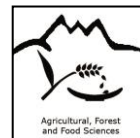




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CICLO: XXXIII

**BIOLOGY AND BIOCOENOSIS OF THREE
EXOTIC PESTS OF THE VINEYARD
AGROECOSYSTEM, WITH PARTICULAR
REFERENCE TO NEW ASSOCIATIONS
INVOLVING PALAEARCTIC PARASITIDS**

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SUMMARY

Some of the most important pests of grapevine in Italy and in Europe belong to the order Hemiptera suborder Auchenorrhyncha and were introduced in our Country in different times. In particular, *Scaphoideus titanus*, a Nearctic species known as the main vector of 16SrV phytoplasmas agents of Flavescence dorée (FD), was reported in Italy in 1963. Similarly, *Metcalfa pruinosa*, which causes indirect damages for the abundant production of honeydew, was introduced from North America in 1979. *Orientus ishidae*, which plays a role as vector of FD phytoplasmas (FDp), was introduced from Asia in 1998.

Scaphoideus titanus is univoltine species and specialist of grapevine (*Vitis* spp). The eggs are laid under the bark of at least a 2-year old wood, where they overwinter. Adults arise from mid-June until mid-October. Recently, new insights on some biological traits have demonstrated that longevity and fecundity were largely underestimated in literature. In fact, males can live over 40 days, whilst females over 60 days and lay an average of about 60–65 eggs. Few data are available on the lifespan and fecundity of *S. titanus*.

The planthopper *M. pruinosa* is a univoltine species as well, overwinter as egg and has a gregarious behavior. It is able to feed on a wide range of plants, and lay an average of 60 eggs. Eggs are laid under the bark from August, hatching takes place from May till July, and adults emerge in the middle of July or early August. In some geographical areas it has been recently reported as responsible for considerable damages to crops, forest and ornamental trees. Moreover, new information emerged on its ability to transmit phytoplasmas belonging to the subgroup 16SrI-B ‘*Candidatus* Phytoplasma asteris’, agents of the Aster yellow disease.

Orientus ishidae has one generation per year and overwinters as egg. Adults live on different host plants, such as hazelnut, hornbeam, willow, and others, often present in the surrounding of the vineyards. It is highly polyphagous and can lay eggs on grapevines even if the nymphs, which can be found from the middle of May up to the middle of July, feed on other plants rather than grapevine, and

adults emerge from mid-May until mid-September. Many aspects of *O. ishidae* biology are still unknown, especially concerning the embryonic and post-embryonic development of nymphs. Moreover, its relations with grapevine are unclear, especially concerning egg-laying and population density of nymphs.

The highest population density of these three species can be detected in the ecological corridors formed by uncultivated areas or woods with wild grapevine, representing a source of re-infestation flows. In such environments, indeed, insecticides are not allowed since they would have serious negative repercussions on the biocoenosis. The lack of effective control strategies against FDp-vector species spread raises the need to consider such environments as an opportunity, rather than a mere issue. In fact, they can represent the ideal habitat for native predator/parasitoids that can limit these three pests.

The general aim of this work is to study biotopes and biocoenosis present in the vineyard agroecosystem, with a particular attention to palaeartic entomophagous (idiobiont and koinobiont parasitoids) adaptation to the three mentioned exotic species. Furthermore, some aspects of the biology of these pests have been investigated.

Therefore, this dissertation has been divided into two parts: the first concerned the study of some new aspects of the biology of *S. titanus* and *O. ishidae* not studied in the literature yet; whilst the second part of this thesis focused on the study of the possible associations between the three exotic leafhoppers and Palearctic parasitoids.

The first work of the first part focuses on *S. titanus*: The principal aim of this research is to assess the lifespan and fecundity of *S. titanus* and investigate the egg maturation dynamics of this species. Adult lifespan and oviposition rate were investigated under semi-natural conditions to assess potential fecundity of this species. Egg load was detected under field conditions in northern Italy. Females lived longer than males, with a *sex ratio* in their favour during the late season when they are still able to lay eggs. Both sexes have a high average lifespan, females can live over 60 days whilst males over 40 days. Furthermore, 25% of the females lived over 70 days, and laid an average of 60–65 eggs. A number of

approximately 70 to 130 eggs was laid by 25% females. The comparison between the egg load trend and the oviposition rate shows that both indices remain constant until September and then decrease in the following months. The presence of the vector was detected until late season in the vineyards. In fact, some females with mature eggs were collected until October 30th, whilst in seminatural condition few individuals were able to lay eggs until the end October-early November. This research shows as *S. titanus* biological parameters have been largely underestimated so far. A serious implication of the high longevity is the prolongation of the adult inoculation period, and a temporal expansion of the risk of infecting the vineyards no longer protected by insecticide treatments. This suggests to reconsider the phytosanitary management of the vineyards after harvesting.

The second research of the first part concerns *O. ishidae*: this research deals with several aspects of the biology of its nymphs: presence of eggs in wood of grapevine; embryonic and post-embryonic dynamics under laboratory conditions; seasonal occurrence of nymphs; presence of nymphs on wild or cultivated grapevine, compared to other elective host plants; and spatial distribution of nymphs on elective host plants, at leaf and shoot levels.

Embryonic and post-embryonic development, spatial distribution, and relationships with grapevine of nymphs were studied under field and laboratory conditions. Egg hatching dynamics and post-embryonic development of nymphs were studied by collecting grapevine wood from managed and unmanaged vineyards and storing it inside rearing cages. Field sampling of nymphs were made on both grapevine and elective host plants of *O. ishidae*, such as hazelnut, hornbeam, willow, and others. More nymphs were obtained from wood collected in unmanaged (3.48-5.63 nymphs/kg wood) rather than managed (0-0.08 nymphs/kg wood) vineyards. Under lab conditions, the embryonic development lasted from 34 to 48 days, whereas the whole post-embryonic development averaged 27 days. Field dynamics of nymphs reflected lab experiments, with early instars having a peak from the middle of May and the beginning of June, and late instars peaking two weeks later. The aggregation patterns decreased from

early to late instars, and from leaves to shoots. Very few nymphs were observed on grapevine leaves.

The second part of this dissertation focused on the study of the possible associations between the three exotic leafhoppers and Palearctic parasitoids.

The first work concerned a survey on a possible Palearctic egg parasitoids adaptation to these three exotic species and the possible interaction with other parasitoids.

Several specimens of egg-parasitoid wasps were obtained from field collected two-year-old grapevine canes. The most prevalent one belonged to *Oligosita collina* group emerged only from *M. pruinosa* eggs with a parasitization rate over 40%. The new association is the first report of such a high level of parasitization on the Flatid planthopper. The parasitization rate mainly relied on the host egg density and the abundance of plants suitable for the oviposition. A second parasitoid generation on the overwintering eggs as well as other hypothesis are discussed. Furthermore, the parasitization rate was higher than the one showed by the Dryinid *Neodryinus typhlocybae*, the control agent introduced in Italy under the biological control strategy, highlighting possible implication in this biocoenosis. We assume that the egg parasitoid adaptation may contribute to *M. pruinosa* control.

The second work focused on the study of an interesting association involving two leafhoppers, *S. titanus* and *O. ishidae*, and the cuckoo wasp *Elampus bidens*.

Cuckoo wasps (Chrysididae) are parasitoids and cleptoparasites using wide range of hosts and oviposition strategies. Although cuckoo wasps are frequently recorded, their reproductive biology is still poorly known. Here we describe for the first time major aspects of the reproductive biology of the cuckoo wasp *E. bidens*. During a research for antagonists of the pest leafhoppers *O. ishidae* and *S. titanus*, both vectors of Flavescence Dorée, we discovered, in up to 25 % of the nymphs of the two leafhopper species in Northern Italy, larvae of a parasitoid wasp. DNA barcoding revealed *E. bidens* as identity of these larvae. Rearing experiments indicated that the endoparasitoid cuckoo wasp larvae were unable to complete their development, a result consistent with the idea that the parasitized

leafhoppers need to be additionally paralyzed and deposited in a nest by a predatory species. Such a “Trojan horse” strategy is known to be applied by species of closely related cuckoo wasp genera. Considering the known host spectrum of Elampine cuckoo wasps and the habitat where we recorded the infected leafhopper nymphs, we hypothesize this predatory species as the apoid wasp *Psen ater*. Search for *E. bidens* larvae in sympatrically occurring leafhopper species provided no evidence in what native host *E. bidens* deposits its eggs. Given that *O. ishidae* and *S. titanus* are major vectors of Flavescence dorée phytoplasmas with few natural antagonists, the high parasitization rate by *E. bidens* in norther Italy are promising and opens up new perspectives for conservative biological control.

This research showed as *S. titanus* biological parameters have been largely underestimated so far, and sheds light on some unknown biological aspects of *O. ishidae* nymphs. Moreover, this work has highlighted some unpublished associations involving the three leafhoppers.

Taking in consideration the consequences deriving from the increase in the use of insecticides in agricultural practices and a concurrent increase in the public demand for sustainable viticulture, a new indigenous natural enemy that live in the uncultivated areas adjacent to the vineyards opens up new prospects of control in an integrated pest management perspective.

We should address further studies upon the native parasitoid community to investigate how a rational habitat management within the vineyard agroecosystem can enhance the presence of the leafhopper predators and limit the negative effect of exotic pests, evaluating the effectiveness of these areas in containing leafhopper re-infestation flows.

1. INTRODUCTION

1.1. EXOTIC SPECIES

Exotic species are organisms introduced, intentionally or accidentally, into a natural environment outside their original natural geographic distribution. This event might have critical effects for the new ecosystem and seriously threaten the biodiversity (Richardson *et al.* 2000; Hulme 2009), since the natural barriers, that have limited the development of flora and fauna in the areas of origin, are overcome. The recent Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES) global assessment estimates that the exotic species number per country has increased by 70% since 1970 across the 21 countries, and has continuously risen, for all groups of organisms on all continents, over the last 200 years. These trends are widely attributed to the increasing international trade, especially in those Regions with high per capita gross domestic product, high human population densities, and large surface areas (Pyšek 2020).

Arthropods are one of the most successful groups of invasive species in the world, and the number of new species introduced every year in Europe is increasing rapidly (Seebens *et al.* 2018; Hulme 2009). Between 1950 and 1974, the exotic invader's introduction rate was 10.2 per year, whilst since 2000 till 2007, it almost doubled to 19.2 species per year (Roques 2010). In 2009, exotic insects registered in Europe were about 1,300 species (Roques *et al.* 2009; Kettunen *et al.* 2009), but only 10 years later, there were more than 3,000 non-native species of terrestrial invertebrates in Europe, and about 2,500 of these were insects (European Commission 2019). Global warming is indicated by recent reviews as an important facilitation of bioinvasions at all stages of the process, including species introduction, colonization, settlement and spread. In fact, climate change can ensure the survival of tropical species arriving in temperate regions improving their chances of permanent settlement (Walther *et al.* 2009), increasing the likelihood of epidemics in the population.

According to DAISIE (Delivering Alien Invasive Species in Europe), Italy, due to the favourable climatic conditions, is one of the most affected European countries by biological invasions. So far, over 1,500 exotic species, a third of which are insects capable of adapting both to thermo-hygrometric variations and to new food and reproductive substrates, are reported. The biotypes may differ, with a greater biotic potential than the original populations, and show, in new environments without effective natural enemies, demographic outbreaks. Italy is also characterized by a relevance international trade and tourism, because of its particular position in the Mediterranean area, so a potential high number of accidentally introduced exotic invaders may find suitable environmental conditions, even the subtropical species. Focusing on phytophagous arthropods, 10 mites and 152 insect species were introduced between 1945 and 2004. The great majority of exotic insects belong to the order Hemiptera (64%), followed by Coleoptera (12%), Lepidoptera (7%), Diptera (6%), Thysanoptera (3%), and Hymenoptera (2%). Mites represent 6% of the entire alien phytophagous species (Pellizzari and Vacante 2005). The 86% of alien insects and mites have the following area of origin: America (37%), Asia (29%), Africa (14%) and Australia (6%). The last 14% is composed of tropical and subtropical species, or insects with uncertain native areas (Pellizzari and Vacante 2005).

1.1.1. Phytosanitary legislation

Several European laws are aimed to prevent, eradicate or mitigate the impact of exotic species that threaten ecosystems, habitats or species through phytosanitary measures. The first regulation of quarantine organisms was brought out in 1951 during an international convention: The International Plant Protection Convention (IPPC). One hundred and sixty six states currently adhere to this treaty, housed at FAO headquarters and periodically updated. The main objective of the agreement is: 'to ensure a common and effective preventive action against the introduction and spread of parasites of plants and plant products and to promote appropriate measures for their control'. The IPPC requires each state to organise National Plant Protection Organizations (NPPOs), which actually are operational tools of the IPPC. These NPPOs are therefore established by each state adhering to the convention and perform various tasks related to phytosanitary surveillance. The Italian NPPO is ultimately the National Phytosanitary Service. The convention also includes the establishment of regional (macro-regional) organizations for plant protection. These Regional Plant Protection Organizations (RPPOs) have a supranational role and perform a coordination and direction function at a macro-regional level.

The European and Mediterranean Plant Protection Organization (EPPO) is the reference RPPO for Italy. Founded in 1951 by 15 states, today it consists of 49 states and the number is still increasing. The adhering states comprises most of the European states, several states bordering the Mediterranean and some Eurasian states, including Russia.

In summary, the main objectives of the RPPOs, including the EPPO, are:

- Plant protection in the area of competence;
- The development of an international strategy against the introduction and spread of parasites and pathogens that can damage cultivated and spontaneous plants within natural or agricultural ecosystems;

- Harmonization between phytosanitary regulations and between all areas of official phytosanitary action;
- The promotion of the use of safe, modern and effective methods of containment of parasites;
- The creation of a documentation service in the phytosanitary field.

One of the objectives of the EPPO is to help the member countries in preventing the entry or spread of harmful parasites (plant quarantine).

The Organization is therefore responsible for identifying parasites that may present a risk, making lists (EPPO A1 and A2 lists) and recommend to member countries phytosanitary measures that should be taken against them. Lists A1 and A2 include the species that the EPPO suggests to member states to include in national phytosanitary legislation as quarantine organisms. These suggestions are based on 'Pest Risk Analysis' (PRA) conducted by groups of experts and on appropriate scientific documentation. For each quarantine organism included in A1 or A2, EPPO recommends to member states the "Pest-Specific Phytosanitary Requirements" (PSPRs), relating to the specific requisites that should be demanded from third countries exporting goods that can carry the very same organism. List A1 groups quarantine organisms not present in the EPPO area of competence yet. List A2 includes quarantine organisms already present in the area but not widely distributed and considered under official control. Until they are transposed into the phytosanitary legislation of the member countries, the organisms of lists A1 and A2 are not quarantined yet, but constitute a kind of waiting list, the so-called Action List. The purpose of the action list is to draw attention of member states to organisms that have only recently been included in the A1 / A2 lists, or of particular importance.

A further list of the EPPO, the Alert List, includes organisms selected by the EPPO Secretariat, mainly from the literature, but also at the suggestion of the NPPOs. All the Alert List organisms are selected on the basis of the occurrence of phytosanitary risks for the EPPO region. The reason in order to consider the

inclusion of these organisms can be of a various kind; parasites that are new to science, recent epidemic facts, news related to spreads, etc. The Alert List is critically reviewed every year by the Panel for Phytosanitary Measures. The organisms included in the EPPO Alert List are not quarantine. The Alert List does not constitute a recommendation for phytosanitary action. The “possible risk” does not arise from implementing a comprehensive PRA. It is a preliminary attempt by the EPPO Secretariat to identify the main elements of risk. Some of the organisms on the Alert List can later be selected as relevant by the Panel and subjected to a complete PRA. Consequently, they can either be included in the A1 or A2 lists of the organisms recommended for phytosanitary regulation (Action List) or, if the PRA indicates that the risk is low, be removed from the Alert List itself.

