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Characteristics of the meconia of European egg parasitoids of *Halyomorpha halys*

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Abstract

Halyomorpha halys is a severe invasive Asian pest worldwide and classical biological control is foreseen as the most promising control method. Egg parasitoids appear to be the most important natural enemies of this pest, especially the Asian hymenopteran *Trissolcus japonicus*. In the invaded areas, only a few egg parasitoid species have been able to adopt *H. halys* as a host. *Anastatus bifasciatus* is the most common native egg parasitoid of *H. halys* in Europe, but reaches only low levels of parasitization, while several other native species are only occasionally found. Recently, adventive populations have been found both in the USA and in Europe of *T. japonicus*, and in Italy of a second Asian species, *Trissolcus mitsukurii*. Species identification based on morphological traits by specialists or by molecular analysis is a crucial step in the management of biological control programs. The ability to identify the genus or species within a narrow guild of egg parasitoids based on adult emergence holes and meconium features can be a simple and useful method to support management efforts. We present here detailed descriptions of the meconium of the most frequent parasitoid species attacking *H. halys* in Europe and the characteristics of their emergence holes of the adult wasps.

Keywords

Acroclisoides sinicus, biological control, hyperparasitoid, scanning electron microscopy (SEM), *Trissolcus japonicus*, *Trissolcus mitsukurii*

Introduction

Halyomorpha halys (Stål) (Hemiptera, Pentatomidae) is an invasive stink bug native to Asia which causes significant damage in agriculture in different countries worldwide (e.g. USA, Italy, Switzerland, Georgia) (Leskey and Nielsen 2018). Biological control with egg parasitoids is viewed as the most promising control method for long-term management, and several studies have focused on these natural enemies in the native range of the pest and in the newly invaded countries of USA and Europe (Haye et al. 2015; Herlihy et al. 2016; Roversi et al. 2016; Zhang et al. 2017). Hymenopteran species of *Anastatus* Motchulsky, *Trissolcus* Ashmead, *Telenomus* Haliday and *Ooencyrtus* Ashmead have been reported to attack eggs of *H. halys* in the native and invaded areas, although with varying degrees of successful development (Zhang et al. 2017; Abram et al. 2017). Unfortunately, in the newly invaded areas, the impact of the native egg parasitoids has not been sufficient to control *H. halys* (Haye et al. 2015; Abram et al. 2017; Dieckhoff et al. 2017; Costi et al. 2019). In Europe, the most common native egg parasitoid is *Anastatus bifasciatus* (Geoffroy) (Hymenoptera, Eupelmidae). Although parasitization is sometimes locally high, the overall parasitism by this species on *H. halys* egg masses laid naturally in the field is less than 6%–7%, too low to provide significant levels of biological control (Haye et al. 2015; Costi et al. 2019; Stahl et al. 2019a; Moraglio et al. 2020). Other native egg parasitoid species that are reported to attack *H. halys* in Europe include the scelionids *Trissolcus kozlovi* Ryakhovskii, *Trissolcus basalis* (Wollaston), *Trissolcus semistriatus* (Nees von Esenbeck) and *Telenomus turesis* Walker, all of which are reared infrequently from *H. halys* at low parasitism levels ranging from 0.1–0.7 % (Moraglio et al. 2020).

In the native range of *H. halys*, its most important natural enemy is the egg parasitoid *Trissolcus japonicus* (Ashmead) (Hymenoptera, Scelionidae). This species causes high levels of parasitism (up to 80%) and is considered a classical biological control agent for potential release in the invaded ranges (Yang et al., 2009; Zhang et al. 2017).

