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# Acceptance and suitability of the box tree moth *Cydalima perspectalis* as host for the tachinid parasitoid *Exorista larvarum*

**Antonio MARTINI, Cinzia DI VITANTONIO, Maria Luisa DINDO**
*Dipartimento di Scienze e Tecnologie Agro-Alimentari (DISTAL), Università di Bologna, Italy*

## Abstract

A laboratory bioassay and anatomical and histological studies were conducted to evaluate the acceptance and suitability of an exotic insect, the box tree moth *Cydalima perspectalis* (Walker) (Lepidoptera Crambidae), as host for the native parasitoid *Exorista larvarum* (L.) (Diptera Tachinidae). The factitious host *Galleria mellonella* (L.) (Lepidoptera Pyralidae) was maintained as control. In the bioassay, *C. perspectalis* and *G. mellonella* mature larvae were separately exposed for 3 hours to *E. larvarum* mated females. Box tree moth larvae were accepted by *E. larvarum* females, but a lower number of eggs were laid on them than on *G. mellonella*. Most eggs hatched, as also shown in the anatomical and histological studies, but no puparia formed in any accepted *C. perspectalis* larva. Two out of six first instar *E. larvarum* larvae penetrated the body of a box tree moth larva and were encapsulated. The encapsulation response turned into the formation of the respiratory funnel by two parasitoid larvae, similarly to what happens in *G. mellonella*. The results obtained in this study showed that the exotic species was unsuitable as host for *E. larvarum*. The mortality following the parasitoid larval activity (independently of successful parasitization) was, however, not significantly different between *C. perspectalis* and *G. mellonella*. The overall results suggest that the mortality of *C. perspectalis* larvae due to the partial development of *E. larvarum* may be useful to regulate the populations of this invasive pest in a context of conservative biological control.

**Key words:** exotic insects, *Cydalima perspectalis*, *Exorista larvarum*, acceptance and suitability, parasitoids, biological control.

## Introduction

The box tree moth *Cydalima perspectalis* (Walker) (Lepidoptera Crambidae), originating from China and widespread in East Asia, was probably introduced in Europe with the trading of *Buxus* seedlings (Kruger, 2008). After its first detection in 2007 in southwestern Germany (Billen, 2007) and the Netherlands (Muus *et al*., 2009), the species rapidly invaded other European countries (Bras *et al*., 2015; 2016). According to Nacambo *et al.* (2014), *C. perspectalis* has the potential to invade all Europe, except the coldest areas. In Italy, it was first detected in 2010 in Veneto and Lombardy (northern Italy) by members of the *Forum Entomologi Italiani* and it is now distributed in other Italian regions (Bella, 2013; Raineri *et al*., 2017).

This moth primarily attacks plants belonging to the genus *Buxus*, mainly *Buxus sinica* (Rehder et E.H Wilson) in China and *Buxus microphylla* Siebold et Zucc. in Japan (Leuthard *et al.*, 2013; Wan *et al.*, 2014). In the areas of introduction *C. perspectalis* has extended its host range to new *Buxus* varieties and hybrids (Leuthard *et al.*, 2013). Other plant species, including *Euonymus japonicus* Thunberg and *Ilex purpurea* Hasskarl have been reported as hosts of *C. perspectalis* in Japan (van der Straten and Muus, 2010; CABI, 2018).

On box trees, the larvae feed on leaves and can also attack the bark, causing defoliation and even death of the affected plants (Leuthardt and Baur, 2013; CABI, 2018). In Europe, *C. perspectalis* represents a threat to *Buxus* plants in public and private gardens and parks (Bella, 2013; Santi *et al*., 2015; Mitchell *et al*., 2018). Serious damage may also occur in natural environments, where the native *Buxus sempervirens* L. is a very important component of unique forest ecosystems, such as in southern France (Di Domenico *et al.*, 2012; Kenis *et al.*, 2013) and in wild areas (code Habitat 5110, protected by the 92/43 EU Directive) of Liguria (Raineri *et al.*, 2017) and Piedmont (Chiara Ferracini, personal communication).

In Europe, *C. perspectalis* overwinters as larva protected in a silk shelter spun among the box tree leaves (Nacambo *et al*., 2014). In the countries of origin and introduction, the number of generations may vary from two to five per year, depending on the climatic conditions (Wan *et al*., 2014). In northeast Italy three generations were found to occur, with flight peaks observed in early June, early August and mid-September, respectively (Santi *et al*., 2015).

In Europe, only two polyphagous parasitoids, *Pseudoperichaeta nigrolineata* (Walker) (Diptera Tachinidae) and *Apechthis compunctor* (L.) (Hymenoptera Ichneumonidae) have so far been found to attack *C. perspectalis* in nature, at a very low rate (Nacambo, 2012, Wan *et al.*, 2014). Moreover, under laboratory conditions, box tree moth eggs proved suitable as hosts for different *Trichogramma* species (Hymenoptera Trichogrammatidae), especially *Trichogramma dendrolimi* Matsumura, but the parasitoid efficacy under field conditions is still unknown (Göttig and Herz, 2016). In the countries of origin, the parasitoid complex of *C. perspectalis* in nature is (as expected) wider and includes hymenopterous oophages, egg-larval, larval and pupal parasitoids as well as the tachinids *P. nigrolineata*, *Compsilura concinnata* (Meigen) and *Exorista* spp., all polyphagous antagonists of lepidopterous larvae (Wan *et al*., 2014).

A species of the genus *Exorista* Meigen, *Exorista larvarum* (L.), native to the Palearctic region, is distributed

throughout Europe and is well known as a parasitoid of lepidopterous defoliators in forest and agricultural environments (Cerretti and Tschorsnig, 2010). *E. larvarum* females lay macrotype eggs on the host integument (Dindo *et al*., 2007). The newly hatched larvae penetrate the host's body, build primary integumental respiratory funnels and grow continuously until pupation, which generally occurs outside the host's remains (Michalková *et al*., 2009; Dindo, 2011). At the Department of Agricultural and Food Sciences (DISTAL; University of Bologna, Italy), a stock colony of *E. larvarum* is currently maintained using the greater wax moth *Galleria mellonella* (L.) (Lepidoptera Pyralidae) as a factitious host (Dindo *et al*., 2003; Benelli *et al*., 2017). In a previous laboratory study (Dindo *et al*., 2013), *E. larvarum* showed potential to adapt to the geranium bronze, *Cacyreus marshalli* (Butler) (Lepidoptera Lycaenidae), another invading lepidopterous species in Europe, although its contribution to pest control appeared to be especially related to host mortality due to incomplete parasitoid development. On this basis, the laboratory experiments described in this article were aimed at studying the acceptance and suitability of *C. perspectalis* as host for *E. larvarum*. Both biological bioassay and anatomical and histological examinations were conducted to evaluate the possibility of adaptation of this native tachinid species to the exotic pest and *G. mellonella* was maintained as a control.