The reference legislative document for the phytosanitary sector of the European Union is Directive 2000/29/EC. This document, which constitutes the fundamental reference text for the phytosanitary sector of the Union reports in Annexes I and II, the lists of organisms, proposed by the EPPO which the European Union has ratified as quarantine for plants and plant products.

The Italian State has implemented Directive 2002/89 / EC (which integrated Directive 2000/29 / EC) with Legislative Decree no. 214/2005 of 19 August 2005. The legislation is very complex and contains articles that provide for prohibitions and restrictions (prohibition of introduction and dissemination) for the organisms included in some annexes to the D.L.

1.1.2. Management and control of exotic species

The phytosanitary measures needed after an accidental introduction of an exotic species are manifold and include many steps (monitoring and management programs).

Chemical control is often required, but not appropriate when operating in the urban context, or because of the detrimental effects, including the potential selection of resistant strains. Moreover, it may negatively influence previous successful Integrated Pest Management (IPM) procedures adopted for other pests and it is inconsistent with the increasing demand for sustainable farming practices by the consumers (Arnò and Gabarra 2011; Biondi *et al.* 2012). Indeed, chemical approach is characterized by multiple side effects which can disturb the role of natural enemies (Desneux *et al.* 2007) and may lead to pest resistance (Shad *et al.* 2012).

Classical biological control, intended as the introduction of exotic natural enemies to control a pest, is considered one of the most efficient method, aiming at permanent control of exotic pests. However, a high initial investment and a long time are required to develop intensive laboratory and field experiments to study the interaction before the final introduction.

Several successful examples of biological control have been conducted over the past years; one of them is about the Nearctic pest *Metcalfa pruinosa* Say (Hemiptera: Flatidae). Its population decreased considerably after the introduction from North America of a specific parasitoid, *Neodryinus typhlocybae* Ashmahead (Hymenoptera: Dryinidae). In fact, the parasitoid was introduced in all countries and regions where the pest was present (Pellizzari and Vacante 2005). This pest will be examined in detail in a separate chapter. Another example concerns *Torymus sinensis* Kamijo (Hymenoptera: Torymidae); this parasitoid is the specific natural enemy of the Chinese pest *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae), commonly known as Chestnut Gall Wasp (CGW), which is considered the world's worst pest of chestnut both

in forests and cultivations (Quacchia *et al.* 2008). CGW is monophagous on many plant species in the genus *Castanea*, and its larvae induce the formation of galls that can be very damaging to the trees. This species was first reported in Italy in 2002, and nowadays it is widespread in Europe. Currently, *T. sinensis* is dispersing successfully alongside the Japanese *D. kuriphilus* populations, showing an effective biological control. In fact, in Northwest Italy, the infestation rate was strongly reduced after about 9 years from the first release of the parasitoid, and no evidence of resurgence in infestation levels are known (Ferracini *et al.* 2018).

The release of exotic natural enemy species, typically used in classical biological control, may cause potential negative effects on non-target species and the ecosystems. At present, many countries are adopting an environmental risk assessment in order to evaluate the exotic species before releasing them (van Lenteren *et al.* 2003; Cock *et al.* 2010; van Lenteren 2012); in particular, an indigenous non-target species test is applied as part of pre-release evaluation programmes for arthropod natural enemies. These procedures are so strict that import and release of exotic natural enemies is extremely difficult. Consequently, when a new exotic pest establishes in a new environment the first approach is nowadays to first look for indigenous natural enemies (van Lenteren 2012). Indeed, a key role in an exotic pest establishment is played by the complex of natural enemies capable of adapting to the new pest and by the parasitization level exerted by indigenous potential parasitoids. A low presence of natural enemies together with a little effectiveness in the new invaded areas can facilitate the success of the invasion in terms of distribution and abundance (Keane and Crawley 2002). Likewise, the conservation biological control could play a key role against exotic pests, involving new indigenous associations, and endorsing a sustainable approach to pest management and the sustainability of crop production as part of an Integrated Pest Management strategy (Begg *et al.* 2017). In this regard, an example of adaptation of indigenous parasitoids and predators to an exotic pest is represented by *Tuta absoluta* (Meyrik) (Lepidoptera:

Gelechiidae). This tomato leafminer is an exotic pest of horticultural crops. It is native of South America and was recorded in Italy during 2008 (Tropea Garzia *et al.* 2012). The damage is due to the trophic activity of their larvae on leaves, stems and fruits (Biondi *et al.* 2018). Many indigenous predators/parasitoids have been able to adapt to *T. absoluta* and some of them showed the potential to be included in an integrated pest management strategy (Biondi *et al.* 2013a; Zappala *et al.* 2013; Ferracini *et al.* 2019).

It is known that potential enemies of invasive species in the newly invaded areas need time to adapt to the new hosts or prey (Cornell and Hawkins 1993). Therefore, it is wise to evaluate the population of indigenous natural enemies adapted to an exotic species even after many years from their introduction.

1.2. EXOTIC SPECIES OF GRAPEVINE

The grape and wine industry is certainly, among all agricultural products, the one that has reached the highest level of production in the last 30 years, becoming a multi-billion dollar global enterprise (Brostrom and Brostrom 2009). Viticulture in Italy represents an agricultural sector of primary importance covering about 705 thousand hectares and producing 55 million hl of wine, placing our country among the top nations for wine production and export. The rapid globalization of grape commodities together with a greater production of fresh grapes to satisfy the industry demand, in addition to the increased demand for rootstocks and cultivars, promote the spread of pests and pathogens in new areas despite the phytosanitary inspections. Machinery relocation might affect pest spread particularly when harvesting machines are moved between hemispheres for two harvests per year.

One of the first cases of exotic species affecting grapevine introduced in Europe, that caused social upheaval, is phylloxera. Grape phylloxera, *Daktulosphaira vitifoliae* (Fitch) (Hemiptera: Phylloxeridae), is a small, invasive, sapsucking insect that causes considerable physical and economic effects on commercial grapevine, *Vitis vinifera* L., production. This pest related to aphids is originally native to the Northeastern United States (Wapshere and Helm 1987) and moved to the major viticultural European centres by means of European American rootstocks, originally introduced to manage grapevine powdery mildew (Gale 2002). The discovery of grape phylloxera in France in 1868 and its subsequent spread over the next decade are well documented. The pest devastated the French wine industry, destroying over 1 million ha of ungrafted *V. vinifera* vineyards by the turn of the century and had other socioeconomic impacts on rural communities (Matta and Alma 2010). Over the past 150 years, grape phylloxera had spread to almost every major viticultural region in the world, including North and South America, Asia, Europe, the Middle East, Africa and Australasia (EPPO 2021a). On suitable indigenous hosts (i.e. American *Vitis* spp.) some grape

phylloxera strains feed on the leaves, causing leaf galls with marginal populations found on the root system. Phylloxera along with Downy mildew and Powdery mildew reduced in just few decades the 70-80% of the European wine production. The main consequences were poverty, migration of agricultural populations from the countryside to the cities, and social movements (Matta and Alma 2010).

We can count several examples of alien species that have been threatening viticulture in recent years, such as *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae). This fruit fly is a pest native to Southeast Asia (India, Bangladesh and Southeast China) (EPPO 2021b). Recently, it was accidentally introduced into the American and European continents. During the 1980s, the pest was recorded on the Hawaiian Islands (Kaneshiro 1983), but its introduction in North America was reported only in 2008 in California (Hauser 2011). In Europe, it was first recorded in Spain during 2008 (Calabria *et al.* 2012). In Italy, *D. suzukii* was reported in Trentino Alto-Adige in 2009 (Grassi *et al.* 2009). Currently, the fly is present in almost all Italian regions (Marongiu *et al.* 2013). This pest has a long, sharp, serrated ovipositor that enables it to infest those grape varieties characterized by softer berries, causing direct damage and indirect by transferring pathogens that can affect wine quality (Ioriatti *et al.* 2015). Mazzetto *et al.* (2020) have demonstrated its preference for cultivars with soft-skinned berries whose susceptibility depends on the skin berry integrity.

Halyomorpha halys (Stål) (Hemiptera: Pentatomidae) the brown marmorated stink bug, is another example. It is a pest native to China, Japan, Korea and Taiwan. It was first introduced into Allentown, Pennsylvania (USA), then it rapidly spread in many other areas of the mid-Atlantic region of the United States. In Europe, *H. halys* was recorded in 2004 in Liechtenstein. Following these initial captures, the stink bug was found in other neighbouring European countries (Haye *et al.* 2014). Adults crowd in grape clusters during harvest, threaten the quality of harvested grapes because of their release of pungent alkaloids (Mohekar *et al.* 2017). Smith *et al.* (2014) pointed out a direct correlation between *H. halys* density and grape yield loss. Its relevance is still

increasing in the eastern United States (Smith *et al.* 2014) and Europe (Wermelinger *et al.* 2008), and since it also is an extremely mobile insect, capable of moving from host to host without suffering disruption in their reproductive processes, the populations are estimated to continue to grow and spread to other areas (Haye *et al.* 2015), so its full economic impact in vineyards has yet to be evaluated.

Another case is *Popillia japonica* Newman (Coleoptera: Scarabaeidae), that is listed in Annex AII of Council Directive 2000/29/EC. Native to Japan it established in the USA in the early 20th century. The Japanese beetle (JB) occurs in the EU in the Azores (Portugal), Lombardy and Piedmont (Italy), near Milan in 2014 (Bragard *et al.* 2018). It feeds on over 700 plant species. Adults attack foliage and fruit surface. They can cause serious injury to tree fruits and soft fruit, vegetable crops, ornamental herbaceous plants, shrubs, vines and trees. Larvae are root feeders regarded as serious pests of lawns and turf, vegetables and nursery stock. *Popillia japonica* can reach very high densities on grapevine (Hammons *et al.* 2010) which is one of its most favoured food plants. Adults feed on leaves and on ripening fruits (Fleming 1972; Mercader and Isaacs 2003; Hammons *et al.* 2008). They aggregate on grapevines in response to aromatic volatile compounds released from JB-damaged leaves, preferring to feed most heavily on European and French–American hybrid varieties, but the basis for such apparent preference has not been clarified yet (Hammons *et al.* 2010).

More than insect feeding damage, the transmission of plant pathogens is the primary concern in most wine grape regions. Key among plant pathogens are the agents of Flavescence dorée phytoplasma, a serious disease of the grapevine in temperate European areas, caused by 16Sr-V group phytoplasmas (subgroups C and D) (Alma *et al.* 2015; 2019). Two exotic leafhoppers are demonstrated to be vectors of FDp: *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) and *Orientus ishidae* Matsumura (Hemiptera: Cicadellidae), described in major entails hereafter.

Some of the most important pests of grapevine in Italy and in Europe belong to the order Hemiptera suborder Auchenorrhyncha and were introduced in our Country in different times. In particular, *S. titanus*, a Nearctic species known as the main vector of 16SrV phytoplasmas agents of Flavescence dorée, was reported in Italy in 1963 (Alma *et al.* 2019; Chuche *et al.* 2014). Similarly, *Metcalfa pruinosa*, which causes indirect damages for the abundant production of honeydew, was introduced from North America in 1979 (Alma *et al.* 2005). *Orientus ishidae*, which plays a role as vector of FDp, was introduced from Asia in 1998 (Guglielmino 2005; Lessio *et al.* 2016).

This research deals with the above-mentioned three exotic pests, some aspects of their biology not explored in the literature yet, and the possible adaptation of Palaearctic parasitoids.

1.2.1. *Scaphoideus titanus*

Scaphoideus titanus belongs to the order Hemiptera, suborder Auchenorrhyncha, infraorder Cicadomorpha, family Cicadellidae, subfamily Deltocephalinae. The genus *Scaphoideus* Uhler is one of the most represented in the Cicadellidae family: in fact, it has more than 150 species located mainly in North America, Asia and Africa, while only nine species were described for the Palearctic realm (Webb and Viraktamath 2007). The species identification mainly relies on the morphology of male genitalia, therefore females and nymphs are often not clearly determinable; moreover, intraspecific variability of the male genital system is not uncommon (Alma 2004).

Origin and distribution

Scaphoideus titanus is a species native to the Nearctic realm, where it is widespread between the 50th and 30th parallel, from Canada to California, more precisely, it is present in five regions of southern Canada and in 38 states of the USA (Barnett 1976).

In the past it was thought that the American grapevine leafhopper European chorology mainly concerned the circle of latitude around the 45th parallel north, from the Atlantic to the Balkan region, and the cold winter temperature required for egg hatching limited it to a continental climate (Vidano 1966). Recent studies have shown that low temperatures favour early and synchronous hatching, even if they are not essential (Chuche and Thiéry 2009). In fact, *S. titanus* has spread from northern France to southern Italy, demonstrating great adaptability over time to different climatic conditions (Chuche and Thiéry 2014; EFSA Journal 2014). Some authors hypothesize an expanding area of its distribution which may extend beyond the 46th parallel north following the global warming (Maixner 2005; Reineke and Thiéry 2016).

The first report in Europe was in France in 1958, where it might be introduced along with propagation material (Bonfils and Schvester 1960). The census of the

family Jassidae, now Cicadellidae, carried out in 1927 did not mention this species, and this would lead to suppose its introduction to be after that year, or its population density very low to be detected (Chuche and Thiéry 2014). In fact, the huge imports of American vines rootstocks from the United States into France, for replanting of 800,000 ha of vineyards to control phylloxera spread, and their subsequent agamic propagation in the southwest France can be an explanation of its introduction (Chuche and Thiéry 2014). The hypothesis of a single introduction is supported by the low genetic variability of the European populations compared to the American ones, even if a different genetic type is present in Switzerland, suggesting a second introduction (Papura *et al.* 2012).

After the first introduction, *S. titanus* spread across Europe from the Champagne region in France to southern Italy, and from Portugal to Ukraine (Chuche and Thiéry 2014; Tothova *et al.* 2015; Mirutenko *et al.* 2018). In the European region it is currently reported in France (including Corsica), Austria, Bosnia-Herzegovina, Bulgaria, Croatia, Hungary, Italy, Portugal, Romania, Serbia, Slovenia, Spain, Switzerland (Chuche and Thiéry 2014) and Slovakia (Tothova *et al.* 2015). In Italy it was identified for the first time in Imperia province (Liguria) (Vidano 1964). Currently it is widespread throughout Italy with the exception of the two major islands, Sardinia and Sicily. In particular, *S. titanus* was recorded in the following regions: Abruzzo, Basilicata, Campania, Emilia-Romagna, Friuli-Venezia Giulia, Liguria, Lazio, Lombardy, the Marche, Umbria, Piedmont, Apulia, Tuscany, Trentino-Alto Adige, Veneto, Aosta Valley (Chuche and Thiéry 2014).

Biology

Scaphoideus titanus is mainly ampelophagous, accomplishing its life cycle on *Vitis* spp. including both European (*Vitis vinifera* L.) and American grapevines, but it was recently reported also on other plant species (Chuche and Thiéry 2014). It is a univoltine species (Vidano 1964), and eggs are laid under the bark of at least a 2-year old wood, where they overwinter, and adults occur from mid-June until mid-October (Chuche and Thiéry 2014).

Few data are available on the lifespan and fecundity of *S. titanus*. Some authors carried out surveys on the seasonal egg maturation in field-collected females (Cravedi *et al.* 1993; Bosio and Rossi 2001; Linder and Jermini 2007), specimens reared in laboratory (Bressan *et al.* 2005; Eriksson *et al.* 2011), the influence of different temperatures on embryonic, post-embryonic development, and hatching dynamic (Chuche and Thiéry 2009; 2012; Falzoi *et al.* 2014). According to the literature, females are known to bear up to 24 eggs (counted in the ovaries) and may have the earliest mature eggs in late July (Vidano 1964). Furthermore, different authors reported different values about the mature eggs mean number throughout the oviposition season, from a minimum average of 6 to a maximum of 11.2 (Cravedi *et al.* 1993; Bosio and Rossi 2001; Linder and Jermini 2007). However, it is worth to be noted that this number is higher in laboratory condition, reaching 14.6 (Bressan *et al.* 2005; Eriksson *et al.* 2011).