Adventive populations of *T. japonicus* were recently found in Europe (Switzerland and Italy), and a second Asian egg parasitoid species, *Trissolcus mitsukurii* (Ashmead) (Hymenoptera, Scelionidae), was found also in Italy (Sabbatini Peverieri et al. 2018; Stahl et al. 2019b). The recent discoveries of these species have highlighted the possibility of effective control of *H. halys* in the near future in Europe, and studies aimed at defining the distribution, establishment and impact of the egg parasitoids are of significant interest. Moreover, recent field studies led to the discovery, both in North America and in Europe, of *Acroclisoides sinicus* (Huang & Liao, 1988) (Hymenoptera, Pteromalidae) (Sabbatini Peverieri et al. 2019). This pteromalid is native to Asia and is thought to be a hyperparasitoid due to its frequent association with *Anastatus* spp. (e.g. *An. bifasciatus* in Europe) and *Trissolcus* spp. (e.g. *T. japonicus* in Europe and Asia) within the same parasitized host egg masses (Clarke and Seymour 1992; Grissell and Smith 2006; Sabbatini Peverieri et al. 2019). All known egg parasitoids of *H. halys* are solitary idiobionts, with generally only one adult egg parasitoid emerging from each host egg. Monitoring the field occurrence of egg parasitoids by collecting and rearing host egg masses, followed by the identification or characterization of emerged parasitoids by taxonomists and molecular biologists (e.g. Talamas et al. 2017) are crucial steps for successful biological control programs. When adult egg parasitoids have already emerged from host eggs prior to their collection, identification by molecular analysis of their DNA remaining in the host eggs is still possible (Gariepy et al. 2014; Stahl et al. 2019c). However, a rapid visual method for species or genus identification is also desirable. For example, the characteristic exit holes of the different species of the *H. halys* egg parasitoid guild in Europe (i.e. *T. japonicus*, *T. mitsukurii*, *Ac. sinicus* and *An. bifasciatus*) were recently described (Sabbatini Peverieri et al. 2019).

In this paper we describe the meconia of the most frequently recorded species of egg parasitoids of *H. halys* in Europe. Meconium is the waste material (feces) excreted by the larvae prior to pupating in the host egg (Gullan and Cranston 2010). Within an egg parasitoid guild, different egg parasitoids produce different kinds of meconia. The meconium can often be clearly seen within the emptied host egg through the exit hole or by dissection if necessary. The morphological analysis of meconia has previously been proposed for species identification within the parasitoid guild of different agricultural and forest pests (Schmidd and Kitt 1994; Mirchev et al. 2004; Sands and Liebregts 2005; El-Heneidy and Adly 2009).

Materials and methods

Collection and rearing of the insects

Halyomorpha halys egg masses (n = 75) were collected from the field at sites in Northern Italy during the 2019 growing season. In the laboratory, single egg masses were reared in glass vials held in a climatic chamber at 26 °C, 65% RH and 16:8 L:D conditions. After parasitoid emergence, egg masses were labelled with site data and preserved in a dry condition until the next step of analysis. Emerged egg parasitoids were separated and transferred to glass vials and labelled with their egg mass origin. The parasitoids were fed with honey and used to establish a lab-reared generation of adults (F1). Parental specimens emerged from field-collected egg masses and adults of the F1 generations were identified to species level with the keys of Talamas et al. (2017) for *Trissolcus* species, Kalina (1981) and Askew and Nieves-Aldrey (2014) for *An. bifasciatus*, and the redescription of *Ac. sinicus* in Sabbatini Peverieri et al. (2019).

To ensure that the meconium-species associations were correct and exclude the possibility of multiparasitism, initial analyses were performed with eggs produced in the laboratory with isolated individual F1 generation female wasps. Fresh egg masses of *H. halys* (< 24 h old) were used to produce the laboratory-reared F1 generation of *T. japonicus*, *T. mitsukurii* and *An. bifasciatus*. For rearing *Ac. sinicus*, due to its hyperparasitoid biology, *H. halys* egg masses previously parasitized by *T. japonicus* and *T. mitsukurii* were offered. Subsequent analyses and identifications of meconia to parasitoid species or genus were performed using egg masses collected in the field.