## Materials and methods

### Insects

A laboratory colony of *E. larvarum* was maintained using *G. mellonella* as a host. The parasitoid adults were kept in Plexiglas cages (40 × 30 × 30 cm) at 25 ± 1 °C, 65 ± 5% RH, 16:8 L:D photoperiod. The flies (50-70 per cage) were fed on lump sugar and cotton balls soaked in a honey and water solution (20% honey). Parasitization occurred by exposing *G. mellonella* mature larvae (the most suitable stage according to Hafez, 1953; Dindo *et al*., 2003) to *E. larvarum* females for about 1 hour. The fly colony was established in 2004 from adults which had emerged from *Hyphantria cunea* (Drury) (Lepidoptera Erebidae) larvae field-collected in the province of Modena (44°10'49"N 10°38'54"E; Emilia Romagna, northern Italy). *G. mellonella* larvae were reared on an artificial diet developed by Campadelli (1987) and maintained at 30 ±1 °C, 65 ± 5% RH and in complete darkness.

Late-instar larvae of *C. perspectalis* were field-collected from *B. sempervirens* plants in the experimental farm of the University of Bologna in Cadriano (44°32'57"N 11°23'15"E; Emilia Romagna, northern Italy) in May 2015. They were transferred to the laboratory, placed in plastic boxes (40 × 30 × 30 cm) with ventilation holes covered with fine mesh and daily supplied with boxwood leaves. Mature larvae (about 3.5 cm long) were used for the experiments. Box tree moths larvae were kept in a climatic chamber at 25 ± 1 °C, 65 ± 5% RH, 16:8 L:D photoperiod.

### Acceptance and suitability bioassay

*C. perspectalis* and *G. mellonella* mature larvae were separately exposed for 3 hours to *E. larvarum* mated females aged 5-12 days (Dindo *et al*., 2007), in 20 × 20 × 20 cm Plexiglas cages (2 larvae per female). The exposure time was longer than in the standard rearing due to the slow oviposition rhythm of *E. larvarum* on *C. perspectalis*. After exposure, the larvae were removed from the cage and the eggs laid on their body were counted. The larvae were considered as "accepted" when at least one parasitoid egg was detected on their integument. For each moth species, an equal number of larvae served as controls and were not exposed to parasitoids. All larvae (either exposed or not) were individually placed in 9-cm diameter plastic petri dishes and observed daily until their death or moth emergence. For the exposed larvae, the formation of parasitoid puparia was registered. The newly-formed puparia were collected and placed singly in glass vials until fly emergence, or death. The trial was conducted at 25 ± 1 °C, 65 ± 5% RH, 16:8 L:D photoperiod.

Four replicates were carried out. The number of larvae per replicate varied depending on the availability of mature *C. perspectalis* larvae. In every replicate, an equal number of *C. perspectalis* or *G. mellonella* larvae was assigned to each treatment and to controls (i.e. 11, 4, 4, 15 larvae in the 1st, 2nd, 3rd, 4th replicates respectively).

To evaluate the results, for the moth larvae exposed to *E. larvarum*, the percentages of accepted larvae (based on exposed larvae), the eggs/accepted larva, the percentages of puparia (based on parasitoid eggs laid on larvae) (= puparium yields) and of adults (based on puparia) were calculated. The percentages of moth adults obtained from *C. perspectalis* or *G. mellonella* larvae exposed or not exposed to *E. larvarum* were also calculated. Moreover, two indices (DI = Degree of Infestation, SP = Success rate of Parasitism) summarizing the host-parasitoid interactions, adapted from Chabert *et al*. (2012), were scored. The DI measured the proportion of host larvae that, following exposure to *E. larvarum*, died due to the parasitoid larval activity (not necessarily resulting in puparium formation). This index was estimated as $(T\text{-}di)/T$, where $T$ and $di$ were the number of moth adults that emerged from control larvae ($T$) or from larvae exposed to *E. larvarum* flies ($di$). The SP measured the probability that an infested host (= a host containing larvae of this gregarious parasitoid) would give rise to at least one adult fly. This was estimated as $pi/(T\text{-}di)$, where $pi$ was the number of moth larvae exposed to *E. larvarum*, which produced at least one adult fly. When $pi$ was > ($T$-$di$), SP was set as 1.

### Statistical analysis

For statistical analysis, the software STATISTICA 10.0 (StatSoft, 2010) was used. The data were analysed by one-way ANOVA or, in case of variance heterogeneity, by Kruskal-Wallis nonparametric test. The percentages of moth adults were analysed separately for the larvae exposed to *E. larvarum* females and for those which were not. Prior to analysis, the percentage values were transformed using an arcsine transformation (Zar, 1984).

**Figure 1.** Overview of *E. larvarum* eggs (arrows) on *C. perspectalis* larva (ventral view).

### Anatomical and histological study

In order to highlight the relationship occurring between *E. larvarum* larvae and *C. perspectalis* host larvae, an anatomical and histological study was conducted. A larva bearing eggs was examined 4 days after oviposition: the larva had stopped eating and was motionless (figure 1). The number of eggs and their fate was recorded. The larva was first dissected in saline (Ringer's solution) by a ventral cut (figure 2) and examined using stereomicroscopy; the entire gut was removed, and the position of the eggs recorded. Selected parasitoid larvae were cleared in KOH saturated solution in order to ascertain their larval instar. In the anatomical study, in order to compare the reaction between the two hosts, a larva of *G. mellonella* bearing three eggs of *E. larvarum* was dissected in the same way, 4 days after oviposition.

In the histological study, following dissection, the *C. perspectalis* larva was fixed in Bouin's solution (Sigma® HT10132) for 12 hours. After fixation, the whole larva was dehydrated in an ascending ethanol series, cleared with xylene, and embedded in Paraplast® Plus tissue embedding medium. Serial frontal sections (6 µm in thickness) were cut off with a rotative microtome (Leica RM2135) and stained with hematoxylin and eosin, and mounted with DPX mountant (BDH Chemical, Poole, UK). The sections were photographed with a Carl Zeiss Axioskop light microscope equipped with an AxioCam digital camera.

