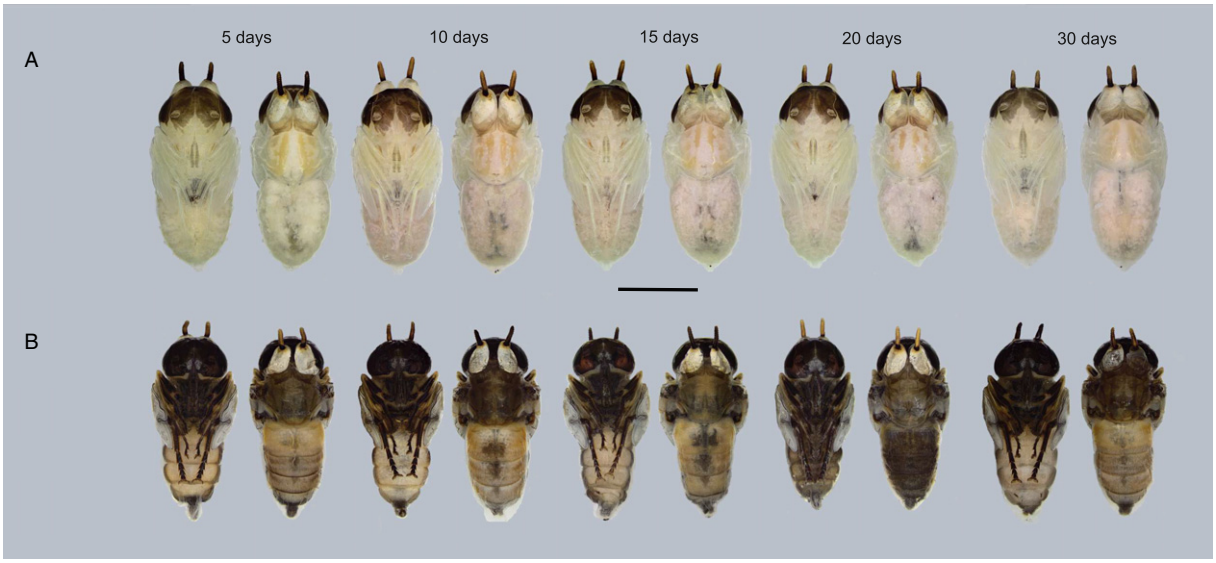

Figure 2 Intra-puparial development of *Eristalis tenax* when stored at 5 °C during increasing periods of time (0–30 days), either (A) shortly after pupariation (early) or (B) just before adult emergence (late). Storage time of 0 days implies under controlled conditions (constant 25 °C). Each treatment shows ventral (left) and dorsal (right) view. Scale bar: 5 mm.

In *E. tenax*, the eyes were dark maroon/black and the whole body, including appendages, was almost completely sclerotised and covered by setae. The abdominal sclerites were well defined. The wings were membranous, and the veins were noticeable. The genital structures were well developed. The pupal cuticle was completely detached from the adult epithelium (Figure 2B).

Table 1 Mean (± SD; sample sizes in parentheses) developmental time (days) of male and female *Eristalinus aeneus* kept under controlled conditions (constant 25 °C) and stored at 5 °C for increasing periods of time (5–30 days), either at 2 days old (early treatment) or at 6 days old (late treatment)

		Cold storage (days)					
Treatment	Sex	0 (control)	5	10	15	20	30
Early	Male	8.14 ± 0.346a,k (131)	13.52 ± 0.514a,k (154)	18.56 ± 0.526a,k (141)	23.77 ± 0.533a,k (132)	28.84 ± 0.416a,k (102)	38.67 ± 0.577 (3)
	Female	8.04 ± 0.192b,y (158)	13.43 ± 0.496a,x* (136)	18.38 ± 0.502b,x (144)	23.43 ± 0.612b,x (142)	28.70 ± 0.459a,x* (108)	38.38 ± 0.518 (8)
Late	Male	8.25 ± 0.571a,k (132)	13.47 ± 0.690a,k (143)	18.61 ± 0.489a,k (147)	23.32 ± 0.436a,l (103)	28.51 ± 0.598a,l (59)	38.50 ± 0.707 (2)
	Female	8.37 ± 0.603a,x (152)	13.29 ± 0.501a,x* (143)	18.30 ± 0.462b,x (138)	23.13 ± 0.362b,y (116)	28.43 ± 0.573a,x* (28)	– (0)

Means within a column followed by the same letter are not significantly different (Wilcoxon rank-sum test: $P>0.05$); comparisons are made pairwise, between sexes within a treatment (a/b), and between treatments within a sex (male: k/l; female: x/y). Asterisks indicate comparisons with a nearly significant difference, which should be considered with care.