Ooencyrtus telenomicida (Vassiliev) (Hymenoptera, Encyrtidae) and *Gryon pennsylvanicum* (Ashmead) (Hymenoptera, Scelionidae) were also studied for comparison.

Oencyrtus telenomicida attacks various European pentatomid species and is also able to attack and successfully develop in *H. halys* fresh sentinel eggs in Italy (Roversi et al. 2016), while *G. pennsylvanicum* is known to be a natural enemy of *Leptoglossus occidentalis* Heidemann (Hemiptera, Coreidae) (Roversi et al. 2011). *Gryon obesum* has been recorded from *H. halys* in the U.S.A. (Tillman et al. 2020) and other *Gryon* species attack pentatomid hosts in North America and Asia (Felipe-Victoriano et al. 2019; Martel et al. 2019). *Ooencyrtus telenomicida* and *G. pennsylvanicum* originated from the permanent colonies maintained at CREA facilities on egg masses of *H. halys* and *L. occidentalis* as hosts, respectively (Sabbatini Peverieri et al. 2012; Roversi et al. 2016).

Microscope observations

Examination of the egg masses from which parasitoids emerged and meconium structure was conducted using a stereomicroscope (SMZ25) equipped with a digital camera (DS-Ri2) and the image acquisition software NIS-Elements (all from Nikon Corporation, Tokyo, Japan). Image postprocessing utilized Gimp (v. 2.10.04, GNU Image Manipulation Program). The samples of meconium were also analyzed by scanning electron microscopy (SEM) to obtain different details; images were taken with a JEOL NEO-SCOPE JCM-5000 equipped with an imaging system. Samples of meconium were prepared following the technique of Raafat et al. (2014). The mounted samples were coated with a thin layer of gold with a JEOL JFC-1300 Sputter Coater. Images were then taken under high vacuum at 10 kv with an enlargement ranging from 60X to 130X.

Results and discussion

A total of 12 egg masses of *H. halys* were collected in the field (298 eggs) which were partially or completely parasitized by at least one of the species of the egg parasitoid guild. Of these, 7.72% emerged in the field prior to collection, 8.05% of the eggs hatched to produce first instar *H. halys* nymphs, and 23.18% eggs died without any emergence. Overall, the parasitized egg masses produced 47 specimens of *T. japonicus*, 89 *T. mitsukurii*, 20 *An. bifasciatus* and 22 *Ac. sinicus*.

Adults that emerged from the collected egg masses were used to establish lab-reared colonies. From these reared colonies, a total of 21 parasitized egg masses of *H. halys* ware randomly selected for further analysis (four egg masses parasitized by *T. japonicus*, four by *T. mitsukurii*, five by *Ac. sinicus*/*T. japonicus*, three by *Ac. sinicus*/*T. mitsukurii* and five from *An. bifasciatus*). In total, 351 parasitized eggs were analyzed which included 104 adults of *T. japonicus*, 105 of *T. mitsukurii*, 97 *Ac. sinicus* (68 associated with *T. mitsukurii* and 29 associated with *T. japonicus*) and 45 *An. bifasciatus*.

Observation of parasitized egg masses revealed the presence of at least one type of meconium in each *H. halys* egg from which an egg parasitoid emerged, and in some cases the meconium (and pupae) could be observed through the intact egg chorion