While in the area of origin it has occasionally been reported on broad-leaf trees (Barnett 1976; Hill and Sinclair 2000), in Europe it feeds only on grapevine and is able to carry out its trophic activity both on the European (*Vitis vinifera* L.) (Alma, 2004) and American vine, in particular *V. rupestris* Scheele and *V. cordifolia* Michx (Maixner *et al.* 1993; Beanland *et al.* 2006).

Egg hatching begins around the third ten days of May and ends about the middle of July, showing a peak in the second half of June. Post-embryonic development occurs through 5 juvenile instars. The newborn leafhoppers colonize mainly the basal leaves of the vine, feeding on the leaf veins (Vidano 1964). Adults appear in early July, with a peak between the end of July and mid-August (Lessio and Alma 2004).

Vector activity

This leafhopper is known to be the main vector of the agents of Flavescence dorée, a serious disease of the grapevine in temperate European areas, caused by 16Sr-V group phytoplasmas (subgroups C and D) (Alma *et al.* 2015; 2019). Due to the grievous economic damage, insecticidal treatments against *S. titanus* and the uprooting of infected plants are mandatory in many European countries (Bianco *et al.* 2019). According to the law, the insecticide application has to be suspended in late summer to avoid residues on grapes. Woodlands with overgrowing rootstocks of American grapevine (AGV) are an important reservoir for this pest, from which they can re-colonize vineyards (Pavan *et al.* 2012; Lessio *et al.* 2014). The AGV, and *S. titanus* nymphs collected on it, may also be infected by FDp, as demonstrated by molecular analyses (Lessio *et al.* 2007; Pavan *et al.* 2012).

Nymphs can generally acquire phytoplasmas by feeding on infected plants and after 4-5 weeks of latency, during which they reach adult stage, they become vectors (Schvester *et al.* 1969). It has been recently demonstrated that *S. titanus* is able to become infective as adult within a short time frame (1-2 weeks), acquiring the FDp from broad beans (*Vicia faba* L.) (Alma *et al.* 2018). Consequently, they can quickly acquire the FDp from infected plants and transmit them to healthy grapevines during their life therefore, adult longevity may play an important role. Moreover, incoming individuals with a high longevity may lay eggs in vineyards also in late season, hence it is very important to assess lifespan and seasonal occurrence of the adults.

Natural enemies

The *S. titanus* low population density in its area of origin, the Great Lakes region of North America (Vidano 1966), raises the hypothesis of a high number of natural limiters able to control it (Nusillard *et al.* 2003). These natural enemies have never been the subject of in-depth studies since the leafhopper in its native area is not a source of particular concern (Arzone and Alma 1994). The main

antagonists found in North America belong to two families of two different orders: Pipunculidae (Diptera) and Dryinidae (Hymenoptera) (Barnett 1976; Nusillard *et al.* 2003). Other parasitoids reported, but not specifically identified, are oophagous mimarids (Nusillard *et al.* 2003).

Dryinidae, natural enemies of many leafhopper species, exert their control not only through parasitism but also through predation, with the exclusion of those belonging to the subfamily Aphelopinae. The predation activity ("host-feeding") is the ingestion or consumption of body fluids that exude from wounds created by an adult female parasitoid, when piercing the body of a host. Adult female parasitoids then feed on the fluids using the host as a food source. Host feeding allows female parasitoids to obtain nutrients that are important for egg maturation and production, which increases their egg load. Predation and parasitism together emphasize the efficiency of the biological control agent. Referring to the subfamilies Anteoninae, Dryininae and Gonatopodinae, the number of parasitized hosts during the female adult life is at least equal to the number of individuals eliminated through predation. However, the number of predated hosts is usually much higher than the parasitized one. Predation activities lead in most cases to the death of the prey, varying according both to the dryinid species and the developmental stage of the host (Guglielmino 2002).

In the two-year period 2000-2002, the INRA of Antibes carried out a research in the area of origin of *S. titanus* aiming to identify any possible antagonists to introduce to France. Five species of dryinids able to parasitize the leafhopper were identified: *Lonchodryinus flavus* Olmi, *Anteon masoni* Olmi, *Gonatopus peculiaris* Brues, *Hexagonatopus perdebilis* (Perkins) and *Hexagonatopus niger* (Fenton). Subsequently, two of these species were collected and farmed: *L. flavus* and *G. peculiaris*. However, the settlement attempt in the new habitat was unsuccessful. After that, studies were carried out on the potential use of an indigenous Gonatopodinae as biological control in southern France: *Gonatopus clavipes* Thunberg. However, even in this case the attempts were unsuccessful as the percentage of parasitized individuals was too low (Nuisillard *et al.* 2003). In

Italy, two species of Palaearctic Gonatopodinae able to parasitize *S. titanus* were detected: *Gonatopus sepsoides* Westwood and *Gonatopus lunatus* Klug. Also in this case the parasitization percentage was modest (<1%) even if, in particular for *G. sepsoides*, a good aptitude for predation was demonstrated in the laboratory with a juvenile mortality rate of 36% (Alma and Arzone, 1994). It is conceivable that these apterous driinids, being parasitoids of other Deltocephalinae species which live on herbaceous plants, occasionally run into the nymphs of *S. titanus* exploiting the possible presence of vine creepers (Alma and Arzone 1994).

In Italy, some oophagous parasitoids adapted to *S. titanus* were reported: *Polynema fumipennis* Walk, *Lymaenon rhacodes* Debauche, *Lymaenon thyride* Debauche, *Lymaenon prope longior* Debauche and *Oligosita collina* Walker. In these cases, the parasitization rate was modest as well (about 0.65%) (Arzone and Alma 1994).

1.2.2. *Metcalfa pruinosa*

Metcalfa pruinosa known as the citrus flatid planthopper (CFP) is a Nearctic species, widely distributed in northeastern America, from Canada (Ontario, Quebec) to the southern USA and Mexico, and the West Indies (Metcalf 1957; Wilson and Lucchi 2001). The genus *Metcalfa* Caldwell and Martorell includes six species and subspecies all known only from the Neotropics (Metcalf, 1957), with the exception of *M. pruinosa*. This species belongs to the family Flatidae Spinola which is one of the largest groups of Fulgoroidea (Hemiptera: Auchenorrhyncha), with 918 species known worldwide (Nault and Rodriguez 1985) and has a prevalent subtropical and tropical geographical distribution (Fletcher 1988). Sixteen genera are known for the Palaearctic region. The two genera with the greatest number of species are *Cyphopterus* and *Phantia* with thirty-two and fourteen units respectively (Nast 1972). In Italy, the Flatidae family includes only two indigenous species: *Cyphopterus difforme* (Spinola) and *Phantia subquadrata* (Herrich-Schaffer).

Origin and distribution

The planthopper was first recorded in Europe 1979 in North-East Italy (Veneto, Treviso and its environs) by Zangheri and Donadini (1980). In Italy it had a rapid diffusion (Duso and Pavan 1987), and the ways of its invasion were studied by Pantaleoni (1989) who concluded that the spreading at long and middle distances was favoured by the road traffic, from some starting points along the highways in much frequented parking places with rich vegetation. *M. pruinosa* went into the surrounding area by self-spreading, across belts of trees and shrubs in the landscape. In 1985, the species was found in South-East France (Provence) where it later became more and more frequent even reaching invasive proportions (Della Giustina 1986). Later on, it spread in Sicily and Sardinia (Italy), Austria, Bulgaria, Croatia, Czech Republic, South-ern France and Corse, Greece, Hungary, Slovenia, Switzerland, and Turkey. This process is expected to continue, as it has been described by Byeon *et al.* (2018). In 2009, CFP was

discovered in Korea in several locations in the southern to central regions of the Korean Peninsula (Lee and Wilson, 2010).

Biology

This planthopper is a monovoltine species and overwinters as egg in the bark of arboreous plants. Oviposition occurs gradually a few hours after mating over 10-15 days. The eggs are placed on the main trunk and various size and aged branches. Each female can lay 60 eggs on average (Santini and Lucchi 1994). In Northern Italy, the eggs hatching generally begins in the second half of May and lasts for over 50 days. The newly hatched nymphs reach the underside of the leaves and the young shoots of the host plants, even away from the wintering sites (Girolami and Conte 1999). *Metcalfa pruinosa* has five post-embryonic instars, all mobile and gregarious. The adults appear at the end of June and can be observed on the vegetation during the whole vegetative period, generally aggregated in colonies. The damage is due to nymph phloem-feeding, which may weaken the host plant and lead to some leaf distortion, and above all to an abundant production of honeydew. This, together with the waxes produced during the juvenile stage, stains plants and any underlying artifacts. The consequent development of epiphytic fungi causes deterioration of the vegetation and aesthetic damages. In the Republic of Korea, it has been recently reported as responsible for considerable damages to crops, forest and ornamental trees (Kim and Kil 2014; Kim *et al.* 2011; Lee and Wilson 2010; Byeon *et al.* 2018).

Metcalfa pruinosa is an extremely polyphagous species feeding on several plants, among which many are cultivated and ornamental. The species is widespread in urban areas especially in parks and gardens and in agricultural areas on the spontaneous vegetation of hedges and uncultivated areas, which often represent a source for the surrounding crops. In the forest environment, CFP appears in the lower layers of the shrub and arboreal vegetation of the hill woods and riparian woods. The number of host plants attacked and damaged by the flatid is very high, accounting for 120 and 330 species in the USA and Europe, respectively (Wilson and Lucchi 2000). Furthermore, in Italy the quality of the grapes has

decreased due to the trophic activity of *M. pruinosa* nymphs as well as soybeans whose quantitative damage amounts to 30-40% (Ciampolini *et al.* 1987).

Vector activity

New information emerged on its ability to transmit phytoplasmas belonging to the subgroup 16SrI-B ‘*Candidatus* Phytoplasma asteris’, agents of the Aster yellow disease, and *Pseudomonas syringae* pv. *actinidiae*, a pathogenic organism responsible for the bacterial canker of kiwifruit tree (Donati *et al.* 2017; Mergenthaler *et al.* 2020). *Metcalfa pruinosa* is considered as non-vector of FDp as it is able to acquire FDp but not to transmit it to healthy plants (Clair *et al.* 2001; Trivellone *et al.* 2019).

Natural enemies

Unlike the other two exotic pests, *M. pruinosa* has been the subject of an important biological control strategy in Italy since 1987, by the introduction of *Neodryinus typhlocybae* (Ashmead) (Hymenoptera: Dryinidae) from North America (Girolami and Camporese 1994), which attacks only the juvenile instars accomplishing two generations per year.

Parasitization occurs through the deposition of an egg upon the insertion of the wing sheaths of the juvenile stages of *M. pruinosa* when these have reached at least the third developmental instar. A larva hatches from the egg and develops by feeding on the host from outside. When the larva has completely emptied the host, it builds a cocoon under its remains, which adheres to the leaf where it overwinters. Part of the larvae that developed in June can accomplish a second (partial) generation in July-August. *Neodryinus typhlocybae* has been introduced in Italy since 1987, as part of research programs of the Entomology Institutes of the Universities of Padua and Udine, with material from the United States (Massachusetts, Ohio, Connecticut) (Girolami and Mazzon 1999).

According to Girolami and Mazzon (2001) and Strauss (2009), females of *N. typhlocybae* parasitize the third, fourth and fifth CFP nymphal instars, and they also prey and feed on the nymphs. *Neodryinus typhlocybae* overwinters as a

mature larva in a silky cocoon, usually fixed on the underside of the leaves. Pupation occurs in late spring, and adults start to emerge in June approximately coinciding with the fourth nymphal instar of the host, as it has been described by Girolami and Mazzon (2001). The parasitoid inserts its egg into the intersegmental membrane below the mesothoracic wingpad of the host. The white-yellowish larva may be observed with its hind part of the body partly protruding from the host body below the wingpad, forming a thylacium. The mature larva penetrates the host body, devours its organs, and finally leaves the host to prepare the whitish, oval, double-walled cocoon under the residues of the dead host (Guglielmino and Bückle 2003). Larvae of the first parasitoid generation may follow two ways: they can enter diapause or pupate in July-August to give a second generation in midsummer.

Several authors reported the extensive release programme and the successful establishment of *N. typhlocybae* in many regions of Italy in the late nineties (Frilli *et al.* 2001). *Neodryinus typhlocybae* was also released in Croatia, France, Greece, the Netherlands, Slovenia, Spain and Switzerland, as reviewed by Girolami and Mazzon (2001) and Strauss (2009;2012). The Dryinid has had a key role in *M. pruinosa* control (Girolami and Mazzon 1999; Malausa 1999; Žežlina *et al.* 2001; Alma *et al.* 2005) and has been able to spread in other countries along with its host, without a direct introduction (Szöllösi-Tóth *et al.* 2017; Véték *et al.* 2019). Some authors are in conflict about the parasitization value achieved in field by the dryinid: some of them observed a parasitization rate over 70% (Girolami and Mazzon 1999; Malausa *et al.* 2006) whilst other ones reported a value of about 30% (Véték *et al.* 2019; Strauss 2009). However, since its introduction, the population of *M. pruinosa* has significantly decreased.

As regard egg parasitoids, scattered notes were reported in literature so far. In North America, only a species within the genus *Telenomus* emerged from its eggs (Conti and Bin 1999), while in Italy only some specimens of *Centrodora livens* (Walker) (Hymenoptera: Aphelinidae) were observed (Raspi and Canovai 2003).

In all cases, the parasitization rate was too low (<1%) for possible biological control.

1.2.3. *Orientus ishidae*

Origin and distribution

The mosaic leafhopper *O. ishidae* is considered an Asian species with a wide range of distribution, reported from Japan, Korea, Taiwan and the Philippines (Metcalf 1967).

It has been first introduced into North America, probably along with the ornamental plant *Aralia* sp. (Felt and Bromley 1941), and Europe. In North America the species was recorded in the USA – New Jersey, New York, Maryland, Pennsylvania, Long Is-land, New Hampshire, District of Columbia, Ohio, Connecticut (Metcalf 1967) – and in Canada (Hamilton 1983). Data on the first record of this species in Europe were provided by Guglielmino (2005), in Switzerland and Northern Italy. It was also found in Piedmont, Veneto, Tuscany, and since its first detection, *O. ishidae* has rapidly spread throughout Europe: Germany, Slovenia, Austria, Czech Republic, France, Hungary, the Netherlands, Great Britain, Poland, Romania and Belgium. Another species, known as *Orientus amurensis* Guglielmino, probably restricted to the Amur region in Russia, and in the Liaoning Province in China, was described, whereas the Nearctic and European populations should be referred to *O. ishidae* (Guglielmino 2005).

The mosaic leafhopper is a polyphagous species. It feeds on many species of trees, bushes and herbs. In Japan it was found on *Malus* spp., in North America on *Carpinus caroliniana*, *Ostrya virginiana*, *Betula* spp., *Crataegus* spp., *Acer* spp., *Quercus palustris*, *Aralia spinosa*, *Tilia cordata*., *Buxus* spp., *Gleditsia triacanthos*, *Prunus virginiana*, *Salix babylonica*, *Salix* spp., *Juglans nigra*, *Malus sylvestris*, *Corylus* spp., and weeds (Felt and Bromley 1941; Rosenberger and Jones 1978; Johnson and Freytag 2001). In Europe it was found on *Salix* spp., *Salix X rubens*, *Salix babylonica*, *Betula pendula*, *Malus domestica*, *Prunus laurocerasus*, *P. domestica*, *Urtica dioica*, *Salix alba*, *Corylus avellana*, *Fagus sylvatica*, *Carpinus betulus*, *Acer campestre*, *Hedera helix*, *Quercus* spp.,

Viburnum tinus, *Vitis vinifera*, *Ulmus minor*, *Chelidonium majus*, *Berberis* spp., *Ostrya carpinifolia*, *Rubus fruticosus*, *Rosa canina*, *Buxus sempervirens*, *Juglans nigra*, *Cornus sanguinea*, *Populus nigra* and *Crataegus oxyacantha* (Guglielmino 2005; Nickel 2010; Lessio *et al.* 2016).