## Results

### Acceptance and suitability bioassay

As expected, all *G. mellonella* larvae were accepted by *E. larvarum* and more than 86% of *C. perspectalis* larvae were accepted as well, with no significant difference between host species. No significant difference was also found for the parasitoid eggs/accepted larva, but higher oviposition was obtained on *G. mellonella* (table 1). No puparia formed in any accepted *C. perspectalis* larva. Conversely, puparia were obtained from *G. mellonella* larvae and all puparia emerged as adults (table 1). The percentage of moths obtained from the larvae exposed to *E. larvarum* was low for both *C. perspectalis* (12.5 ± 7.2) and *G. mellonella* (10.2 ± 5.3). The difference between the moth species was not significant (H = 0.09; N = 8;

**Figure 2.** Larva of *C. perspectalis* dissected ventrally.

**Table 1.** Acceptance and suitability of *C. perspectalis* and *G. mellonella* for the parasitoid *E. larvarum*: accepted larvae (%) based on moth larvae exposed to parasitoids, *E. larvarum* eggs/accepted larva, *E. larvarum* puparia (%) based on parasitoid eggs laid on larvae, adults (%) based on parasitoid puparia (means ± SE). Means in a column followed by the same letter are not significantly different (one way ANOVA). (1) No statistical analysis was performed.

| Host species | Accepted larvae (%) | *E. larvarum* eggs/accepted larva (n.) | *E. larvarum* puparia (%) (1) | *E. larvarum* adults (%) |
|---|---|---|---|---|
| *C. perspectalis* | 86.1 ± 11.1a | 4.2 ± 2.6a | 0 | ---- |
| *G. mellonella* | 100 a | 7.3 ± 1.8a | 15.5 ± 5.4 | 100 |
| F (1,6) | 2.41 | 3.81 | | |
| *P* | 0.17 | 0.98 | | |

**Table 2.** Indices (%) summarizing host-parasitoid interactions per host species. DI: Degree of Infestation; SP: Success rate of Parasitism.

| Host species | DI ± SE | SP [1] |
|---|---|---|
| *C. perspectalis* | 91.7 ± 8.4a | 0 |
| *G. mellonella* | 95.7 ± 2.5 | 100 |
| H (N) | 0.00 (8) | |
| *P* | 1.0000 | |

[1] no statistical analysis was performed.

**Table 3.** The fate of eggs (see figure 3) laid on a *C. perspectalis* larva four days after oviposition, on the basis of the anatomical and histological examinations. The eggs E4 and E8 had detached during the dissection

| Egg | Embryo development | Hatching | Larval stage | Encapsulation | Respiratory funnel |
|---|---|---|---|---|---|
| E1 | + | + | ? [1] | + | |
| E2 | + | + | ? [1] | + | |
| E3 | − | | | | |
| E4 | − | | | | |
| E5 | + | + | L1 | − | + |
| E6 | + | + | L1 | partially | |
| E7 | + | + | L1 | + | |
| E8 | + | + | L1 | − | + |
| E9 | + | − | L1 | | |

[1] not detected during sectioning.

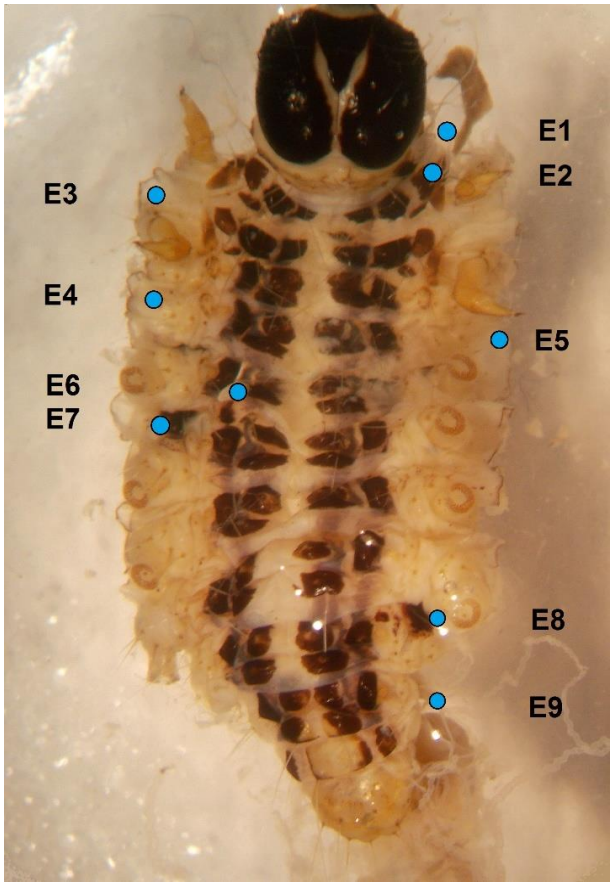


**Figure 3.** The position of the 9 eggs on *C. perspectalis* integument.

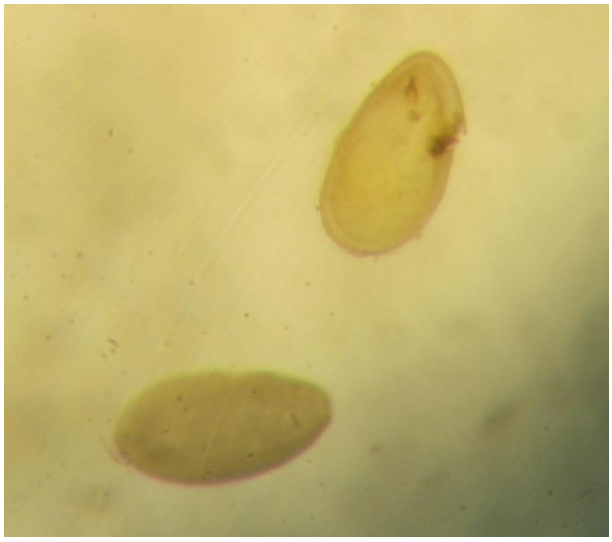

**Figure 4.** Unhatched eggs: **E9** (upper) with a developed L1 (hook visible) and **E4** (lower) with the embryonic development not completed.

*P* = 0.09, Kruskal-Wallis test). The percentage of moths that emerged from larvae not exposed to parasitoids was 77.9 ± 1.8 for *C. perspectalis* and 96.1 ± 2.3 for *G. mellonella* and, for this parameter, the difference was significant (F = 35.18; df = 1,6; *P* = 0.001, one-way ANOVA). Regarding the indices summarizing the host-parasitoid interactions, the DI exceeded 90% and was not significantly different between the host species *C. perspectalis* and *G. mellonella.* The SP was, however, 100% in *G. mellonella* and 0 in *C. perspectalis* (table 2).