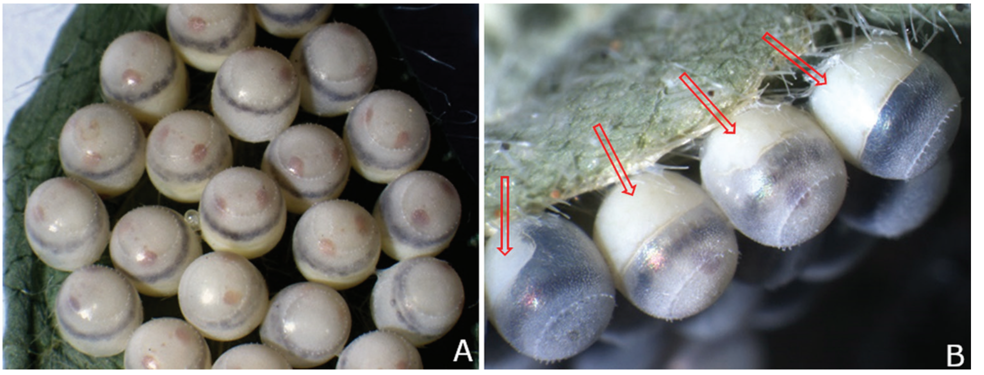

Figure 1. *Halyomorpha halys* eggs parasitized by *Trissolcus mitsukurii*: red eye spots (**A**) and meconium (arrows) (**B**) are clearly visible through the chorion.

Figure 2. Exit holes of egg parasitoids of *Halyomorpha halys* in Europe and hyperparasitoids: *Acroclisoides sinicus* (**A**), *Trissolcus mitsukurii* (**B**), *Anastatus bifasciatus* (**C**), *Acroclisoides sinicus* partly emerged (**D**), *Trissolcus japonicus* (**E**), *Ooencyrtus telenomicida* (**F**, only from sentinel eggs); hatched *Halyomorpha halys* egg (**G**).

prior to emergence of the adult (Fig. 1). According to the descriptions in Sabbatini Peverieri et al. (2019), different features of exit holes of the parasitoid guild of *H. halys* in Europe can be useful for species identification (Fig. 2). Unparasitized *H. halys* eggs that produced nymphs exhibited semitransparent white chorions with partially removed or absent operculae and egg bursters often present; these egg shells were clearly empty (Figs 2G, 3A, B).

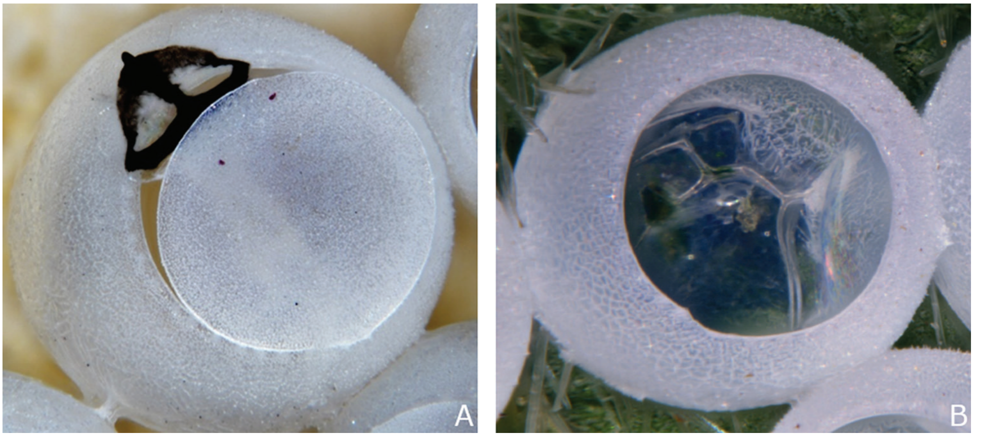

Figure 3. Hatched *Halyomorpha halys* egg with black egg burster visible (**A**) and empty egg shell (**B**).

Meconium description

Anastatus bifasciatus produced meconium with generally elongate brown or dark brown pellets packed in a compact mass where single fecal pellets were still clearly observable (Figs 4A, 5A, 6A, 7A). The meconium was frequently recognizable through the circular exit holes; however, for more detailed observation partial or full dissection of the host egg is required (Fig. 5A).