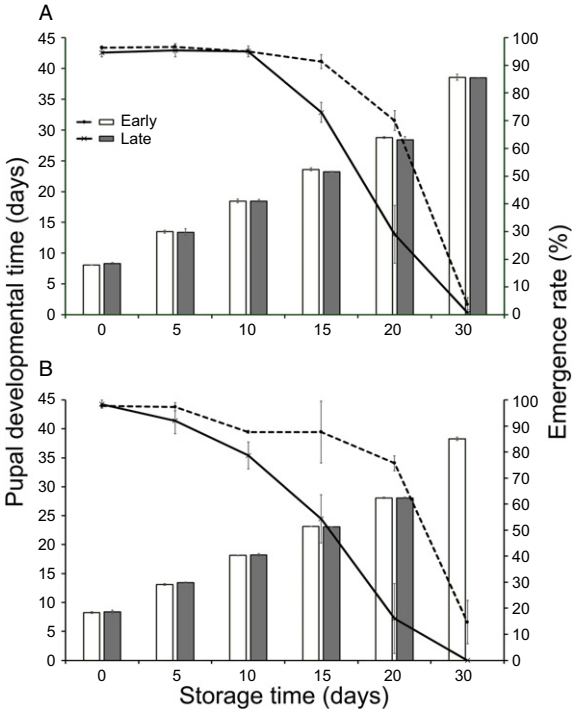

Figure 3 Mean (± SD) developmental time (days) and emergence rate (%) of (A) *Eristalinus aeneus* and (B) *Eristalis tenax* when stored at 5 °C during increasing periods of time (0–30 days), either shortly after pupariation (early) or just before adult emergence (late). Storage time of 0 days implies under controlled conditions (constant 25 °C).

Cold storage

The mean pupal development time under controlled conditions lasted around 8 days in both species. The lengthening of this stage observed in the treatments was directly proportional to the cold storage duration (Tables 1 and 2), showing that at 5 °C pupal development was completely stopped. This phenomenon was similar in both early and late treatments, and in the two studied species. In the longest treatments (30 days of cold storage), adults of both *E. aeneus* and *E. tenax* emerged after 38 days, but survival was much reduced (4.3 and 14.7%, respectively; Figure 3).

In general, the survival rate decreased as cold storage was extended. *Eristalinus aeneus* survival declined after 15 days of cold storage, more pronounced in the late (73%) than in the early treatment (91%). Survival was negligible after 30 days of cold storage in both early (4%) and late treatments (<1%) (Figure 3A). *Eristalis tenax* provided similar results, but this species was slightly more sensitive to cold exposure than *E. aeneus*. Early treatments of *E. tenax* showed the first major drop in survival after

Table 2 Mean (± SD; sample sizes in parentheses) developmental time (days) of male and female *Eristalis tenax* kept under controlled conditions (constant 25 °C) and stored at 5 °C for increasing periods of time (5–30 days), either at 2 days old (early treatment) or at 7 days old (late treatment)

		Cold storage (days)					
Treatment	Sex	0 (control)	5	10	15	20	30
Early	Male	8.22 ± 0.414a,k (124)	13.1 ± 0.384a,l (135)	18.08 ± 0.275b,k (135)	23.14 ± 0.348a,k (122)	27.98 ± 0.779a,k (103)	38.07 ± 0.258 (15)
	Female	8.28 ± 0.449a,x (169)	13.09 ± 0.286a,y (157)	18.2 ± 0.398a,x (128)	23.18 ± 0.383a,x (141)	28 ± 0.776a,x (124)	38.52 ± 0.509 (29)
Late	Male	8.29 ± 0.455a,k (94)	13.29 ± 0.453b,k (140)	18.16 ± 0.370a,k (87)	23.01 ± 0.116a,l (75)	28 ± 0a,k (19)	– (0)
	Female	8.38 ± 0.486a,x (201)	13.54 ± 0.501a,x (136)	18.24 ± 0.425a,x (149)	23.08 ± 0.272a,x (88)	28.03 ± 0.186a,x (29)	– (0)

Means within a column followed by the same letter are not significantly different (Wilcoxon rank-sum test: $P>0.05$); comparisons are made pairwise, between sexes within a treatment (a/b), and between treatments within a sex (male: k/l; female: x/y).