Biology

Orientus ishidae has a single generation per year and overwinters in the egg stage (Nickel 2010; Lessio *et al.* 2016). Nymphs appear at the end of May and are present until the beginning of August, while adults are active from the end of June until the beginning of October (Lessio *et al.* 2016). These latter are usually aggregated at the edges of vineyards, feeding on host plants such as hazelnut, hornbeam, willow, and others (Lessio *et al.* 2016, Alma *et al.* 2019).

Vector activity

The mosaic leafhopper is a recognised vector of Flavescence dorée phytoplasmas. The first evidence occurred in 2010, when some adults tested positive to 16SrV phytoplasmas in Slovenia (Mehle *et al.* 2010), subsequently confirmed in Northern Italy (Gaffuri *et al.* 2011). Meanwhile, the interest for this species increased, and its presence was confirmed throughout Europe (Koczor *et al.* 2013, Chireceanu *et al.* 2017, Klejdysz *et al.* 2017).

Adult specimen ability in transmitting the plant-pathogen was demonstrated by a successful inoculation of 16SrV phytoplasmas to grapevines, after an acquisition as nymph from infected broad beans (*Vicia faba* L.) and a latency period on hazelnut, under laboratory conditions (Lessio *et al.* 2016). However, the detection of FDP-related genotypes in several wild plant taxa (*Alnus glutinosa*, *A. incana*, *Clematis vitalba*, *Ailanthus altissima*, *Salix* spp. and *Corylus avellana*) suggest that this disease is characterized by an open transmission cycle (Filippin *et al.* 2009, Casati *et al.* 2017; Malembic-Maher *et al.* 2020).

Despite *O. ishidae* is commonly widespread in the vineyard agroecosystem, it seems that it may not move frequently inside vineyards. Although, it is a confirmed vector of 16SrV phytoplasmas to grapevine under laboratory

conditions, even with a low efficiency, its role in the field and the sources of inoculum in nature are not known to date.

Natural enemies

Regarding potential parasitoids able to adapt on *O. ishidae*, to date hardly any information is available in the literature.

1.3. VINEYARD AGROECOSYSTEM IN NORTH-WEST ITALY: UNCULTIVATED AREA, ARE THEY A MERE ISSUE?

In Piedmont (Northwest Italy), the agroecosystem consists of land abandonment and intensive farming largely covered by vineyards. In details, vineyard agroecosystem in Piedmont is represented by conventional or organic vineyards or hobbyists interspersed with ecological corridors formed by uncultivated areas or woods with wild grapevine. A trait of NW Italian vineyards is the fragmented habitat, rich of many plant species potential hosts of the three exotic pests. In such environments insecticides are not allowed since they would have serious negative repercussions on the present biocoenosis (Lessio *et al.* 2011).

Metcalfa pruinosa and *O. ishidae* are polyphagous, so able to feed on numerous plant species, meaning that the greatest population densities are found in ecosystems beyond the vineyards, mainly in forests (Nickel 2010; Mehle *et al.* 2011). *Metcalfa pruinosa* is extremely ubiquitous and present with high population density in the wooded areas of the vineyard agro-ecosystem from which can colonize the grapevines (Duso 1984; Vidano 1988).

Orientus ishidae can complete its life cycle on many wild or cultivated trees and shrubs within the vineyard agro-ecosystem. Lessio *et al.* (2016) stated that *O. ishidae* is widespread in the vineyard agroecosystem, adults are usually aggregated at the edge of the vineyards and it is able to move into the vineyards, even if not frequently.

As regard *S. titanus* in North America, it was shown that its populations are more abundant in those woods with a high presence of American vine plants than in cultivated vineyards, and a possible migration of adults towards the vineyards at the end of the season was also supposed (Beanland *et al.* 2006). Also in Italy a possible edge effect was hypothesized, caused by the proximity of uncultivated American vines to cultivated vineyards: in fact, a greater number of catches on yellow sticky traps in the rows adjacent to the woods with American vines were observed, suggesting a possible migration of the adults towards the vineyards

(Pavan *et al.* 2012). In the past, *S. titanus* was considered a species with little migratory aptitude, and its movements generally linked to upward air currents. The weak updraughts usually occur in the evening and morning hours, i.e. the two moments of the day in which flights are concentrated (Alma *et al.* 2019). However, recent studies, based on marking techniques showed that such movements really exist (Lessio *et al.* 2014).

Due to the lower insecticide pressure, the population densities of *S. titanus*, *O. ishidae* and *M. pruinosa* in these habitats are very high, representing the main source of re-infestation flows (Beanland *et al.* 2006; Pavan *et al.* 2012; Lessio *et al.* 2014). Should these habitats be considered only a problem or a potential resource for Palearctic entomophagy of these three exotic pests?

Spontaneous vegetation is a central element of the biodiversity of an agroecosystem, and can perform various ecosystem service (Altieri 1982; Altieri 2004; Burgio *et al.*, 2004; Tschardtke *et al.* 2012). Landscapes characterized by high edge density with high proportions of semi-natural habitat present a greater abundance of arthropods and natural enemies (Martin *et al.* 2019). These natural habitats can offer adequate resources to enhance the presence of natural enemies and improve the pest control as long as they are large enough to allow a substantial increase in within-field enemy abundance (Holland *et al.* 2016). Moreover, the landscape and habitat structures alteration and engineering have been suggested as means to influence both insect pests and their natural enemies' dynamics (Jonsson *et al.* 2010). The absence of effective control strategies against FDP-vector species spread raises the need to consider such environment as an opportunity, rather than a mere issue. In fact, they can represent the ideal habitat for native predator/parasitoids that can limit this pest.

2. AIM OF THE WORK

The general aim of this work is to study biotopes and biocoenosis present in the vineyard agroecosystem, with a particular attention to palaeartic entomophagous (idiobiont and koinobiont parasitoids) adaptations to the three mentioned exotic species. Furthermore, some aspects of the biology of these pests have been investigated.

Specifically, the present research was aimed at:

- Evaluating lifespan and fecundity parameters of *S. titanus* and their possible implications in its integrated control.
- Assessing some aspects of the biology of *O. ishidae* nymphs such as embryonic and post-embryonic dynamics, seasonal occurrence, spatial distribution on different host plants, and their relationships to grapevine.
- Studying the biocoenosis present in the Piedmontese vineyard agroecosystem, paying particular attention to a possible Palaeartic parasitoid adaptation to these three exotic species and the possible interaction with other parasitoids.
- Evaluating the parasitization rate and other crucial aspects for a possible application in biological control.

3. SURVEYS ON BIOLOGY AND ECOLOGY OF TWO EXOTIC LEAFHOPPERS

3.1. NEW INSIGHTS ON *SCAPHOIDEUS TITANUS* BIOLOGY AND THEIR IMPLICATIONS FOR INTEGRATED PEST MANAGEMENT

3.1.1. Introduction

Few data are available on the lifespan and fecundity of *S. titanus*. A crucial factor, which determines the insect population growth and its potential spread, is the fecundity (Graziosi and Rieske 2014). Fecundity is generally considered a function of time, and related to body size, food quality and environmental temperatures (Kindlmann *et al.* 2001). Both egg and time limitation can reduce the chance of reproduction, therefore females tend to avoid the risk of becoming egg- or time-limited to maximize lifetime fecundity (Minkenbergh *et al.* 1992; Rosenheim 1996; Heimpel and Rosenheim 1998; Rosenheim *et al.* 2008). Egg limitation is related to oviposition and egg maturation (Papaj 2000; Richard and Casas 2012), being this latter associated with external factors such as food resources, or internal features such as egg load. Egg load, in case of synovigeny, varies during lifespan since new eggs are continuously produced after oviposition (Dieckhoff and Heimpel 2010), highlighting again the importance of the longevity for these insects. The maximum amount of *S. titanus* eggs laid during the season, and the seasonal dynamics of oviposition, have not been investigated yet.

The principal aims of this research is to assess the lifespan and fecundity of *S. titanus* and investigate the egg maturation dynamics of this species.

3.1.2. Materials and Methods

Lifespan

The survey was conducted in 2016 and 2017. Two-year old grapevine canes were collected from brushwood with wild grapevine in different Piedmontese grapevine growing areas (NW Italy) in the following municipalities: Albiano, Borgiallo, Caluso, Mazzè and Prascorsano in the Torino Province; Mombercelli, Perletto, Portacomaro and Vinchio in the Asti Province; Canale, Dogliani, Farigliano and Montà in the Cuneo Province. The canes were stored during the winter under out-door condition in a rearing cage (insect-proof polyethylene and nylon mesh 100 x 100 x 75cm high) at Grugliasco in Turin Province, with two potted healthy wild grapevine plants (Kober 5BB) to feed the insects. This American hybrid, *Vitis berlandieri* x *Vitis riparia*, was chosen because it is largely widespread in Piedmont and because *V. riparia* was preferred by *S. titanus* compared to the cultivated vines, *Vitis vinifera* (Maixner *et al.* 1993).

Forth to fifth-instar nymphs were randomly collected every ten days, using a mouth aspirator for five seconds per leaf on ten leaves per plant, and then caged together. They were monitored daily in order to collect newly emerged adults. Each newly emerged naive female was isolated together with a single virgin male into a glass tube (15cm long x 2cm diameter) for mating. A wild grapevine leaf was provided for each tube, and changed regularly, to feed the insects. Subsequently, each insect pair was raised on a cut wild grapevine shoot inside a cylindrical Plexiglas cage (20cm high x 12cm diameter) with a fine mesh on the top, in outdoor conditions. The vine shoots were placed into a vial with water to keep them alive and replaced regularly. The total lifespan was recorded monitoring the specimens since they emerged as adult until their death, every day. A total of 41 females and 33 males were used in the year 2016 whilst a total of 55 females and 39 males were used in 2017. Females were arranged in 4 different age-cohorts, using eclosion time as criteria. Being a proterandric species, the

majority of males eclosed at the beginning of the season (Lessio *et al.* 2009), thus a grouping in cohorts corresponding to the females was not feasible.

Fecundity

The same rearing for longevity were used for oviposition study. A portion of 2-year old vine shoot (1.5 to 4cm length), previously sterilized in water at 100°C for 10 minutes, was placed in each cylinder, and replaced every 5-10 days, to let the leafhoppers laying eggs until death. All these twigs were inspected by gently removing the bark by means of a lancet in order to observe and count the laid eggs under a stereomicroscope (LEICA MZ16A). The daily oviposition egg rate was calculated.

Egg load

In 2016 and 2017, insects were field-collected, by sweep netting in two important vine-growing areas in Piedmont (Northwestern Italy), located in Monferrato (Asti Province) and Canavese (Torino Province). Five sites per area were chosen. The collection was carried out every ten days, since the beginning of July until the end of October, on 5 grapevines randomly chosen, for 3 minutes per plant per sampling site. Leafhoppers were subsequently dissected by means of micro-pinneedles in the laboratory.

Scaphoideus titanus females were killed with ethyl acetate in the lab before dissection. The specimens were placed onto a microscope slide (25 × 75 × 1 mm) into a phosphate buffered saline (PBS) solution for ovary dissection. Egg load was assessed by dissecting each female under a stereomicroscope at 25× magnification. The abdomen was separated from the rest of the body using a couple of needles. Ovaries were removed from the abdomen by means of micro-pin needles and spread on a slide for egg load counts as described in Picciau *et al.* (2017; 2019). Mature eggs were defined by the presence of a clear apical pedicel, complete yolk deposition, and no nurse cells (Fig. 1). The number of mature eggs was counted under microscope at 80× magnification.



Figure 1. Ovary dissection of *Scaphoideus titanus*: mature eggs, elongated with a curved tapering apex (white arrow indicating the apex).

Statistical Analysis

Median survival rates and survivorship curves for each sex were analysed using the Kaplan-Meier survival estimator (Kaplan and Meier 1958). Cox proportional-hazard regression (Cox 1972) model was used to assess the effects of season (Year) and species sex (Sex) on the hazard ratio of death at a given time. The proportional hazards assumption was checked using the Chi square test on the Schoenfeld residuals (Schoenfeld 1980) using the `cox.zph` function in survival package (Therneau and Lumley 2015). As survival and hazard functions for years 2016 and 2017 were not proportional, analyses were stratified by factor “year”. The Likelihood Ratio Test and Wald chi-square tests (Crawley 2005; Faraway 2005) were used to select the appropriate minimal model. A different analysis using the log-rank test (Harrington and Fleming 1982) was due to compare the

longevity and oviposition timespan of the four different females age-cohorts for both years.

Data of the oviposition and the egg load rate were analysed by fitting Generalised Linear Mixed Models (GLMMs) with the `glmmTMB` package (Brooks *et al.* 2017). Over- and under-dispersion and zero inflation were checked in the models by using the `testDispersion()` and `testZeroInflation()` functions from the `DHARMA` package (Hartig 2019, Brooks *et al.* 2019). In each analysis, models with different error distributions (e.g., Poisson, negative binomial etc.) and effect interactions were considered and compared to each other using the lowest small-sample-size corrected Akaike information criterion (AICc). The models with AIC <2 from the one with the lowest AIC were considered as valid (Burnham and Anderson 2002) (Tab. S6). For the most parsimonious model chosen by AICc, the residuals were examined using the function `testUniformity()` from the `DHARMA` package (Brooks *et al.* 2019). To verify the significance of the fixed effects, the fitting model was compared to "null" models, (including only the random effects), by checking the differences in deviance between the corresponding models and in AICc values (Burnham and Anderson 2011; Bolker *et al.* 2009; Zuur *et al.* 2009) using `r` package `bbmle` (Bolker and R Core Team 2017). Pairwise comparisons between the different periods were conducted using Tukey contrasts via the `R` `multcomp` package (Hothorn *et al.* 2008).

Regarding the oviposition rate, count data were analyzed using a Generalized Poisson (GP) error distribution with the first order autoregressive correlation structure (`corAR1()`) to model the temporal dependence among observations and to accommodate the over-dispersed Poisson distributed data (Zuur *et al.* 2009; Crawley 2005, Brooks *et al.* 2019). In the conditional part of the full model, the response variable (the daily eggs laying rate) was fitted as a function of the fixed variables: Dates (12 levels) and Year (2 levels) as categorical variables as well as their interaction, and Temperature as covariate. Subject was included as a random effect in our model to take into account the repeated measures for each sample. In order to consider the number of days in which the wooden pieces were

replaced, an “offset variable” was included. The dispersion part of the model was modelled including the interaction Date x Year.

To assess the difference in the daily oviposition rate among the 4 age-cohorts a GLMMs, with GP error distribution and the first order autoregressive correlation structure (`corAR1()`), was performed using the `glmmTMB` package. The model included Cohort (four levels), Time (11 levels) as fixed effects, and Subject as random effect. Data were analysed aligning the beginning of the oviposition of the 4 groups.

The seasonal dynamics of egg maturation was analysed by a zero-inflated mixed model GLMMs in order to take into account the absence of eggs recorded at beginning and end of season owe to the female age. The GP error distribution was used instead of the Poisson distribution because over-dispersion was present (Brooks *et al.* 2019). The response variable was the count of mature eggs in the ovary. The full model included Area, Year, their interaction, Dates (category variables), and Temperature as fixed factors, whilst the interaction between Area and Sites was included as a random intercept effect to take into account the repeated measures for each site. These factors were included in both the conditional and zero inflation part of the model. Marginal means were estimated from the minimum adequate model using the `emmeans` package (Lenth, 2019).