In contrast, the meconium of *T. japonicus* and *T. mitsukurii* appear very different from the packed fecal pellets of *An. bifasciatus*. The meconium of these *Trissolcus* species appeared as a crescent-shaped mass of a creamy brownish or dark-grey in color (Figs 4B, C, 5B, C, 6B, C, 7B, C) and individual fecal pellets were not clearly recognizable. In a few cases, after dissection of the meconium and with careful lighting, it was possible to observe the shape of individual fecal pellets pressed together to form the whole mass (Fig. 6F). Apparently, mature *Trissolcus* larvae produce feces of a more liquid consistency than those of *An. bifasciatus*, although the meconium tends to harden with time. Slight movements of the mature larvae or pre-pupae inside the host egg is perhaps responsible for pushing the semiliquid meconium to the borders of the egg and molding it to the shape of the egg wall. This aspect of meconium may be common in scelionids, since meconia of *G. pennsylvanicum* display the same features and similarly assumes part of the shape of its host egg (*Leptoglossus occidentalis*) (Fig. 8A–C).

Meconia of *Ac. sinicus* appear as droplet-like fecal pellets (similar to sesame seeds), brown or dark grey in color (Figs 4D, 5D, 6D, E, 7D, E). In contrast with *Trissolcus* species or *An. bifasciatus*, pellets of *Ac. sinicus* do not form a single compact mass but are instead more loosely distributed along a transverse line on the inner of the egg shell, forming a discontinuous layer of feces. The meconium of *Ac. sinicus* is thus clearly distinguishable from that of the primary parasitoids of *H. halys* considered here. In all cases when *Ac. sinicus* emergence holes were investigated, its meconium was associated