Figure 4 Mean (± SD) percentage of morphological alterations in (A) *Eristalinus aeneus* and (B) *Eristalis tenax* when cold stored at 5 °C during increasing periods of time (0–30 days), either shortly after pupariation (early) or just before adult emergence (late). Storage time of 0 days implies under controlled conditions (constant 25 °C).

10 days of cold storage (88%), dropping further after 20 (76%) and 30 days (15%). If treated late, survival decreased progressively with longer storage until 30 days of storage, when none of the adults emerged (Figure 3B).

A slight variation was found in the developmental time between sexes in both species, with males of *E. aeneus* and females of *E. tenax* showing longer developmental times (Tables 1 and 2). This tendency was observed in all the treatments except for the late control of *E. aeneus* and the early 5 days of cold storage of *E. tenax*. In *E. aeneus*, significant differences were found between males and females within the same treatment in both early and late treatments after 10 and 15 days of cold storage; however, this difference was also observed in the early control (Table 1), which means that it cannot be just attributed to the effect of cold exposure. In general, pupae of *E. aeneus* stored at the beginning of their development displayed a slightly longer developmental time than those stored at the end, being significantly longer in several treatments, especially in females (and sometimes nearly significantly) (Table 1).

In the case of *E. tenax*, most of the comparisons were not significantly different (Table 2). As a result, no difference in cold storage effects between the sexes could be established. Statistical differences between treatments after 30 days of cold storage were not analysed as mortality was generally too high and the samples of live flies were too small. Because differences between males and females within each treatment were not abundant or could not be attributed to an effect of cold storage, males and females were not separated in the graphs (Figure 3A,B). In general, *E. aeneus* showed a balanced sex ratio (Table 1), whereas in *E. tenax*, female adults were more abundant. However, the same tendency was observed in the control treatments (Table 2), suggesting that this difference is probably not due to the cold exposure.

After cold storage, some emerged adults showed various types of morphological alterations: general body malformations, lack of pigmentation on the eyes, and atrophy of the wings. The number of affected adults rose with storage duration, especially in late treatments. In *E. aeneus*, all adults belonging to early (20 and 30 days) and late treatments (15, 20, and 30 days) presented some kind of alteration (Figure 4A). In *E. tenax* also all emerged adults from early (20 and 30 days) and late treatments (10, 15, and 20 days) were affected (Figure 4B). Treatments with a shorter exposure only had a minimal proportion of affected insects, assumed to be the norm for a mass-rearing process. Furthermore, it is remarkable that all *E. tenax* adults of the early treatment were unable to fly after 15 days of cold storage, despite the absence of any kind of external malformation in most flies (Figure 4B). Thus, cold storage of pupae just after the appearance of the pupal spiracles is generally less detrimental than cold storage of pupae close to adult emergence, with *E. tenax* being more sensitive than *E. aeneus*.

Discussion

The results show that at 5 °C the pupal development time can be controlled and extended for up to 18 days in the case of *E. tenax* and 23 days in the case of *E. aeneus* without compromising the survival and quality of the adults. Despite these positive results, it would be desirable to increase the length of storage time of both species.

Following the terminology proposed by Fraenkel & Bhaskaran (1973) and the description of the intra-puparial development of *E. aeneus* and *E. tenax* under controlled conditions provided by Campoy et al. (2020b), it can be concluded that pupae in early treatments were morphologically at the beginning of the pharate adult phase (between 42 and 72 h), when the adult development takes place (see figures 3b and 4b in Campoy et al., 2020b). This is frequently divided in sub-phases, usually defined by the colour of the eyes (Fraenkel & Bhaskaran, 1973; Pujol-Luz & Barros-Cordeiro, 2012). It naturally follows the pupal-adult apolysis, which is difficult to identify using optical microscopy (Martín-Vega et al., 2016), but the pupal cuticle is still tight to the imaginal epidermis. In the case of late treatments, pupae of *E. aeneus* were at the end of the pharate adult phase (120–168 h) (figure 3f in Campoy et al., 2020b), whereas pupae of *E. tenax* showed an intermediate morphology between the end of the pharate adult phase and the adult stage (144–192 h) (figures 4f and 5c in Campoy et al., 2020b). This comparison supports the fact that, at 5 °C the pupal development was utterly stopped.