## Anatomical study

The specimen of *C. perspectalis* supported nine eggs (figure 3). Their fate is summarized in table 3: two eggs detached during dissection; both were unhatched, one with a developed first instar larva (L1) (**E9**) and the other with embryonic development not completed (**E4**) (figure 4); all the other eggs remained attached to the host's body and all hatched, with the exception of **E3** (figure 5). A brown to black spot (figure 6) indicated the area of parasitoid larva penetration as seen in *G. mellonella* (Valigurova *et al.*, 2014). However, given the natural pigmentation of *C. perspectalis* larva, this was not always so evident.

One first instar larva of *E. larvarum* (from **E5**) was freely moving its head in the host hemocoel (figure 7), but remained with its posterior end in the primary respiratory funnel as observed by Michalková *et al.* (2009). On the contrary the larvae from **E6** and **E7** were almost completely and partially encapsulated, respectively (figure 8). After opening the capsule corresponding to **E6**, it was possible to observe a healthy L1 inside (figures 9 and 10). The larvae from the remaining hatched eggs were maintained *in situ* for the histological examination.

The larva of *G. mellonella* dissected 4 days after oviposition showed the entrance hole of the parasitoid just in proximity to the egg; the host melanisation seemed less strong in *G. mellonella* than in *C. perspectalis* (figure 11). Two L1 (figure 12) were detected with their

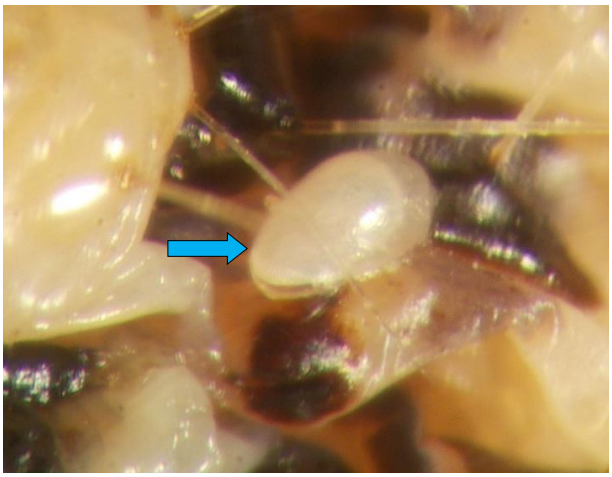

**Figure 5.** Hatched dehiscent egg (arrow) (**E6**).

**Figure 6.** The intense cuticle melanisation indicating the penetration of the parasitoid larva (**E8**).

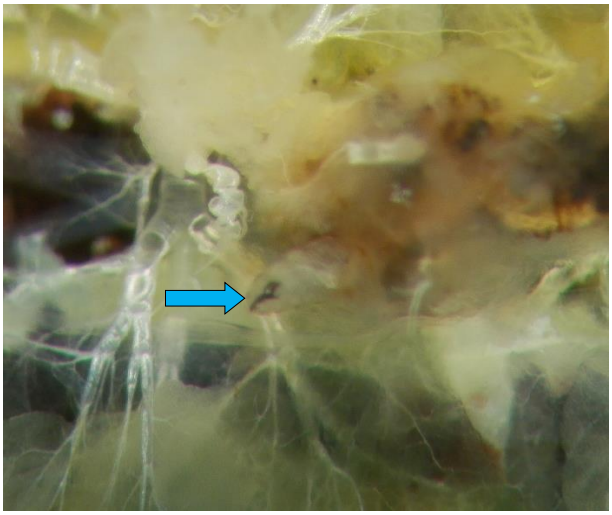

**Figure 7.** The L1 (arrow) of *E. larvarum* from **E5**, within the hemocoel.

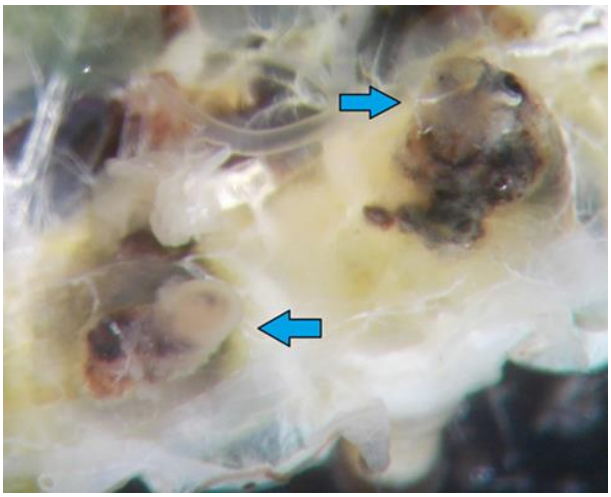

**Figure 8.** The two L1 (arrows) partially (**E7**, left) and almost completely (**E6**, right) encapsulated.

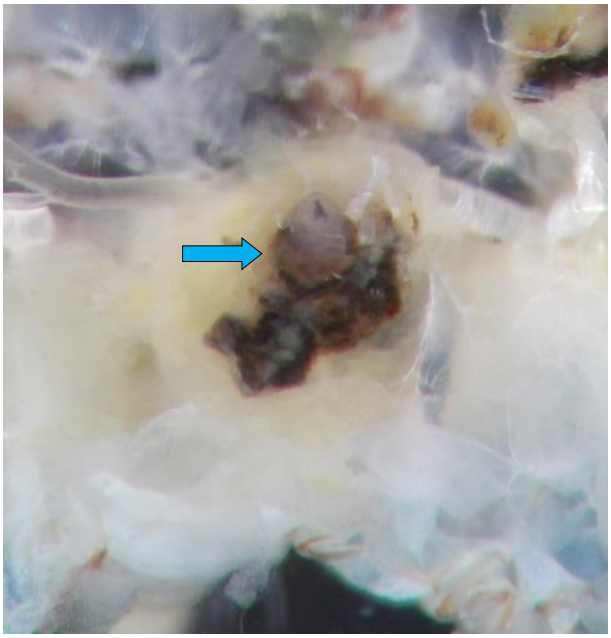

**Figure 9.** The L1 (arrow) from **E6** inside the dissected capsule.

head free in the perivisceral sinus and their abdomen enclosed in the respiratory funnel (figure 13). After staining with Methyl blue (Sigma® M5528) dissolved in saline, a weak sheet of hemocytes was highlighted (figure 14). The third larva was confined in the same way in the pericardial sinus. This confirms the limited activity of the first instar larva as reported by Hafez (1953).

## Histological study

The eggs of *E. larvarum* were deposited upon the body surface of the *C. perspectalis* larva and were glued with a mucilaginous material that fastened the eggs to the host's body (Clausen, 1940) (figure 15).