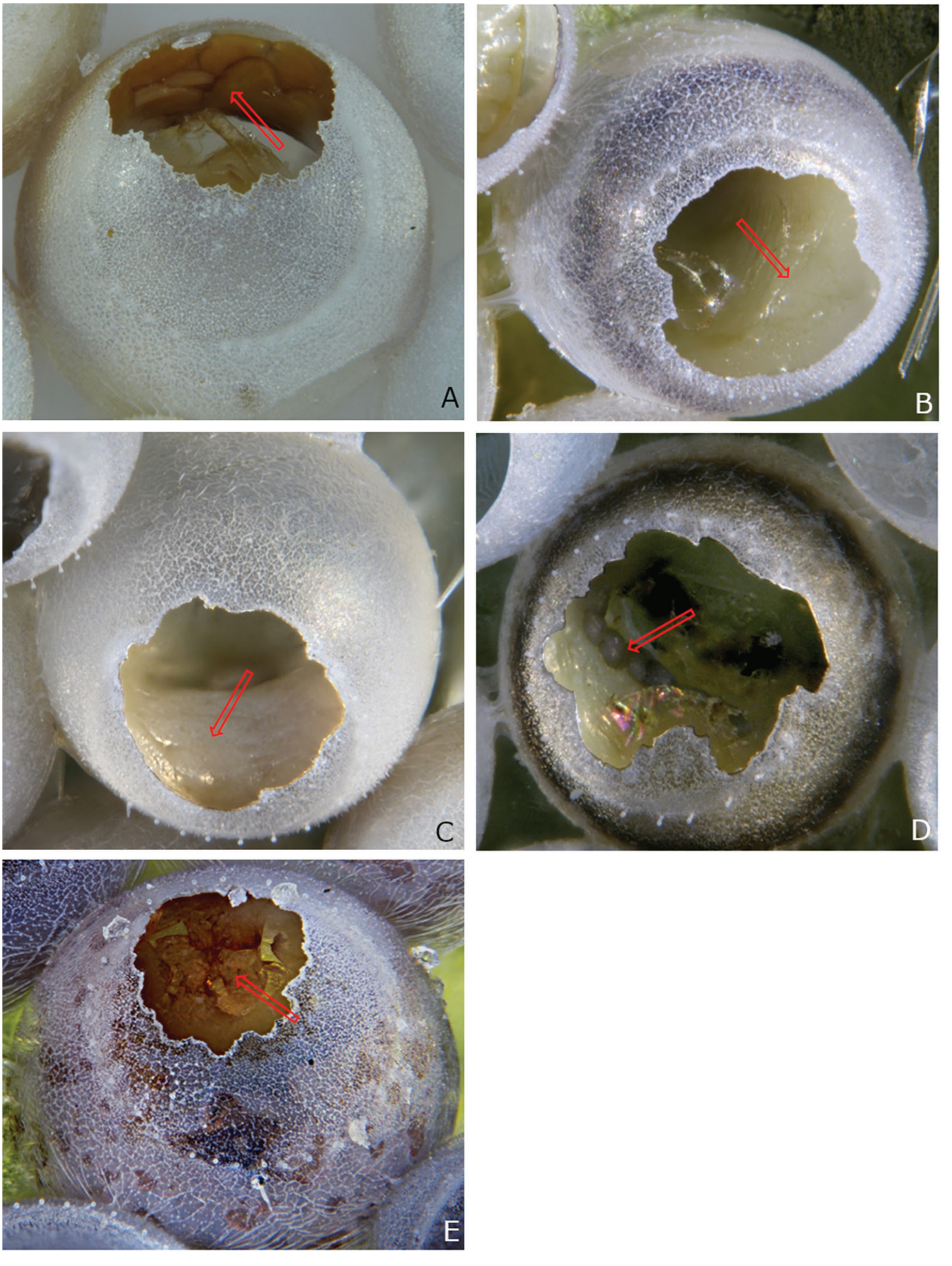

Figure 4. Adult exit holes and meconium (arrows) of egg parasitoids of *Halyomorpha halys*: *Anastatus bifasciatus* (**A**); *Trissolcus mitsukurii* (**B**); *Trissolcus japonicus* (**C**); *Acroclisoides sinicus* on previous parasitized egg by *Trissolcus mitsukurii* (**D**); *Ooencyrtus telenomicida* (**E**).

Figure 5. Meconium (arrows) of egg parasitoids of *Halyomorpha halys* visible through partially dissected host eggs: *Anastatus bifasciatus* (**A**); *Trissolcus mitsukurii* (**B**); *Trissolcus japonicus* (**C**); *Acroclisoides sinicus* on previous parasitized egg by *Trissolcus mitsukurii* (**D**); *Ooencyrtus telenomicida* (**E**).

Figure 6. *Halyomorpha halys* egg parasitoid meconium extracted from the host egg: *Anastatus bifasciatus* (**A**); *Trissolcus japonicus* (**B**); *Trissolcus mitsukurii* (**C**); *Acroclisoides sinicus* (red arrows) on meconium of *Trissolcus japonicus* (**D**); *Acroclisoides sinicus* (red arrows) on meconium of *Trissolcus mitsukurii* (**E**); detail of meconium of *T. japonicus* (view from the bottom (**F**); *Ooencyrtus telenomicida* (**G**). Scale bars: 500µm.