The ability of *E. aeneus* and *E. tenax* to stop development in response to cold exposure could be explained as a dormancy response or merely as quiescence. A dormant stage is an immediate developmental arrest caused by crossing a physiological threshold, usually responding to adverse conditions (Sømme, 1995; Koštál, 2006). Diapause is a more profound and centrally mediated arrest of development which usually requires prior conditioning of the insect before the beginning of adverse conditions, as well as a post-diapause stage of recovery (Dallwitz & Wardhaugh, 1984; Danks, 2002). The studied specimens were not exposed to any conditioning, and the cessation and reactivation of the development occurred instantly when cold storage started and finished, respectively.

Some studies have reported that adults of *E. tenax* can overwinter (Bressin & Willmer, 2000); in fact, Nicholas et al. (2018) developed a long maintenance rearing method for this species based on this biological attribute, but in the case of *E. aeneus* this behaviour is unknown. Both species are present in South Europe, adults of *E. tenax* can be observed between February and November, but their activity decreases during the warmer months, in which *E. aeneus* seems to be more active. Both species probably spend the colder months as third instars or pupae (*E. tenax* also as adults). This fact would suggest that immatures of *E. aeneus* need to spend a longer period of the year under suboptimal conditions (based on the activity period displayed by this species in the wild), which could explain why its cold resistance shown in this experiment was higher than that of *E. tenax*.

The cold tolerance of any insect species is influenced by a wide range of factors that need to be known and controlled to increase the efficiency of a rearing system. Some of the most relevant factors influencing the outcome are related to temperature, the duration of cold exposure, and how it is applied. Some studies have reported that problems associated with constant exposure to low temperatures could be alleviated by applying a fluctuating thermal

regime: periodic exposure to short recovery periods at an optimal temperature allowing physiological repair, reducing the speed and severity of chilling injury (Renault et al., 2004; Koštál et al., 2007). It enhances not only survival chances but also the fitness of post-storage insects (Leopold, 1998; Colinet & Hance, 2009). Additionally, gradual exposure to experimental temperatures (from optimal to sub-optimal and vice versa) usually improves the cold-hardening opportunities, increasing thermal tolerance (Chown & Nicolson, 2004).

Variability among species is very common and generalizations should be made cautiously, even when comparing taxonomically close species (Leopold, 1998; Colinet & Hance, 2010). In our results, both species were alike regarding pupal developmental time in response to cold storage, but differed in mortality and chilling injury thresholds. The variable cold sensitivity between species may be explained in various ways. Genetic differences could exist in responses to natural climatic fluctuations or to artificial selection during the laboratory rearing process, based on common detrimental effects, such as inbreeding, genetic drift, and bottlenecks, reducing their adaptation and stress tolerance after several generations (Hopper et al., 1993; Colinet & Boivin, 2011; Francuski et al., 2014), or selecting those strains that are better adapted to optimal (i.e., laboratory) conditions (Khosa & Brar, 2000). Other factors with an influence on cold tolerance are endogenous. Food quality influences the uptake of vital nutrients, and the resulting lipid reserves are extremely important in enabling stored insects to survive cold exposure and subsequently regain proper fitness levels and achieve high biological activity. These reserves are partially consumed during the storage period, and their lack would result in starvation and death (Colinet et al., 2006; Liu et al., 2007). Both species in this study were reared using the same larval medium based on soaked decaying oat grains. Although this medium has provided good results in their artificial rearing, *E. aeneus* is better adapted to it than *E. tenax* in terms of survival (Campoy et al., 2020a). This could imply that *E. aeneus* pupae have more energy reserves than *E. tenax*, which would explain the slight differences in survival and cold tolerance displayed between the species.