As observed in previous studies on *G. mellonella* (Michalková *et al.*, 2009; Valigurová *et al.*, 2014), the first instar of the parasitoid larva induced a hemocyte defence of the host: figure 16 shows the hatched egg


**Figure 10**. Detail of the L1 from **E6** at microscope after clearing in KOH.

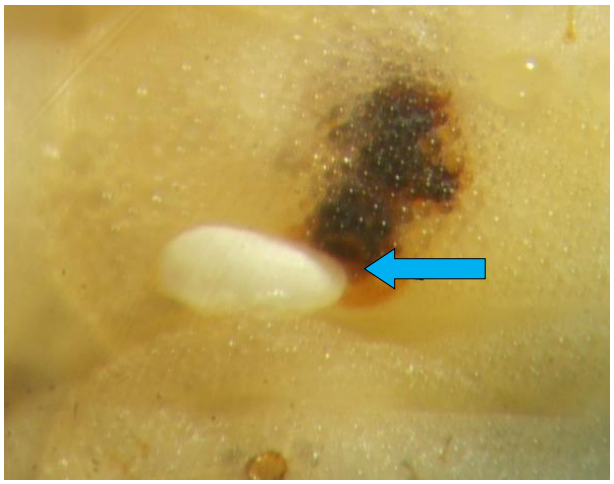

**Figure 11.** The dehiscent egg of *E. larvarum* on *G. mellonella* with cuticle melanisation due to penetration of the larva. Arrow: the entry hole.

(**E6**) upon the host's larval integument and a section of the respiratory funnel (the larva was removed: figure 10). In the same section, on the opposite side, it is possible to see the entry hole of the larva that emerged from **E5** surrounded by melanised host cuticle (figure 17). A deeper section evidences the capsules built around parasitoid larvae from **E6** and **E7** (figure 18). In an even deeper section a transverse section of the respiratory funnel and the abdominal part of the larva that emerged from **E7** are visible (figure 19). Inside the aborted egg

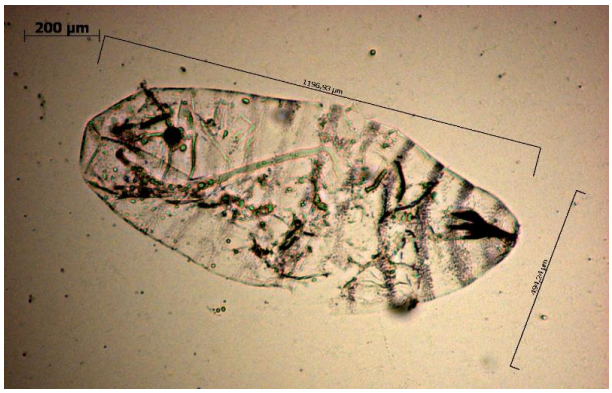


**Figure 12.** A L1 of *E. larvarum* from *G. mellonella* four days after oviposition.

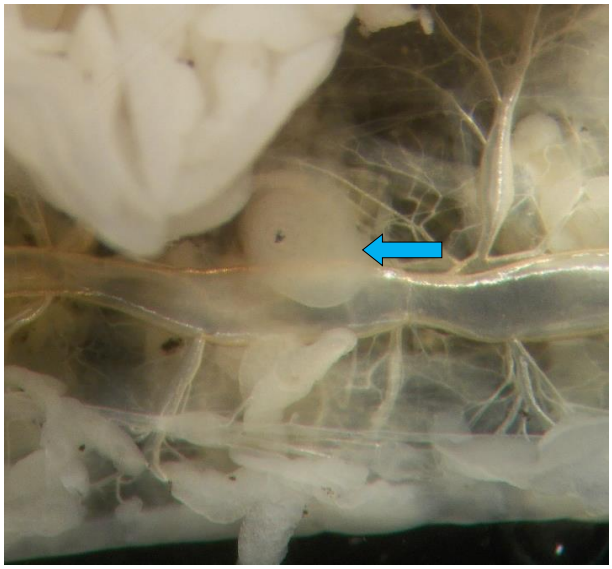

**Figure 13.** Respiratory funnel (arrow) surrounding a L1 in *G. mellonella* four days after oviposition.

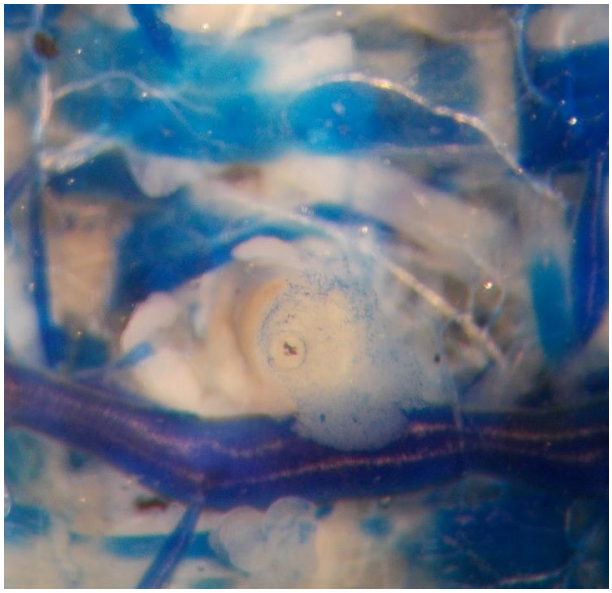

**Figure 14.** The same funnel shown in figure 13 after staining cellular sheet (hemocytes) with Methyl blue saline.

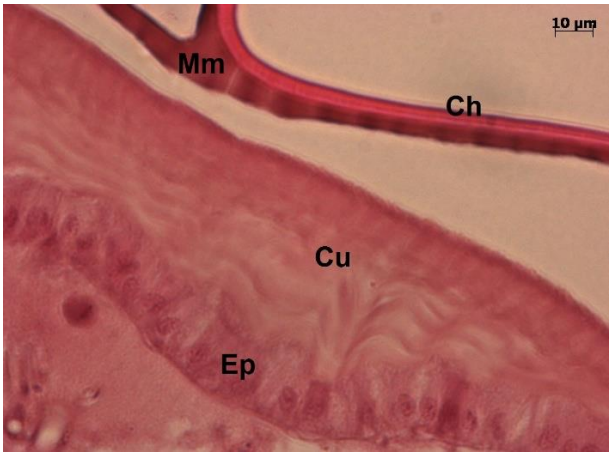


**Figure 15.** Detail of the ventral surface of the egg upon *C. perspectalis* cuticle. **Ep**: epidermis; **Cu**: host cuticle; **Ch** chorion; **Mm**: mucilaginous material.