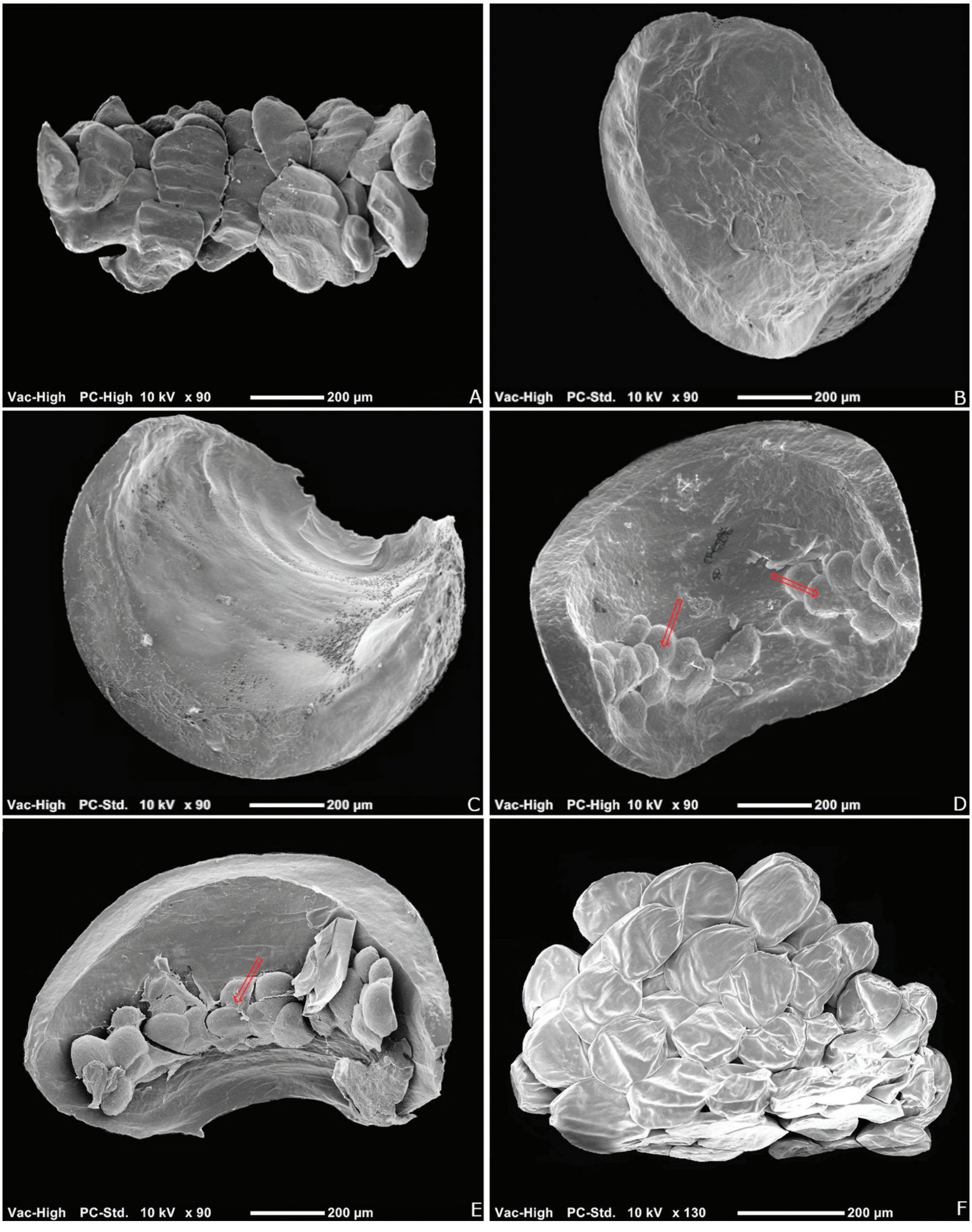

Figure 7. Meconium of *Halyomorpha halys* egg parasitoid at SEM: *Anastatus bifasciatus* (**A**); *Trissolcus japonicus* (**B**); *Trissolcus mitsukurii* (**C**); *Acroclisoides sinicus* (red arrow) on meconium of *Trissolcus japonicus* (**D**); *Acroclisoides sinicus* (red arrow) on meconium of *Trissolcus mitsukurii* (**E**); *Ooencyrtus telenomicida* (**F**).

strictly with the presence of the meconium of *Trissolcus* specimens: the meconium is not randomly voided in the host egg, but is placed onto the meconium of the primary parasitoid (i.e. its "host"). This suggests that hyperparasitism of *Trissolcus* by *Ac. sinicus*