Another important difference was found between early and late treatments, the first of which in general provided better outcomes. The variability in survival was more evident after 15 days of cold storage, although differences between early and late treatments were evident in *E. tenax* in all cold storage periods. Leopold et al. (1998) observed that stored mid-aged pupae of *Musca domestica* L. displayed higher survival than those exposed either just after pupariating or at the end of the pupal stage. Some of the most critical developmental processes occur in these sensitive phases. After pupariation, there is an intense remodelling of the larval tissues through histolytic and histogenetic processes plus the assembly of new structures to form the adult. Before adult emergence, several metabolic and behavioural processes are activated for ecdysis and the future adult life (Bucher et al., 1948; Leopold et al., 1998). Mid-aged pupae apparently had a lower respiration rate than post-pupariating and pre-emerging pupae. Insects with lower respiration rates were able to deal with cold storage in a better way, probably by eliminating toxins produced during stressful situations such as long-term chilling (Rojas & Leopold, 1996). This matches our results, where insects stored close to adult emergence had lower survival than those stored at the beginning of the pupal stage. It is important to highlight that early stages were subjected to cold storage after the pupal–adult apolysis, when the pharate adult phase began (Fraenkel & Bhaskaran, 1973), and thus most of the critical remodelling processes of the larva were over. Additionally, the similar survival found at 5 and 10 days of cold exposure in either early or late treatments, followed by a clear differentiation in the successive treatments, shows the cumulative effect of chilling injuries, ultimately lethal in the longest treatments.

Apart from the direct effects of cold storage on the survival of treated insects, we have observed that various morphological affections may be caused by the chilling. Some adults of both *E. aeneus* and *E. tenax* displayed morphological alterations, wing deformity, and lack of pigmentation in the eyes (this last trait was particularly evident in *E. aeneus*), probably as a result of disturbed tissue remodelling, structural assemblage, and hormonal imbalance (Sehnal, 1991; Hewa-Kapuge & Hoffmann, 2001). Some individuals also presented abnormal locomotion and incapability of leaving the puparium or flying induced by neuromuscular dysfunction or starvation during storage (Koštál et al., 2006; Colinet & Boivin, 2011). Such alterations affecting morphology and locomotion are relevant as they affect adult emergence rate and pollination efficiency. Cold storage may also result in differential mortality between the sexes, distorting the sex ratio (Leopold & Chen, 2007; Chen et al., 2008); however, this effect was not seen in *E. aeneus* and *E. tenax*.

Some negative effects of cold storage are not detectable just after emergence, for example, shorter longevity due to reduced fat reserves during storage (Colinet et al., 2006), or reproductive problems due to affected mating behaviour or reduced production of viable gametes (Lacoume et al., 2007; Colinet & Hance, 2009), or malformations in the reproductive organs and slow maturation of eggs (Hanna, 1935; Denlinger & Lee, 1998). Correlation between body size, sex, and developmental time under optimal/suboptimal conditions has never

been studied; however, various authors reported that females of *E. tenax* tend to be bigger than males (e.g., Francuski et al., 2011). These post-emergence effects should be assessed in detail in future studies.

To conclude, in this study, we found that both *E. aeneus* and *E. tenax* can be successfully cold stored, which delays adult emergence and benefits their use as commercial pollinators. However, further research is needed to improve the cold storage protocol. It is important to detect the upper threshold temperature at which development can be stopped, as a higher temperature would cause lower mortality. Additionally, a fluctuating thermal regime should be tested as a beneficial tool to extend the pupal stage, while limiting losses. Apart from the analysis of morphological anomalies, we need to study the effects on adult fitness and function, specifically on fecundity, longevity, and pollination activity.

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Author Contributions

Andrés Campoy: Conceptualization (equal); Data curation (equal); Funding acquisition (lead); Investigation (lead); Methodology (lead); Software (equal); Visualization (equal); Writing-original draft (lead). Olga Egea-Casas: Conceptualization (supporting); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (lead); Software (lead); Writing-review & editing (supporting). Celeste Pérez-Bañón: Conceptualization (lead); Data curation (lead); Methodology (supporting); Resources (equal); Supervision (lead); Validation (lead); Visualization (equal); Writing-review & editing (lead). Santos Rojo: Conceptualization (supporting); Funding acquisition (equal); Project administration (lead); Resources (equal); Software (lead); Supervision (equal); Validation (lead); Visualization (equal); Writing-review & editing (equal).

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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