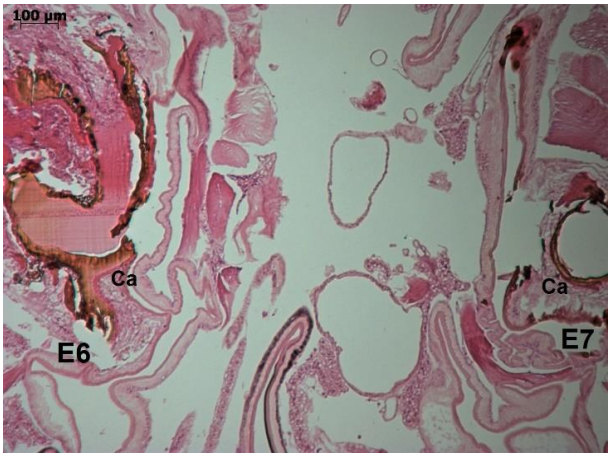


**Figure 18.** Longitudinal and transverse sections of the capsules built around the larvae from **E6** and **E7**, respectively. **Ca**: capsule.

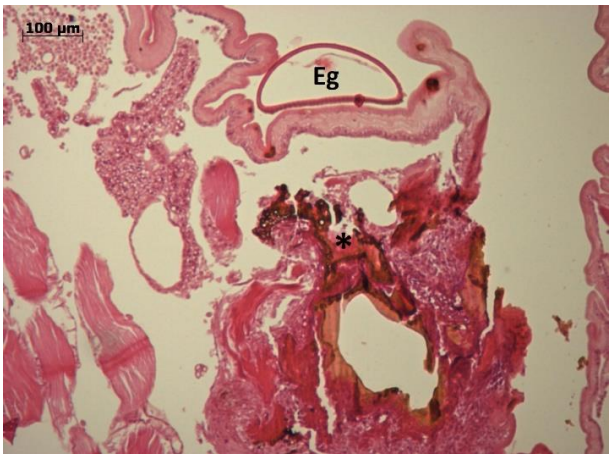


**Figure 16.** Transversal section of the egg (**E6**; **Eg**) and respiratory funnel (**asterisk**) (**E6**).

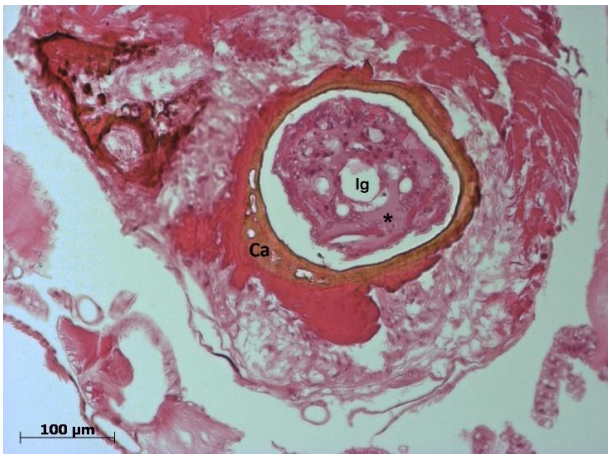


**Figure 19**. Portion of capsule surrounding the abdomen of the larva that emerged from **E7**. **Asterisk**: parasitoid larva; **lg**. larval gut; **Ca**: capsule.

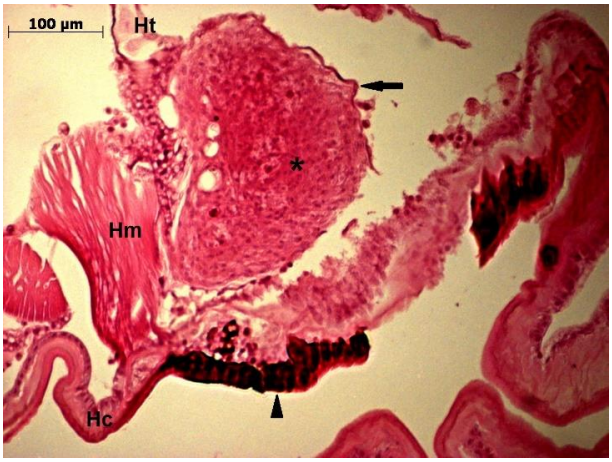


**Figure 17.** The entry hole of the larva from **E5**; **arrowhead**: melanised edges; **asterisk**: parasitoid larva; **arrow**: parasitoid cuticle; **Hc:** host cuticle; **Hm**: host muscles; **Ht** host trachea.

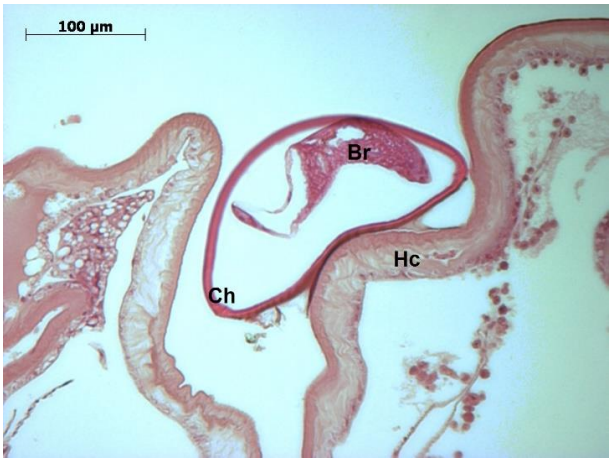


**Figure 20.** The aborted egg **E3** with a remnant of blastoderm inside. **Hc**: host cuticle; **Ch**: chorion; **Br**: blastoderm remnants.

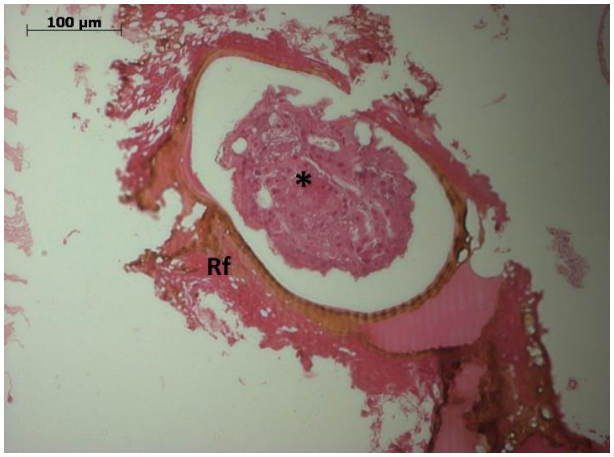


**Figure 21**. Respiratory funnel around the larva that emerged from **E8**. **Asterisk:** larva. **Rf**: respiratory funnel.