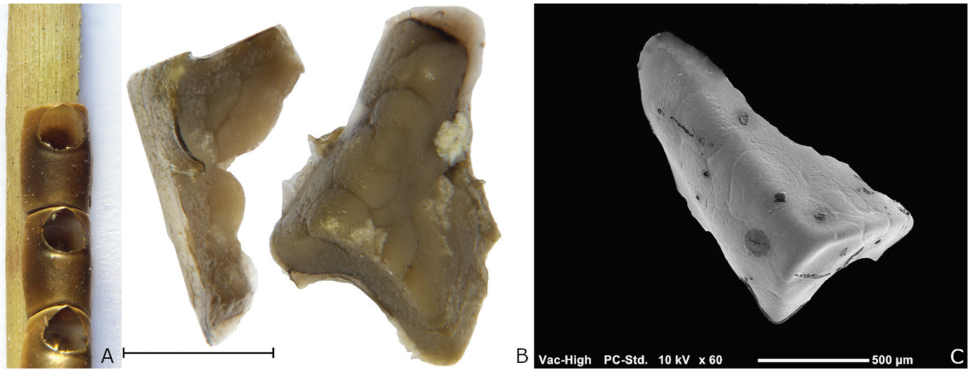

Figure 8. *Leptoglossus occidentalis* eggs parasitized by *Gryon pennsylvanicum* (**A**) and meconium recognizable in the host egg (**B**) and its SEM image (**C**). Scale bar: 500µm (**B**).

occurs during a late stage of development of its host, when the larvae were mature, during the larvae-pupae transition, or at the pupal stage.

The meconium of *O. telenomicida* is different from all the other species of egg parasitoids previously considered, appearing as a mass of feces comprised of amber-brown discs that are randomly distributed inside the egg host (Figs 2, 4E, 5E, 6G, 7F). In contrast with the meconium of the egg parasitoids described above, the individual pellets of *O. telenomicida* can easily be separated by breaking apart the mass with a brush (Fig. 6G).

Our results show that the meconia produced by different species within the egg parasitoid guild of *H. halys* in Europe are family-specific. Because a limited number of genera can develop in *H. halys* eggs, this can be used to help identify the parasitoids responsible for parasitism in the absence of an adult specimen. Surveys have identified three species of egg parasitoids that emerge with the greatest frequency from *H. halys* eggs in Europe (one eupelmid and two scelionids). The comparative rarity of other species means that most identifications will be of *Trissolcus* and *Anastatus*, which can be distinguished from each other based on the meconium. Although the meconia of *T. japonicus* and *T. mitsukurii* are similar in appearance, distinguishing between the two is facilitated by features of the emergence hole (Sabbatini Peverieri et al. 2019). The characteristics of the meconia reported here for *T. japonicus*, *T. mitsukurii*, and *Ac. sinicus* were comparable to those of meconia observed in representative specimens of parasitized *H. halys* eggs used to rear *T. japonicus*, *T. cultratus* and *T. mitsukurii* in USDA-ARS laboratory culture in Newark, DE, USA, in reared material from Asian field collections of *T. japonicus*, *T. mitsukurii*, *T. cultratus*, *Anastatus* spp., and *Ac. sinicus*, and in several *Anastatus* species that attack *H. halys* in the USA. However, a definitive species identification can be provided only by a taxonomist based upon adult specimens or through molecular analysis. Furthermore, the species-level identity of parasitoids that attacked host eggs but did not mature can be obtained only through molecular analysis.

In summary, the exit holes and meconia of *H. halys* egg parasitoids are easily recognizable, and examination of parasitized eggs from which adult wasps have already emerged can be helpful for identifying the parasitoid that emerged from the host eggs.

Although definitive species identifications within genera require molecular analysis of insect remnants, by considering the relatively short list of common egg parasitoids of *H. halys* reported in the invaded areas in Europe, a rapid view of the probable parasitoid complex can be obtained. Moreover, by monitoring egg parasitoids in *H. halys* biological control programs, the cumulative impact of the egg parasitoids on eggs or egg masses of the pest can be defined even if some of the adults have already emerged in the field prior to collection.

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