3.1.3. Results

Lifespan

Scaphoideus titanus longevity differed significantly, between sexes, in both years (Fig. 2). Females were able to reach an average of 61.60 ± 3.36 days ($n = 41$; median survival time = 65; interquartile range IQR, 44 – 77), and 62.65 ± 3.33 days ($n = 55$; median survival time = 63; IQR, 47–80) in 2016 and 2017, respectively. Moreover, 25% of the females lived 77 to 102 days in 2016 and 81 to 123 days in 2017. Concerning males, an average of 49.30 ± 2.94 days ($n = 33$; median survival time = 51; IQR, 38 – 57), and 45.20 ± 4.15 days ($n = 39$; median survival time = 39; IQR, 22 – 63.5) in 2016 and 2017 respectively, was recorded. Moreover, 25% of the males lived 57 to 84 days in 2016 and 63 to 101 days in 2017. Concerning the Cox's proportional hazard there are no censored data, being all specimens died by the end of the trial. Neither the Year factor (coefficient 0.88, SE 0.21, $z = -0.59$, $P = 0.55$) nor the Year * Sex interaction did not significantly influence the longevity (coefficient 0.93, SE 0.32, $z = -0.21$, $P = 0.83$), in contrast to the Sex factor (coefficient 1.87, SE 0.24, $z = 2.62$ $P < 0.01$). The appropriate minimal model is the one including the Sex factor, as confirmed by the Likelihood Ratio Test (LRT) (Tab. S1). Based on this model, females lived significantly longer than males in both years. Furthermore, males were 92% more likely to die than females through their lifespan (coefficient 1.92, SE 0.16, $z = 4.02$, $P < 0.001$) (Tab. S2).

No significant differences in longevity (year 2016: $\chi^2 = 4.7$, 3 df, $P = 0.2$; year 2017: $\chi^2 = 2.1$, 3 df, $P = 0.5$ Log-rank test) and oviposition timespan among the 4 age-cohorts of females were found in both years (year 2016: $\chi^2 = 6.2$, 3 df, $P = 0.1$; year 2017: $\chi^2 = 0.3$, 3 df, $P = 0.9$ Log rank test).

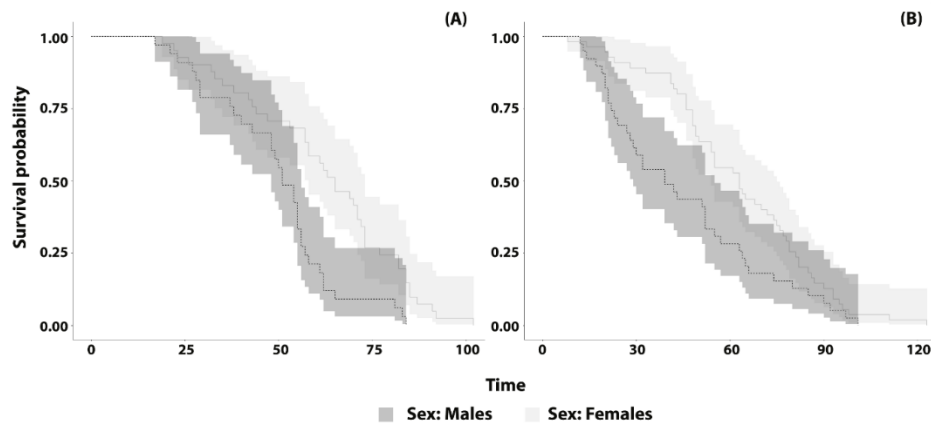


Figure 2. Kaplan-Meier survival curves with 95% confidence intervals for *Scaphoideus titanus* females and males. (A): year 2016 (41 females, 33 males); (B) year 2017 (55 females, 39 males). Lines show expected survival to a given time; dashed lines show 95% confidence intervals.

Fecundity

In 2016, females laid an average of 65.85 ± 4.32 eggs (median = 64; IQR, 53 - 85) in a period of 47.22 ± 4.44 days (median = 50; IQR, 34.5 - 64). The maximum number of eggs laid by a female was 131. The 25% of the females were able to lay a number of eggs between 85 and 131, during the season in a period ranging between 64 and 86 days. The oviposition began at an average of 13.59 ± 0.57 days (median = 14; IQR, 12 - 15.75) after adult emerging. In 2017, females laid an average of 60.25 ± 5.18 eggs (median = 55; IQR, 30 - 75) in a period of 42.71 ± 3.61 days (median = 42.5; IQR, 25 - 61). The maximum number of eggs laid by a single female was 177 (outlier value). The 25 % of females laid a number of eggs between 75 and 141, and the oviposition period ranged between 61 and 89 days. The oviposition began at an average of 11.83 ± 0.87 days (median=11.5; IQR, 9.75–13.25) after adult emerging. The comparison between the “null” model (without the fixed term “Dates*Year”) and the model with this interaction, showed this latter to have a significantly better fit, as demonstrated by the chi-

square test for Δ deviance ($\chi^2 = 224.56$, $df = 46$, $p < .000$), and the AIC value (3066.1 with, vs. 3198.7 without). The model minimizing information loss (lowest AIC) indicated that there was a time trend in egg-laying in 2016 and 2017 (Tab. S3). In 2016, the oviposition rate was high from the beginning of August until the first ten days of September. The daily oviposition rate peak was recorded at the end of August. The rate then gradually decreased until the beginning of November, when few females (5%) were still alive (Fig. 3). In 2017, the oviposition rate was high from the beginning of August until the second ten days of September and no significant difference was recorded during the two-month period. The rate decreased from the end of September until the end of October, as in 2016, when a lower number of females (7.27%) were still alive until the first ten days of November (Fig. 3). Concerning the comparison between the two years, only in three dates there were significant differences (Figs. S1, S2). No significant difference in the daily oviposition rate among the 4 age-cohorts were found in both years (2016: $\chi^2 = 11.74$, $df = 12$, $p = 0.47$; 2017: $\chi^2 = 8.8$, $df = 12$, $p = 0.71$) (Tab. S4).

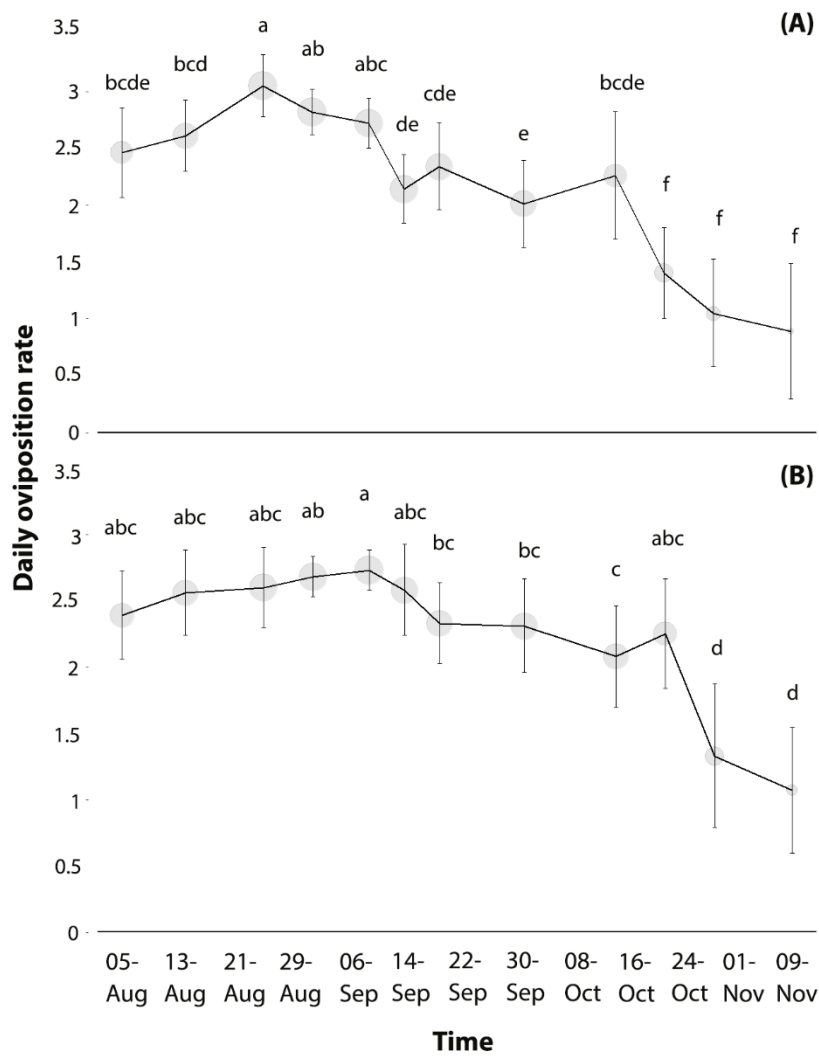


Figure 3. Daily oviposition rate in 2016 (A) and 2017 (B), graphs of the time trend of the modelled predicted values. Within each subfigure, mean values were separated through Tukey post hoc test when significant at $P \leq 0.05$ (different letters indicate significant difference among dates of the daily oviposition rate). In both graphs the size of the circles is proportional to the number of samples used to generate the estimate, ranging from 2 (smallest circle) to 38 (largest circle). Data are presented as predicted values on the log scale with 95%

confidence intervals (CI). Model coefficients are provided in the supplementary material.

Egg load

A total of 247 and 350 specimens were dissected in 2016 and 2017, respectively. The egg load was compared along 14 weeks between the 2 areas during the two years. In 2016, the first mature eggs detected in the specimens from Asti Monferrato were on 20th July with a mean egg load of 1.25 ± 0.96 , the mature eggs were found in 25% of the collected females. Ten days later the percentage of females with mature eggs increased reaching 70% showing a mean egg load of 5.86 ± 0.57 . The highest average number was recorded on September 30th with 7.81 ± 0.89 , with a maximum value per individual of 16 eggs observed on July 30th. The females collected on October 30th showed an average of 1.57 ± 0.53 of mature eggs. In the leafhoppers from Canavese the first mature eggs were found on July 20th with an average egg load of 2.30 ± 0.98 , the mature eggs were found in 15 % of the collected females. From the end of July, all the females presented eggs inside the ovaries. The highest average number was recorded on August 30th with 9.5 ± 0.67 . In October the average number gradually decreased with a minimum of 2.14 ± 0.75 recorded in the last captured individuals (30th October). The maximum number of mature eggs was 14 in the months of August and September.

In 2017, the first mature eggs detected in the specimens from Monferrato were on July 20th with a mean egg load of 1.70 ± 0.83 , the mature eggs were found in 60% of the collected females. The highest average number was recorded on September 10th with 8.78 ± 0.50 , the maximum value per individual (14) was observed on August 20th. The last females, collected on October 30th, bore also mature eggs (2.11 ± 0.70).

The first mature eggs detected in the specimens from Canavese were on July 20th with a mean egg load of 2.87 ± 1.26 , the mature eggs were found in 50% of the collected females. The highest average number was recorded on September 20th

with 9.5 ± 1.12 , on August 20th the maximum value per individual (14) was observed. Even in this locality the last females, collected on October 30th, presented mature eggs (2.5 ± 0.41). The comparison of the proportion of mature eggs between the two areas is shown in Fig. S3.

The most parsimonious model indicated that in the conditional part of the model, the interaction between Dates and Areas was significant, whilst the zero-inflation part of the model specified only the variable "date" as predictor of the probability to find an observation in the all-zero component of the model. Thus, the factors "year" and "temperature" have not influenced the egg load trend (Tab. S5, S6). At the first date there is the higher chance to observe an extra zero in the model compared to the other dates. The comparison of the first sampling data (July 10th) was not considered in the model because an enough female number was not found during both years and in all studied areas. In both areas the comparison of the average egg load among all collection dates showed high values until the end of September. Since the beginning of October the mean values start to decrease significantly (Fig. 4). Concerning the comparison between the two areas, only in two dates there were significant differences (Figs. S4, S5).

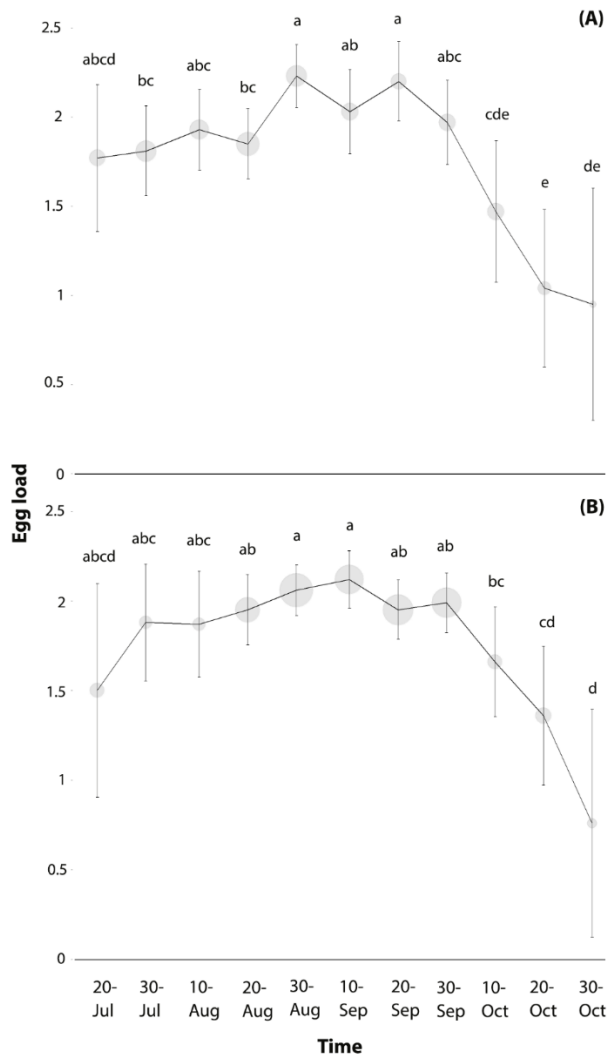


Figure 4. Egg load trend in Canavese (A) and Asti Monferrato (B) areas during 2016 and 2017 of the modelled predicted values. Within each subfigure, mean values were separated through Tukey post hoc test when significant at $P \leq 0.05$ (different letters indicate significant difference among dates of the egg load trend). In both graphs the size of the circles is proportional to the number of samples used to generate the estimate, ranging from 11 (smallest circle) to 57 (largest circle). Data are presented as predicted values of the conditional model on the log scale with 95% confidence intervals (CI). Model coefficients are provided in the supplementary material.

3.1.4. Discussion

Lifespan and fecundity are the basis of the reproductive success of a species especially for those ones which relies on a synovigenic oviposition strategy like *S. titanus*. The knowledge of these biological parameters is essential when developing vector control programs and predictive mathematical models of vector infestation. In case of *S. titanus*, as per many insect species, these biological traits can influence the potential size reached by the population (Chuche and Thiéry 2014) in a particular environment.

The studies we conducted on adult *S. titanus* longevity show that this biological parameter was so far underestimated. In fact, it is known that the duration of the adult activity is about 30 days in males and slightly longer in females (Vidano 1964; Chuche and Thierry 2014). In disagreement with the literature, in our results the longevity is significantly higher in both sexes. Males were able to live an average of 45 days, and 25% from 50 to 70 days, whilst females an average of 60 days, with 25% from 70 to 100 days. Vidano (1964) explains in his work that he collected data both in greenhouse rearing and in the field. He probably refers to the former while writing about lifespan and to the latter when presenting fecundity data such as the egg load. Considering the hot and dry environmental conditions of greenhouse, it is likely to imagine why the leafhoppers could not live longer, even if raised onto potted grapevine plants. Female lifespan is clearly longer than males, in accordance with the data obtained in lab conditions with other cicadellid species (Guglielmino and Virla 1997; Van Nieuwenhove *et al.* 2016; Grosso *et al.* 2017). The high longevity of the females explains a *sex ratio* in their favour during the late season since the end of August as previously hypothesized by Lessio *et al.* (2009). In fact, this is the first study in which *S. titanus* lifespan is monitored in semi-natural conditions. Longevity tests showed that *S. titanus* has a remarkable plasticity by the biological point of view. In fact, some females were collected in field during the second half of October, still active and with mature eggs. Furthermore, the evidence of longevity tests makes

plausible to expect, with greater frequency over the years, catches in October and potentially in the first ten days of November, depending on the weather conditions.