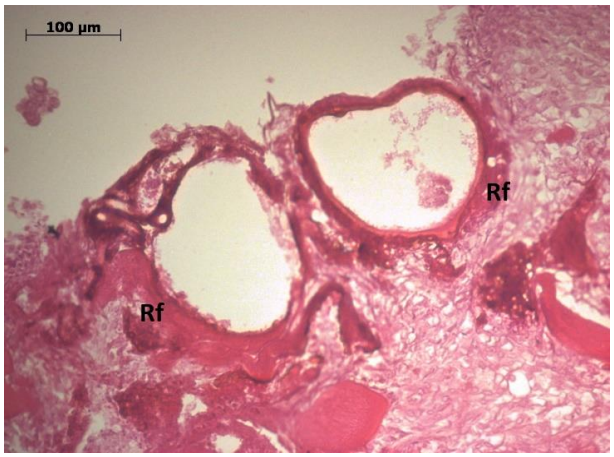


**Figure 22.** Two adjacent respiratory funnels built by the larvae that emerged from **E1** and **E2**. **Rf**: respiratory funnel.

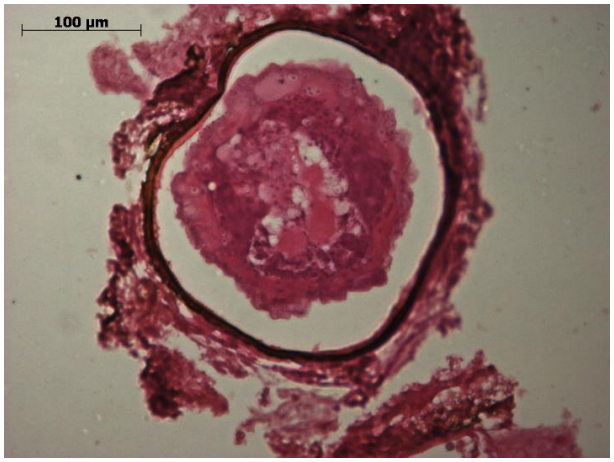


**Figure 23.** The encapsulated larva from **E7**, sectioned transversally at the level of the cephalic ganglion.

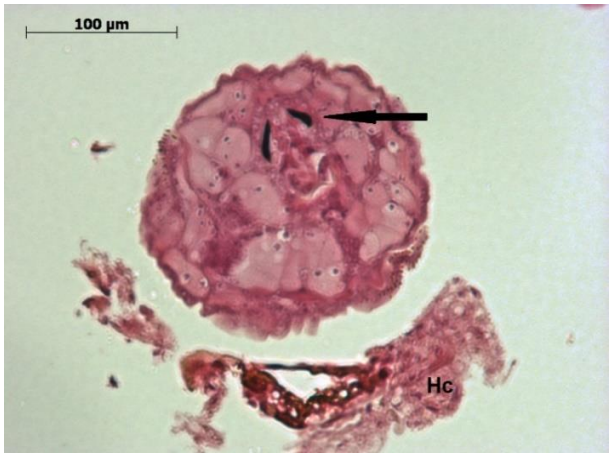


**Figure 24.** Distal portion of the cephalic region of the larva that emerged from **E5**. **Hc**: host's hemocytes; arrow: hook.

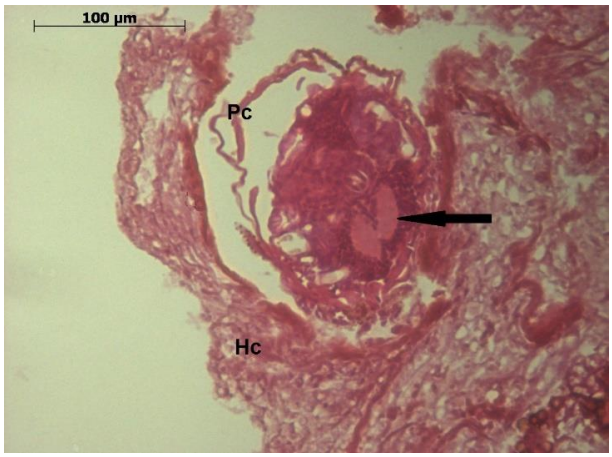


**Figure 25.** The larva from **E8** sectioned transversally at the level of cephalic ganglion (**arrow**). **Hc**: host's hemocytes; **Pc**: parasitoid cuticle.

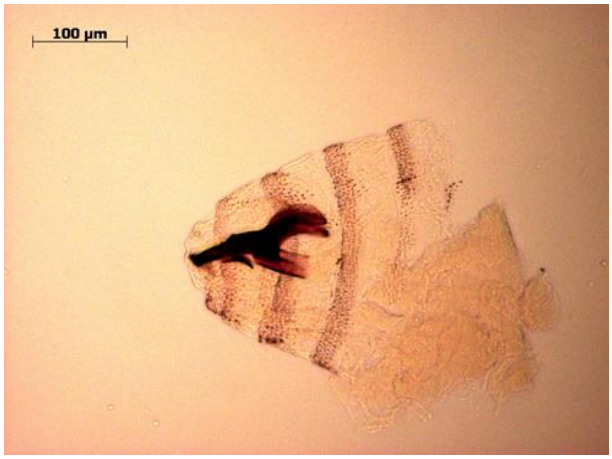


**Figure 26.** The cephalic region of L1 from **E8** showing its hook.

(**E3**), glued onto the host's integument, it is possible to observe the remnants of blastoderm (figure 20). In the same section the respiratory funnel around the larva that emerged from **E8** can be observed (figure 21). A subsequent section shows the two respiratory funnels corresponding to larvae that emerged from **E1** and **E2** (figure 22). The encapsulated larva that emerged from **E7** is visible in figure 23 at the level of the cephalic ganglion. The larva that emerged from **E5** was sectioned transversally at the level of its hook (figure 24). The larva that emerged from **E8** was serially sectioned up to its cephalic region (figure 25). The section shows a weak layer of hemocytes around the larva and no melanisation. After that, the paraffin block was dissolved in xylene, the cephalic region was cleared and mounted on a slide in order to ascertain the larval instar (L1) by observing the hook under a microscope (figure 26).

## Discussion

In the laboratory, box tree moth larvae were accepted by *E. larvarum* females. A lower number of eggs were laid on *C. perspectalis* than on *G. mellonella*, but the difference between the two moth species was not significant, although a quite long (3 hours) exposure time was necessary for oviposition. We can speculate that, although not the preferred host, *C. perspectalis* may be accepted by *E. larvarum* also in nature. In our study most eggs hatched, as also shown in the anatomical and histological studies. Two out of the six first instar larvae penetrated the host body and were encapsulated. The encapsulation response turned into the formation of the respiratory funnel by two *E. larvarum* larvae, similarly to what happens in *G. mellonella* (Valigurová *et al.*, 2014) and in other suitable hosts of tachinid species (Mellini, 1991). Other tachinid species, however, escape the immune response of their hosts and successfully develop until pupation by using different mechanisms (Ichiki and Shima, 2003; Yamashita *et al.*, 2019).

In the acceptance and suitability bioassay, no accepted *C. perspectalis* larvae produced any *E. larvarum* adult (Success rate of Parasitism = 0). Not even the puparium stage was reached by any parasitoid larva in this exotic lepidopterous species, which thus proved to be unsuitable as host for *E. larvarum.* An old association between this tachinid and *C. perspectalis* might however exist in nature. Shi and Hu (2007) reported a mortality of 32.7% of box tree moth larvae following parasitization by *Exorista* spp. in the region of Xinyang (Henan province, China), an area of origin of *C. perspectalis*. We may speculate that the species, or one of the species, found in this area was *E. larvarum,* which is well distributed in China (O'Hara *et al.*, 2009), but further investigation is necessary either to confirm or to exclude this possibility.

*G. mellonella* was confirmed to be well accepted and suitable for *E. larvarum* (Success rate of Parasitism = 100). The puparium yield (based on the number of eggs laid on larvae) was, however, lower than that obtained in previous studies (Dindo *et al.*, 2006; Benelli *et al.*, 2018). This low yield was possibly related, at least in part, to the need to expose the larvae of both moth species to flies for a long time (i.e. 3 hours), due to the slow oviposition rhythm of *E. larvarum* on *C. perspectalis* (a further proof of the relatively low acceptance of the box tree moth as host for this tachinid species in our laboratory study). For *G. mellonella*, this exposure time resulted in an excessive number of eggs per host larva (more than 7 on average, when the optimal number is 3-5) and, as a consequence, in an excessive superparasitism. As shown by Mellini and Campadelli (1997), excessive superparasitism negatively affects parasitoid development, i.e. a high number of *E. larvarum* larvae die before pupation due to insufficient space availability in the host, which often leads to the detachment of the parasitic larvae from the respiratory funnels and, as a consequence, their death for asphyxia. Further research will be aimed at comparing the acceptance (and suitability) of *C. perspectalis* vs. *G. mellonella* under choice conditions, with a shorter exposure time.

About 78% of control (not exposed to flies) *C. perspectalis* larvae developed to adult. This percentage was significantly lower compared to *G. mellonella* control larvae. Under laboratory conditions, therefore, *C. perspectalis* larvae had less survival than *G. mellonella* larvae even without the exposure to *E. larvarum* flies, for unknown reasons. When *C. perspectalis* or *G. mellonella* larvae were exposed to flies and accepted, however, the mortality following the parasitoid larval activity (not necessarily resulting in puparium formation) was not significantly different between the two moth species. As proof, the Degree of Infestation (e.g. the proportion of host larvae that, following exposure to *E. larvarum*, died due to the parasitoid larval activity) was not significantly different between the two host species. This result suggests that *E. larvarum* increased the mortality of *C. perspectalis* larvae, despite the unsuitability of the exotic insect for the parasitoid development. It is commonly recognized that, in invaded countries, the formation of the complex of local natural enemies, able to adapt to an exotic species (e.g. *C. perspectalis*), is a very slow process (Liu and Stiling, 2006; Benelli *et al.* 2015). It is possible that the number of antagonists able to regulate the box tree moth in the introduction areas will increase over time. Nonetheless, it is worth noting that the known natural enemies of this species are relatively few also in the countries of origin, possibly due to the aposematic coloration and the toxic alkaloids sequestered by the larvae from their host plants, which limit their predation by birds and entomophagous insects (Leuthard *et al*., 2013). Future studies may investigate if toxic alkaloids have a direct effect either in deterring oviposition or in disrupting the larval development of *E. larvarum.* The mortality of *C. perspectalis* larvae due to the partial development of *E. larvarum* (and other parasitoids) may, however, be useful to regulate the populations of this invasive pest in a context of conservative biological control, as previously suggested for other lepidopterous species, both exotic (Dindo *et al*., 2013) and native (Depalo *et al*., 2012). Augmentative release of *E. larvarum* against *C. perspectalis* may also be considered, since this tachinid can be easily reared under controlled conditions. Mass rearing capability is one of the key factors that influence the choice

of a parasitoid species for applied biological control (Dindo and Grenier, 2014). Further tests aimed at investigating the possibility to exploit *E. larvarum* for conservative or augmentative biological control should, however, involve field-collected flies, because the low suitability of the box tree moth shown in this study might be the consequence of an adaptation of the parasitoid to the factitious host *G. mellonella*. As shown by van Lenteren *et al.* (2003), the efficiency of a natural enemy may decrease following a long-term use of a non-permissive host or prey for its production.

The potential of *P. nigrolineata* and *A. compunctor* as biocontrol agents also deserves to be investigated, in order to increase knowledge on the overall effect exerted on *C. perspectalis* by the complex of its natural enemies native to Europe. Both parasitoids (as well as *E. larvarum*) are polyphagous, but the fact that they are native and well widespread in Europe (www.faunaeur.org) may minimize their impact on non-target lepidopterous species, even in case of augmentative release. In an IPM strategy, to protect *Buxus* plants in gardens and hystorical parks the sprays of broad spectrum insecticides should be avoided. A suggested alternative is the use of *Bacillus thuringiensis* spp. *kurtstaki* preparations at correct timing, following monitoring by sex pheromone traps (Santi *et al.*, 2015). Finally, due to *E. larvarum* polyphagy, it is unlikely that, in nature, *C. perspectalis* (even if unsuitable for the complete parasitoid development) becomes a "sink" for the eggs of this tachinid, as suggested, for other associations, by Bjegovic (1967). In fact, *E. larvarum* may attack a variety of suitable hosts, better accepted than the invasive moth species.

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**Authors' addresses:** Maria Luisa DINDO (corresponding author: marialuisa.dindo@unibo.it), Antonio MARTINI, Cinzia DI VITANTONIO, Department of Agricultural and Food Sciences, *Alma Mater Studiorum* Università di Bologna, viale G. Fanin 42, 40127 Bologna, Italy.

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