Previous surveys on *S. titanus* biology highlighted how some information about female fecundity and mature egg trend during the season were discordant with each other (Cravedi *et al.* 1993; Bosio and Rossi 2001; Linder and Jermini 2007; Eriksson *et al.* 2012).

The present studies show that oviposition begins approximately 12 - 14 days after eclosion, around 20th of July in agreement with Vidano (1964). The results of the oviposition test (over 60 eggs laid on average per female in both the years, in over 40 days), show that the reproductive potential of this species is considerably higher than what estimated in the literature (Vidano 1964; Chuche and Thiéry 2014). The laid egg rate trend was similar in both years showing significant difference only in three dates, and remains high throughout the season till late October and then begins to decrease. The drop in the oviposition rate is probably attributable mainly to the age of the females because no significant differences among different cohorts, neither in terms of oviposition trend nor in longevity and oviposition timespan, were found. In fact, some studies conducted on leafhoppers and other insects in laboratory conditions at fixed temperatures highlighted that the oviposition trend gradually decreases over time (Valle *et al.* 1986; Huang *et al.* 2008; Krugner 2010). However, despite the mean number of laid eggs progressively decreased in September and October, in both years few females were still able to lay eggs until the end of October or even the beginning of November. The mean temperatures occurred in autumn during this two-year survey were rather mild (Plant Protection Service of Regione Piemonte, data not shown). In fact, in the recent past a distinct increase in the growing-season mean temperatures was recorded in Europe (Fraga *et al.* 2012). Thus, an influence of the temperature on longevity and fecundity, being insects poikilotherm animals, should not be excluded (Bale *et al.* 2002; Van Lenteren *et al.* 2006; Krugner 2010; Inward *et al.* 2012). In all species there is a thermal optimum that corresponds to

the maximum fertility value. Some studies on different leafhopper species, showed that the fertility seems to be lower when exceeding this value (Valle *et al.* 1986; Tokuda and Matsumura 2005; Huang 2008). The thermal optimum of *S. titanus* is currently not known, however it can be hypothesized that it might be able to tolerate low winter temperature for short time since the origin area of the species is characterized by a continental climate.

Concerning the egg load trend, the results show a considerable variability in mature egg number per female both for different date and collecting site. This can be explained by the gradual hatching and the subsequent coexistence of females of different age and degree of egg maturation during the season (Vidano 1964; Chuche and Thiéry 2009). Furthermore, there are more females with no eggs at the beginning and the end of the season because of their age, the former due to the high number of newly emerged females, and the latter for a high number of old ones. According to some authors, the oviposition occurs between August and September (Chuche and Thiéry 2014; Mazzoni and Lucchi 2014). In the present research, in both years and the two investigated areas, the first specimen with mature eggs was found on July 20th, as previously reported by Vidano (1964). This means that *S. titanus* begins to lay eggs and thus can potentially settle shortly after the middle of July. In accordance with previous surveys (Bosio and Rossi 2001; Linder and Jermini 2007) and unlike to what was stated by Cravedi *et al.* (1993), the present study has shown that the average number of the mature eggs remains high for almost all the season and then decreases in October. The egg load trend showed no significant differences between the two surveyed years, while significant differences were highlighted between the areas only in two dates. Therefore, the general egg load trend is similar between areas as well. Differences at singular dates could be explained, as stated by some authors (Chuche and Thiéry 2009; Chuche and Thiéry 2014; Chuche *et al.* 2015), with a non-simultaneous hatching period which can be translated into a different timing for both developmental stages and egg maturation. The asynchronic phenology can be induced by different temperature values perceived by the overwintering

eggs (Chuche and Thiéry 2009; Falzoi *et al.* 2014), maybe owing to different microclimatic conditions. In fact, we were able to collect some *S. titanus* fifth instar nymphs in Asti Province during both years in the first half of September. We believe that their presence might be due to a very prolonged hatching period, even if Bernard *et al.* (1988) hypothesized a second generation because of a possible non-wintering eggs existence.

In contrast with Cravedi *et al.* (1993) and Bosio and Rossi (2001), who observed the peak of mean egg load in August and at the beginning of September, the mature egg number remains constantly high throughout the season until the beginning of October in the three surveyed vineyards, when the insecticide treatments are no longer performed. Moreover, being the eggs protected by the cork (Vidano 1964; Bagnoli and Gargani 2011; Gargani *et al.* 2013; Lessio and Alma 2013), chemical control cannot be effective, leaving them alive until the next spring.

Scaphoideus titanus egg load mean was till now considered about 8-10 eggs per female in case of field collected data (Cravedi 1993; Bosio and Rossi 2001; Linder and Jermini 2007), and about 13–15 eggs per female in studies performed under laboratory conditions (Bressan *et al.* 2005; Eriksson *et al.* 2012) with a maximum of 24 eggs, completely laid within 10 days (Vidano 1964). Our results are closer to the lowest egg load mean values of the field collected data, resulted from a dissection of uneven-aged specimens, but much lower of the values obtained in laboratory conditions where coetaneous specimens were used. As previously commented, Vidano probably linked his observations on maximum egg load to the shortdetected lifespan, and hypothesized that they all can be laid during the short oviposition period. The tests conducted in the present research showed that these data were again largely underestimated.

The comparison between the egg load curves and the oviposition rate shows that both indices remain constant until September and then decrease in the following months. During the test in seminatural conditions few females continued to lay until end October-early November, and the last females with mature eggs were

caught in the field at the end of October. Conversely, the laying period is very long (on average more than 40 days) and the overall number of laid eggs is considerable, sometimes exceeding 100. Consequently, it is reasonable to expect an increase in the population size in the vineyard during the next year. Moreover, the prolonged oviposition period could explain the difference between the *sex ratio* obtained *in vitro* and that one obtained in field by yellow sticky traps (Lessio *et al.* 2009), probably because females fly less during the oviposition activity, as hypothesized by Bosco *et al.* (1997), and males are more active for female localization behaviour (Mazzoni *et al.* 2009).

Chuche *et al.* (2012) observed that different winter temperatures, which the overwintering eggs are subjected to, affect weight and size only of the hatched nymphs from third to fifth instar, but not the adults. However, a possible effect of different climatic conditions occurred during the oviposition and overwintering season on the longevity and fecundity of *S. titanus* adults emerged the following year, should be taken into consideration. Eggs laid at different times during the oviposition period may be subjected to the same effects, raising questions about the embryonic development, or the possible different genotypes of females able to produce eggs with different temperature hatching demand as hypothesized by Chuche *et al.* (2012). However, further specific studies are needed to clarify these aspects.

These new insights on *S. titanus* biology might be attributable to a previous incomplete monitoring activities in the field or, as hypothesized by Maixner (2005), and Reineke and Thiéry (2016), to a warmer late autumn. Being poikilotherm, a prolongation of summer and a mild autumn could affect its physiology (Bale *et al.* 2002; Inward *et al.* 2012). It is known, in fact, how different climates can induce changes in phenology and voltinism. Indeed, some species have diversified the biological cycles depending on the latitude. Within the order Hemiptera, *Hyalesthes obsoletus* Signoret (Fulgoromorpha: Cixiidae) is considered monovoltine in Palaearctic area and bivoltine in Israel (Klein *et al.* 2001), like other cixiid species in the genera *Cixius* and *Tachycixius* present in

Lebanon (Tedeschi *et al.* 2015; Picciau *et al.* 2016). Therefore, it could be hypothesized that a possible increase in temperatures, induced by climate change, could increase adult *S. titanus* longevity, especially those ones emerged in the late season. Moreover, some authors (Chuche *et al.* 2015) presume that the gradual egg hatching is associated with an adaptation to the phenology of the host plant. Grapevine may be influenced by temperature as well, delaying the leaf fall (Bertin 2008). This condition would allow the last adults to fly and feed during the late season.

Adults still active at the end of the season could acquire phytoplasma and move to vineyards, which are no longer protected by insecticide treatments for food safety regulations.

Thus, this can be considered a very critical period, which is even worsened by the risk of possible migratory flows of fecund females (Pavan *et al.* 2012; Lessio *et al.* 2014), along with their still high potential fecundity. Besides the FDP inoculation, a serious risk of settlement of new *S. titanus* populations arises.

3.2. DEVELOPMENT, SPATIAL DISTRIBUTION, AND PRESENCE ON GRAPEVINE OF NYMPHS OF *ORIENTUS ISHIDAE*, A NEW VECTOR OF FLAVESCENCE DORÉE PHYTOPLASMAS

3.2.1. Introduction

Many aspects of *O. ishidae* biology are still unknown, especially concerning the embryonic and post-embryonic development of nymphs. Moreover, its relationships with grapevine are unclear, especially concerning egg-laying and population density of nymphs.

Given the lack of basic knowledge about this emerging pest, this research deals with several aspects of the biology of *O. ishidae* nymphs: presence of eggs in wood of grapevine, depending also on the management of vineyards; embryonic and post-embryonic dynamics under laboratory conditions; seasonal occurrence of nymphs; presence of nymphs on wild or cultivated grapevine, compared to other elective host plants; and spatial distribution of nymphs on elective host plants, at leaf and shoot levels.

3.2.2. Materials and Methods

Study area

Samplings took place in 2017 and 2018, in vine-growing areas settled within the following districts of Piedmont, North-western Italy: Caluso (45.31796°N; 7.88061°E), Mazzé (45.309539°N; 7.934383°E), Borgiallo (45.399622°N; 7.667674°E), Portacomaro (44.962311°N; 8.258457°E), Mombercelli (44.820415°N; 8.302749°E), and Vesime (44.644452°N; 8.226433°E). Within these areas, we selected eight experimental sites: four managed (organic) vineyards; two abandoned vineyards (that is, let unpruned and unmanaged for a period of 2–5 y, with many weeds but without overgrown trees or vine rootstocks) (Camerano and Terzuolo 2015); and two woods mainly consisting in wild hazelnut (*Corylus avellana* L.) and hornbeam (*Carpinus betulus* L.) trees, with overgrowing American rootstocks of *Vitis berlandieri* × *riparia* (e.g. Kober 5bb, SO4) and *V. berlandieri* × *rupestris* (e.g., 1003 Paulsen) (Camerano and Terzuolo 2015), which are also a source of 16SrV phytoplasmas although often symptomless (Lessio *et al.* 2007). All of the sites were small sized (1,500–4,000 m²). The vineyards were settled within a heterogeneous landscape consisting of many patches of broadleaf woods and edges of spontaneous hazelnut and hornbeam trees, which are two elective host plants of *O. ishidae* (Lessio *et al.* 2016).

In organic vineyards, two insecticidal sprays were made by farmers with natural pyrethrum at the middle and end of June in both years, according to Regional Phytosanitary Service guidelines.

Egg-laying and hatching dynamics

To quantify egg-laying by *O. ishidae* on grapevine (*Vitis* spp.), and to study egg-hatching dynamics, wood was randomly collected in winter time from the each of the experimental sites (Tab. 1). Two year (or more) old grapevine canes and

branches were cut into pieces (15–20 cm), stored into a cool chamber (+5°C), and periodically sprinkled with water to avoid dehydration of eggs.

At the end of January, the wood was placed indoors at an average temperature of $T = 22 \pm 1^\circ\text{C}$, to start egg-hatching. Each field collected sample was weighted and placed inside a separate insect proof cage (110 cm × 110 cm × 80 cm) made of mesh and aluminium frame, along with potted hazelnut and broad bean plants to provide food for nymphs. A layer of vermiculite was placed on the bottom of the cage to preserve humidity, and the cages were periodically sprinkled with water. The cages were inspected daily, and the emerged nymphs of *O. ishidae* were counted, removed, and kept alive for the post-embryonic development experiments.

Table 1. Wood of grapevine (*Vitis* spp.) collected in different experimental sites

Site	District	Management	<i>Vitis</i> species	Year	Wood collected
1	Mazzé	Wood	American	2017	3.71
				2018	1.00
2	Caluso	Abandoned	<i>V. vinifera</i>	2017	1.50
				2018	1.00
3	Borgiallo	Abandoned	<i>V. vinifera</i>	2017	2.00
				2018	6.90
4	Portacomaro	Wood	American	2017	4.00
				2018	1.15
5	Mongardino	Organic	<i>V. vinifera</i>	2017	2.00
				2018	6.00
6	Caluso	Organic	<i>V. vinifera</i>	2017	5.00
				2018	9.00
7	Mazzé	Organic	<i>V. vinifera</i>	2017	5.00
				2018	4.00
8	Vesime	Organic	<i>V. vinifera</i>	2017	5.00
				2018	5.00

Post-embryonic development

The post-embryonic development of *O. ishidae* was studied under the same conditions of the previous experiment. Newly hatched nymphs were retrieved from the rearing cage and placed singularly inside Plexiglas cylinders (h = 20 cm; diameter: 12 cm) closed with a fine mesh on the top. Two circular holes (diameter: 2 cm) were made on the walls of each cylinder and covered with the same mesh to allow a better circulation of air. Each cylinder was placed onto a potted broad bean plant, and a disk of filter paper was placed on the soil to decrease humidity and for a better detection of any dead insect. Cylinders were

inspected daily to observe molts up to the adult stage, and after each moult, the corresponding exuvia was removed from inside.

Sampling of nymphs on host plants

Nymphs of *O. ishidae* were sampled on European grapevine, on wild American rootstocks, and on surrounding spontaneous hazelnut and hornbeam plants, in all of the experimental sites. On the whole, N = 79 plants were observed (European grapevine: 20; American rootstocks: 18; hazelnut: 33; hornbeam: 8). Samplings took place from the middle of May to the end of July (2017–2018). On hazelnut and hornbeam, for each plant, we observed 50 leaves distributed on 10 shoots (5 leaves per shoot). On grapevine species, we inspected 50 leaves close to the trunk or to wooden canes. Leaves were gently turned upside down, and nymphs were counted under a lens without being removed, assigning them to I–II instar (N1 and N2: winglets absent), and III–V instar (N3, N4 and N5: winglets present).

Data analysis

Nymphs emerging from wood collected in different types of vineyards (cultivated or wild) in different years were compared with a generalized linear model (GLM) procedure. The number of nymphs was used as dependent variable; the type of vineyard (either managed or unmanaged), the species of *Vitis* (either *V. vinifera* or American rootstocks), and the year of collection were the factors; and the weight of collected wood was the offset variable. To overcome problems due to zero values, we used a Tweedie mixed distribution, which manages properly both over-dispersion and zero-inflation, with a Log link function.

A cumulative distribution function was used instead to describe egg-hatching dynamics. Finally, we calculated the descriptive statistics (mean, SD, and confidence limits) of the duration for both the embryonic development of nymphs

emerged from different wood lots and post-embryonic development of each life instar.

The seasonal distributions of early (N1–N2) and late (N3–N5) instar of field-collected nymphs between the two sampling years were compared with a chi-square test with 4 df (five sampling dates, from the middle of May to the middle of July, at a bi-weekly step). Counts of nymphs on plants were analyzed with a GLM procedure testing the effect of plant species (European grapevine, American rootstocks, hazelnut, hornbeam). In this case, we did not separate early from late instars nor we considered sampling date. The number of sample units per date and plant species was considered as an offset variable. A Poisson distribution of data was assumed, and the Log link function was used.

Taylor's Power Law (TPL) (Taylor 1984) was used to analyze nymph distribution on leaves and shoots of hazelnut and hornbeam. The general equation of TPL is:

$$\log_{10} S^2 = \log_{10} a + b \cdot \log_{10} m$$

where, having counted a given object (e.g., nymphs) on a given number of sampling units, S^2 and m are the sample variance and mean, respectively. While the coefficient a (intercept) depends just on the sampling method, the coefficient b (slope) is typical for the species considered: $b < 1$ indicates randomness, $b = 1$ uniformity, and $b > 1$ aggregation. In this case, we considered first the leaves and then the shoots as a sampling unit, calculating therefore the mean and variance for each tree. In this case, we excluded counts on grapevines (no or too few nymphs detected, see results): therefore, a dataset of $N = 41$ was available. On the other hand, given the similar plant architecture (small broadleaf trees or bushes, with a relatively similar leaf size and distribution of leaves on the branches), we did not distinguish between counts on hornbeam or hazelnut. Data were analyzed separately depending on life instar (N1 and N2; N3, N4 and N5), and were previously \log_{10} transformed according to Taylor (1984) after adding a 0.1 constant to avoid transformation problems in zero values. Finally, a linear

regression was run between the sample variance (dependent variable) and the sample mean (independent variable).

All of the statistical analyses listed were performed with SPSS 25.0 statistical package (IBM Corporation, Armonk, NY).

3.2.3. Results

Collectively, 142 nymphs of *O. ishidae* were obtained from wood of grapevine, ranging 0.33–14.8 per kg of wood in single collections.

Overall, significant differences were found in the number of nymphs hatched from different kinds of wood collected in differently managed sites and during different years (GLM: $\chi^2 = 40.01$; $df = 3$; $P < 0.001$). When considering single factors, the type of vineyard had a significant influence on the number of nymphs hatched ($\chi^2 = 16.86$; $df = 2$; $P < 0.001$): organic vineyards were characterized by significantly lower nymph density than the other two kinds, whereas no differences were detected between abandoned vineyards and woods with American rootstocks (Fig. 5).

No differences were found between the 2 y of wood collection ($\chi^2 = 0.81$; $df = 1$; $P = 0.37$).

The wood collected in 2017 in the sites of Caluso (abandoned vineyard; nymphs/kg wood: 14.78; total: 17), Mazzé (wild American grapevine; nymphs/kg wood: 7.53; total: 29), Borgiallo (abandoned vineyard; nymphs/kg wood: 8.83; total: 72), and Portacomaro (wild American grapevine; nymphs/kg wood: 3.38; total: 13) provided enough nymphs to describe egg-hatching dynamics. Hatching started from 2 to 4 wk (13–30 d) after the beginning of incubation, and lasted 2–7 wk (14–51 d). The mean hatching time was minimum in Portacomaro (34 d) and maximum in Mazzé (48 d) (Tab. 2; Fig. 6).

Data of post-embryonic development under laboratory conditions were obtained from a set of $N = 20$ newly hatched nymphs, which were successfully reared up to the adult stage (other specimens died before becoming adults and were therefore excluded from analysis). Developmental times increased along with life stages: N1 lasted approx. 4–5 d, whereas N5 lasted 6–7 d. Overall, the postembryonic development lasted 27–29 d (Tab. 3).

Fig. 5. Nymphs of *O. ishidae* (mean \pm SE per kg of wood) hatched from different kinds of grapevine's wood. Different letters indicate significant differences in wood type (GLM, $P < 0.05$).

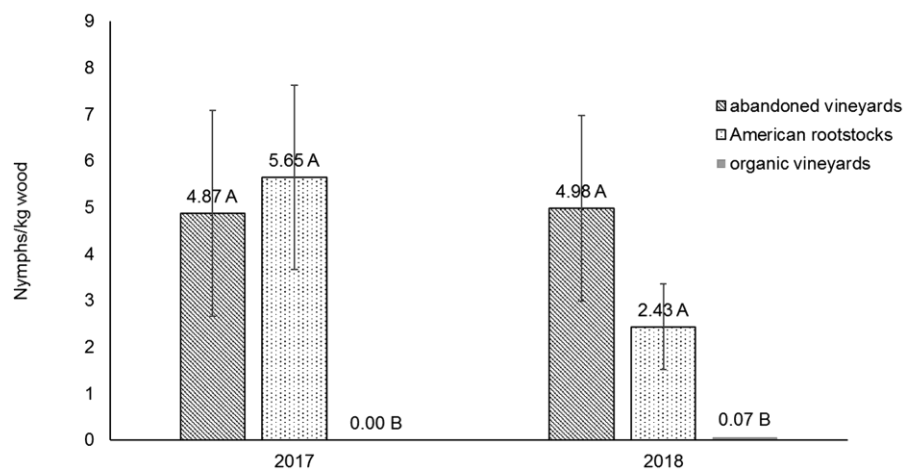


Table 2. Time of embryonic development (TD, in days) in *O. ishidae* at T = 21–23°C

Site	Year	N	TDmin	TDmax	HD	TDmean	SE	Lower CI	Upper CI
1	2017	30	25	59	34	48.87	0.34	48.20	49.54
2	2017	17	30	50	20	39.76	0.30	39.17	40.36
3	2017	72	13	64	51	46.36	0.15	46.08	46.65
4	2017	13	28	42	14	34.46	0.39	33.69	35.23

N: number of hatched nymphs; TD min and TD max: time of development of the first and last hatched specimen; HD: hatching duration (HD = TD max - TD min). Site 1: Mazzè (unmanaged, American grapevine); site 2: Caluso (unmanaged, European grapevine); site 3: Borgiallo (unmanaged, Eur. grapevine); site 4: Portacomaro (unmanaged, Am. grapevine).

Fig. 6. Cumulative frequency distribution of egg-hatching in *O. ishidae* obtained from grapevine wood at $T = 21\text{--}23^\circ\text{C}$.

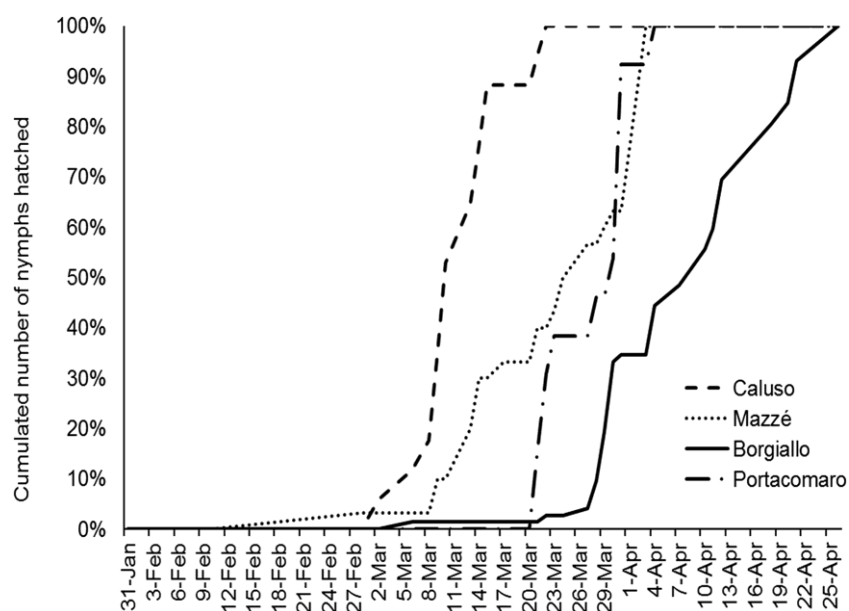


Table 3. Duration of post-embryonic development (in days) in *O. ishidae* ($N = 20$) at $T = 21\text{--}23^\circ\text{C}$

<i>Life stage</i>	<i>Mean</i>	<i>SE</i>	<i>Lower CI</i>	<i>Upper CI</i>
<i>N1</i>	4.45	0.18	4.09	4.81
<i>N2</i>	5.30	0.46	4.40	6.20
<i>N3</i>	5.30	0.29	4.73	5.87
<i>N4</i>	5.85	0.17	5.52	6.18
<i>N5</i>	6.95	0.34	6.28	7.62
<i>Total</i>	27.85	0.55	26.76	28.94

Seasonal dynamics of nymphs on host plants

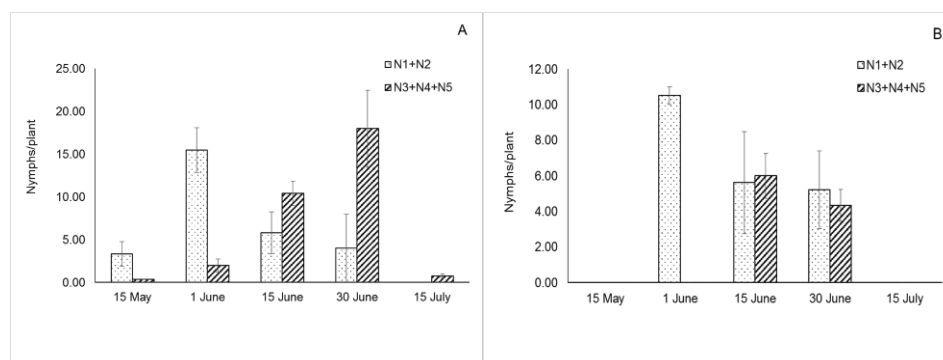
Collectively, 507 nymphs were observed on hazelnut and hornbeam plants throughout different sampling periods (N1–N2: 285; N3–N5: 222). The first nymphs (N1–N2) appeared at the middle of May, whereas N3–N5 appeared between the end of May and the middle of June. The peak of N1–N2 occurred at the end of May in both years, whereas N3–N5 peaked at the end of June in 2017 and at the middle of June in 2018. At the end of July, no nymphs were found. Data are shown in Fig. 7. Referring to the distribution among five sampling dates (from the middle of May to the middle of July), no differences were found between years, neither for early stages ($\chi^2 = 0.22$; $df = 4$; $P = 0.05$), nor for late ones ($\chi^2 = 0.42$; $df = 4$; $P = 0.09$). On the other hand, only three specimens were observed on grapevine (abandoned grapevines: one early instar on 31 May 2017, and one late instar on 14 June 2017; wild grapevine: one late instar on 16 June 2018). A significant difference was observed in the number of nymphs detected on different plant species (GLM: $\chi^2 = 3971.05$; $df = 3$; $P < 0.001$). The number of nymphs per plant was significantly higher on hazelnut with respect to all other. As well, it was significantly higher on hornbeam with respect to both species of grapevine, whereas no significant differences were found between European and American grapevine (Tab. 4).

Table 4. Nymphs of *O. ishidae* counted on different host plants

Plant species	Nymphs (mean \pm s.e.)	χ^2 (d.f.)	P
<i>Corylus avellana</i> L	13.00 \pm 1.32 a	3971.05 (3)	<0.001
<i>Carpinus betulus</i> L.	9.38 \pm 2.68 b		
<i>Vitis vinifera</i> L.	0.10 \pm 0.07 c		
American rootstocks	0.10 \pm 0.07 c		

Different letters indicate significant differences (GLM, $P < 0.05$).

Fig. 7. Seasonal distribution of nymphs in *O. ishidae* collected on host plants (hazelnut and hornbeam): (A) 2017 and (B) 2018.



Aggregation patterns of nymphs

The aggregation patterns of *O. ishidae* nymphs, calculated with TPL, resulted highly significant and changed depending on the instar and the plant organ considered as sample unit (Table 5). When considering leaves, early-life instars were moderately aggregated ($b = 1.29$), whereas late ones resulted less crowded ($b = 1.16$).

The same trend was observed for spatial distribution at a shoot level; however, both early and late instars were less aggregated with respect to leaves. In particular, late instars on shoots had an almost random spatial distribution ($b = 1.07$).

Table 5. TPL regressions on nymphs of *O. ishidae* counted on leaves and shoots of broadleaf host plants

<i>Source</i>	<i>R</i> ²	<i>ANOVA</i>			<i>Coefficients</i>			
		<i>F</i>	<i>P</i>	<i>B</i>	<i>t</i>	<i>P</i>		
<i>N1</i>	+	0.9	894.7	0.000	<i>Interce</i>	0.52	9.72	0.00
					<i>Slope</i>	1.29	29.9	0.00
<i>N3</i>	+	0.9	726.3	0.000	<i>Interce</i>	0.29	5.41	0.00
					<i>Slope</i>	1.16	26.9	0.00
<i>N1</i>	+	0.9	510.0	0.000	<i>Interce</i>	0.25	5.03	0.00
					<i>Slope</i>	1.16	22.5	0.00
<i>N3</i>	+	0.9	528.3	0.000	<i>Interce</i>	0.13	2.92	0.01
					<i>Slope</i>	1.07	22.9	0.00

3.2.4. Discussion

The present research confirmed that *O. ishidae* is capable of laying eggs on grapevine. This behavior may be promoted by the proximity of trees to vineyards or by the co-habitat of wild grapevine and other plants in woods. In fact, more nymphs hatched from wood collected from abandoned vineyards and/or wild rootstocks, where hazelnut and hornbeam plants were generally closer to the edges or, in the case of woods, mingled with overgrowing grapevine rootstocks.

Another reason may be the greater presence of older wood (2 y, or more) due to no pruning. Although we did not take into account 1-y old wood for the presence of eggs, it is likely that *O. ishidae* prefers older grapevine wood just like *S. titanus* (Lessio and Alma 2013, Chuche and Thiery 2014). Therefore, females might have fed and developed on other plants and only afterwards exploited grapevine wood for egg-laying.

However, while this species was very abundant on hornbeam and hazelnut, very few nymphs were found on grapevine leaves. Although in organic vineyards pyrethrum was sprayed on grapevines after the middle of June, nymphs were not found even before treatments. Therefore, there must be another reason to explain such a discrepancy. Perhaps, grapevine is used just for egg-laying, and nymphs move elsewhere after hatching. Among leafhoppers in the same subfamily (Deltocephalinae), *Anoplotettix fuscovenosus* (Ferrari) exhibits a similar strategy: eggs are laid under the bark of grapevine, but nymphs move to the weeds in the inter-row (Alma 1995). Sometimes, *Phlogotettix cyclops* (Mulsant and Rey) lays eggs under the bark of grapevine canes too (Chuche *et al.* 2010). It is not surprising therefore that females of *O. ishidae* exploit grapevines for egg-laying, given also their frequent drifting behavior in vineyards (Lessio *et al.* 2016). However, it is not clear if egg-laying on grapevines represents a biological strategy or just a casual occurrence. In any case, although nymphs of *O. ishidae* are capable of acquiring 16SrV phytoplasmas from grapevines (Lessio *et al.*

2016), this aspect does not seem important from an epidemiological point of view, as very few specimens have been found on grapevines.

The embryonic development of *O. ishidae* lasted 34–48 d at $T = 21\text{--}23^{\circ}\text{C}$. A similar trend has been observed in *S. titanus*, which has a mean hatching time of 30 and 45 d at constant temperatures of 20 and 22°C , respectively (Falzoi *et al.* 2014). The discrepancies observed in the egg-hatching dynamics among sites may be due to cold or mild winters. In fact, it has been demonstrated that *S. titanus* eggs hatch faster when exposed to cold rather than mild winter temperatures (Chuche and Thiery 2009). Concerning the post-embryonic development, late instars of *O. ishidae* take longer to develop than that early ones: this is similar too to *S. titanus* (Falzoi *et al.* 2014), and is related to the fact that body size has an effect on developmental time (Gillooly *et al.* 2002).

Seasonal dynamics of nymphs observed in the field were partially in accordance with the data presented by Valley and Wheeler Jr (1985), who found them from the beginning to the end of June on ornamental honey locust in Pennsylvania. In our research, nymphs were found from the middle of May up to the middle of July. These differences may be due to different temperatures between Pennsylvania and Northern Italy, to increasing temperatures over the last three decades, to differences in host plants, or simply to sampling discrepancies. With regard to the influence of host plants, it is possible that *O. ishidae* populations have adapted to differences in bud break among plant species. This synchrony has been detected concerning grapevine and *S. titanus* (Chuche *et al.* 2015), which however is a monophagous species and is probably co-evolving with its own host plant. *Orientus ishidae* is highly polyphagous (Nickel 2010, Lessio *et al.* 2016, Alma *et al.* 2019) and therefore it is less likely that populations specialize on one host plant: in fact, the success in this species is probably due to its plasticity.

The spatial distribution of nymphs was affected by the life stage and the sampling unit. Dispersal increased from early to late instars, and from leaves to shoots. Late instars are less aggregated probably because they disperse due to overcrowding

on leaves. This aspect was not observed in *S. titanus*, which rarely builds up great densities on grapevine leaves. In fact, the nymphs of this species are aggregated (Lessio and Alma 2006) and have also an aggregative feeding behavior under laboratory conditions (Chuche *et al.* 2009). This could be due to differences in plant architecture. In fact, grapevine (especially if row-shaped) has few shoots sprouting from the trunk: nymphs of *S. titanus* hatching from eggs laid under the bark have therefore less shoots to colonize. On the other hand, nymphs of *O. ishidae* have more opportunities of reaching sprouts when eggs are laid on the trunk and on the branches of broadleaf trees. Another reason may be the different feeding habits between these two species. *Orientus ishidae* causes severe stunting on leaves (Felt and Bromley 1941, Lessio *et al.* 2016), probably because of a cell rupture feeding behavior. In fact, damages resemble in some way marginal burning caused by *Empoasca vitis* (Goethe), a typical cell rupture feeder (Jin *et al.* 2012); therefore, overcrowding may cause a decrease of food resources. On the other hand, *S. titanus* usually probes in one point producing salivary sheaths (Chuche *et al.* 2017), without affecting directly grapevine leaves.

The similarity of life cycles between *S. titanus* and *O. ishidae* may be the reason why both are vectors of 16SrV phytoplasmas to grapevine, although with different efficiency and therefore importance. However, phytoplasma sources for nymphs of *O. ishidae* are less certain. No nymphs collected on many host plants tested FDP positive, although they are capable of acquiring from infected grapevines (Lessio *et al.* 2016). Recently, some host plants (e.g., willow, hazelnut) have been found positive to 16SrV phytoplasmas in Switzerland (Casati *et al.* 2017). Another possibility is that adults of *O. ishidae* acquire phytoplasmas directly on grapevine. In fact, it has recently been proved that *S. titanus* is capable of acquiring 16SrV phytoplasmas in the adult stage and transmitting them within only 2 wk (Alma *et al.* 2018). Given the biological similarities between *S. titanus* and *O. ishidae*, this matter should be further investigated.

4. SURVEYS ON PALEARCTIC PARASITOIDS OF THREE EXOTIC PESTS

4.1. PALAEARCTIC EGG PARASITOIDS INTERACTION TO THREE GRAPEVINE EXOTIC PESTS IN NORTH WESTERN ITALY: A NEW ASSOCIATION INVOLVING *METCALFA PRUINOSA*

4.1.1. Introduction

The egg is the most vulnerable stage for leafhoppers (Waloff and Jervis 1987) and egg parasitoids are considered the most important natural enemies of Auchenorrhyncha (Denno and Roderick 1990; Waloff and Thompson 1980). In particular, since *M. pruinosa*, *S. titanus* and *O. ishidae* spend a large part of their cycle at the egg stage, it would be essential to identify any egg parasitoids able to adapt to them. Nevertheless, limited information is currently available on egg parasitoids adapted to these exotic species (Arzone and Alma 1994; Girolami and Camporese 1994; Malausa *et al.* 2003; Raspi and Canovai 2003). Both *M. pruinosa* and *S. titanus* lay an average of about 60 eggs per females (Santini and Lucchi 1994; Bocca *et al.* 2020a), whilst for *O. ishidae* no information is available.

One of many important factors that could affect the egg parasitoids activity in field is the host density and its interaction with the ecological context (Stiling 1987; Walde and Murdoch 1988; Gunton and Pöyry 2016). Foraging parasitoids tend to prefer areas where their hosts are present at a higher density and adapt their foraging behavior to host density variation (Hassell and Southwood 1978; van Alphen and Jervis 1996; Wajnberg 2006). Nevertheless, in some cases parasitization rate can be independent or inversely-dependent from the host density (Stiling 1987; Walde and Murdoch 1988; Pacala and Hassell 1991), even if a recent meta-analysis suggested that positive spatial density dependence is more common than a negative correlation (Gunton and Pöyry 2016). Habitat

complexity and its interaction with the host density is another factor which could drive the field parasitization rate (Andow and Prokrym 1990; Cronin 2003; Thies *et al.* 2005). As far as the egg parasitoids of plant-leafhoppers are concerned, conflicting results regarding the dependence on host density according to different spatial scales and different patches are reported (Stiling 1980; Segoli 2016; Torres-Moreno and Moya-Raygoza 2020).

The aim of this work is to study the biotopes and biocoenosis present in the vineyard agroecosystem. Furthermore, we pay particular attention to a possible Palaearctic egg parasitoid adaptation to these three exotic species and the possible interaction with other parasitoids. The parasitization rate, in percentage, on different plant species and some variables such as the host egg density and the vegetation cover are evaluated as well as other crucial biological aspects for a possible application in the biological control strategy.

4.1.2. Materials and Methods

This research was carried out over three years: during the first year an extended survey was performed by means of sentinel eggs to assess the possible entomophagous adaptation to the three pests. Based on these results, in the following two years an experimental design in two important areas of the Piedmontese vineyard agroecosystem was set up to evaluate the parasitization rate of the parasitoid identified from *M. pruinosa* and the possible influence on the parasitization rate of plant species, host egg density and plant cover. During the same years we carried on preliminary studies upon the voltinism of the egg parasitoid and its interaction with *N. typhlocybae*.

Survey on *Metcalfa pruinosa*, *Scaphoideus titanus* and *Orientus ishidae* egg parasitoids complex and host parasitization specificity

One and two-year-old canes of grapevine, *Corylus avellana* L., *Ulmus* spp., *Acer* spp., *Cornus sanguinea* L. and *Sambucus nigra* L. were collected from brushwood in three Piedmontese grapevine growing areas. The collection was carried out in 20 localities of Torino, Asti and Cuneo provinces during winter 2016. Portions of vine shoots were inspected by gently removing the bark by means of a lancet. Then, cane sections hosting eggs were incubated at 25°C in falcon tubes, provided with damp cotton-wool and closed with an insect-proof-net.

During 2017 summer, sleeve-cages (1.6 mm x 1.6 mm to allow egg parasitoids to enter and prevent the leafhoppers from escaping), containing vine canes sterilized in an autoclave and living leafhoppers (ten females of one species in each cage) were placed in Piedmontese viticulture areas where the three mentioned pests were present at high population density and several oophagous were found out the previous year, to verify the specificity of the egg parasitoids. Five cages for each species, for a total of 15, were placed in every area. At the

end of the winter, the canes were incubated at 25°C in falcon tubes, provided with damp cotton-wool and closed with the insect-proof net.

Parasitization rate, voltinism and emergence curves

In order to investigate the difference in terms of egg-parasitization rate on *M. pruinosa* among areas and years, a survey was conducted on the three most common plant species present in the vineyard agroecosystem of two Piedmontese areas, located in the provinces of Asti (Area 1) and Cuneo (Area 2) (see Fig. S6 and Tab. S8 in Supplementary material). These environments are characterized by agricultural lands, with a high density of vineyards and uncultivated areas or brushwood with wild grapevine. Five sites per area were randomly chosen in uncultivated areas, woods with wild grapevine or abandoned vineyards. For each site, a surface of about 1500 m² was sampled by subdividing each site in sampling units. Five kilograms of two-year-old grapevine canes (*Vitis* spp.), hazelnut (*C. avellana*) tree and dogwood (*Cornus* spp.) were randomly collected during the winters of 2017 and 2018. Portions of twigs from each plant species were inspected, to sort out the eggs of each pest or the canes containing eggs, and then incubated as previously described. In each site the host egg density per plant species was calculated as the total number of eggs (parasitized + not parasitized) counted for the total amount of woods per plant species collected in each site, divided by the total number of the same plant species. The influence of the habitat complexity on the parasitization rate, interpreted as the homogeneous diffusion of plants suitable for *M. pruinosa* oviposition, was evaluated in each site considering the vegetation cover percentage of the three aforementioned plant species. The plant cover was quantified as the number of the plant species (count) divided by the total number of trees present in each site.

In order to evaluate voltinism and emergence curves, approximately 500 parasitized eggs were preserved in falcon tubes provided with damp cotton wool and closed with the insect-proof net, under out-door condition conditions, in spring 2019. The tubes were constantly monitored; the parasitization rate and the

flight curves were calculated. Moreover, 300 parasitized eggs of *M. pruinosa* were isolated and incubated at 25°C to verify the number of egg-parasitoid emerging from each egg.

Comparison between the parasitization rate of *Neodryinus typhlocybae* and *Oligosita collina* group on *Metcalfa pruinosa*

The study was carried out in eight sites in Turin province during 2019 (see Fig. S6 and Tab. S9 in Supplementary material). Each site was subdivided into subunits according to a stratified sample. The nymphs were counted for a length of time of 5 minutes in each subunit. During the previous winter two-year-old grapevine canes, and twigs of other tree species were randomly collected in each subunit for an amount of 10 kilos of wood per site. Cane sections hosting eggs were investigated and incubated as previously described. The parasitization rate of *N. typhlocybae* in each site and each subunit was calculated as follow: a first count on first generation cocoon at the end of July when the species shows its peak in northern Italy (Alma *et al.* 2005), and a second count on the overwintering cocoons at the beginning of September.

Statistical analysis

Data analyses were conducted in R version 3.5.2 (R Core Team 2018). *Metcalfa pruinosa* eggs parasitization was analyzed by Generalized linear mixed model (GLMM) with a logit-link and assuming a logistic distribution for the error of the latent variable. The analysis was conducted using the `glmer` function in the `lme4` package in R (Bates *et al.* 2015). The response, originally recorded as parasitization success or failure (parasitization rate was calculated by counting parasitoids and hosts emerged and calculating the ratio between “parasitoid” and “total of emerged insects”), was linearly modelled using the log odds transformation. The fixed effects included in this model were Plant species, Host eggs density, Plant cover and Year together with some interaction between them. Specifically, the interaction between Plant species and Plant cover, between Plant species and Host eggs density, between Year and Plant species, and finally among

Plant species, Host eggs density and Plant cover were considered. Year (two levels), Plant species (*Vitis* spp., *C. avellana*, *Cornus* spp.) were modelled as categorical variables whilst standardized Plant cover and standardized Host eggs density was used as continuous variables. GLMM was fitted using Site nested within Area as random effects to take into accounts the dependencies among measures in each Area. The analysis unit of the Host egg density calculated in this survey, represents an aggregated data for each site (the sampling took place on a specific quantity of wood collected per site, but not on a specific number of plants determined in advance.) Therefore, the spatial scale (*sensu* Dungan *et al.* 2002) of host density was not comparable to a grain size of plant but rather to a larger grain size like the site scale. Furthermore, the effect on area extent could not be calculated since areas were too large to be treated as patch (Stiling 1987).

The best subset selection method was used to select the best model for this purpose: all possible models are compared using a subset of aforementioned predictors (Hastie 2009). Log-Likelihood ratio test (LRT) (Neyman and Pearson 1933) and the Akaike's information criterion (AIC) was used to evaluate the candidate models, the selected model was chosen also according to the best results interpretability.

Based on the results of the chosen model, a further model to inspect the influence of the variables Year, Plant species and Plant cover on *M. pruinosa* oviposition was proposed. Therefore, a GLMM with a negative binomial distribution for the response was performed using the `glmer.nb` function in the `lme4` package in R. This distribution was preferred over other models based on the comparison of the AIC values. The response variable was number of *M. pruinosa* eggs per plant in each site. The model included Year (two levels), Plant species (three levels), Plant cover (continuous variable) and the interaction between Plant species and Plant cover and between Plant species and Year as fixed effects; Site nested within Area was considered as random effect. Year was considered as a fixed factor since the number of levels was not sufficient to consider it as a random factor (Crawley 2002; Bolker *et al.* 2009). In order to consider the number of plants on

which the wooden pieces were collected, an offset variable was included. As described for the previous model, the model was chosen by means the best subset selection method.

LRT test was used to verify the significance of the fixed effects and the overall significance of the model, comparing the fitting model to "null" models (including only the random effects). Full model results and overall significance tests for models are presented in supplementary material.

For the selected model chosen by AIC, the residuals were examined using the function `testUniformity()` from the DHARMA package (Hartig 2020) (see Fig. S8 in supplementary material). Marginal means and contrast were also estimated using the `emmeans` package (EMMs) (Lenth 2020) and graphically depicted in Figs. 10 and 11.

Concerning the survey on *N. typhlocybae* and *O. cf collina* parasitization, three variables have been taken into account: the number of *M. pruinosa* eggs/nymphs parasitized and not parasitized; Parasitoid species (two levels) and Site (eight levels). Two hypotheses were tested: marginal independence, namely if Parasitization and Species are independent; conditional independence: if their relationship can be explained by Site, specifically if exist the independence of the variables Parasitization and Species given the variable Site. The hypothesis of marginal independence between Species and Parasitization variables ignoring the Site variable was verified using the chi-square (χ^2) test. The Cochran-Mantel-Haenszel test for 8 partial 2x2 contingency tables controlled for Site was performed to verify the independence of the Parasitization and Species variables given by the Site variable (conditional independence model).

4.1.3. Results

Survey on *Metcalfa pruinosa*, *Scaphoideus titanus* and *Orientus ishidae* egg parasitoids complex and host parasitization specificity

During 2017, a total of 343 *M. pruinosa*, 746 *S. titanus* and 134 *O. ishidae* nymphs were counted. From the incubation of parasitized eggs of *M. pruinosa*, two species of egg parasitoids emerged: a total of 191 females and 157 males which belonged to *Oligosita collina* group (Hymenoptera: Trichogrammatidae) (*Laudonia, in litteris*) (*sex ratio* 1:0.9; females to males) hereafter named as *O. cf collina*, and 127 females of *Centrodora livens* (Walker) (Hymenoptera: Aphelinidae). No egg parasitoids emerged from the incubation of the eggs of *S. titanus* and *O. ishidae*. Only two old apparently parasitized *S. titanus* eggs, each one with a jagged hole, were observed during the inspection of grapevine canes.

As regards to the host parasitization specificity trial, in 70% of sleeve-cages with *M. pruinosa* eggs emerged specimens of *O. cf collina* while no specimens of *C. livens* were observed. No egg parasitoids emerged from canes with eggs of *S. titanus* and *O. ishidae*.

Parasitisation rate, voltinism and emergence curves

During winter 2017/2018, the parasitization rate was 41 % \pm 6.05 (mean \pm standard deviation) in Area 1 (median = 41%; interquartile range IQR, 40 – 44; *sex ratio* 1:1.4), 44.6 % \pm 5.80 in Area 2 (median = 46%; IQR, 44 – 48; *sex ratio* 1:1.9). During winter 2018/2019, the parasitisation rate was 39 % \pm 19.3 in Area 1 (median = 40%; IQR, 37 – 53; *sex ratio* 1:1.6), 36.7 % \pm 17.3 in Area 2 (median = 39%; IQR, 20 – 47; *sex ratio* 1:1.2).

Metcalfa pruinosa eggs parasitization model

The selected model included the fixed predictors Host egg density, Plant cover, Plant species and the interaction between Host egg density and Plant cover. While no significant differences (using $\alpha < 0.05$) in the parasitization percentage

concerning the predictor Year, the interaction between Year and Plant species, the interaction between Plant species and Plant cover, and the interaction among Plant species, Plant cover and Host egg density were found (see Tabs. S10, S11 in Supplementary material). Therefore, the latter predictors were dropped from the chosen model. The selected model featured the best residual diagnostic among the top five models of the best subset selection. This model indicated that when the factor Plant species referred to *Cornus* and the value is 0 for the factors Host egg density and Plant cover, the baseline odds of the parasitization rate is 0.34. The baseline odds decrease by 25 % when the Plant species is *Corylus* and increase by 30% when the Plant species is *Vitis*. All else being equal, a unit change in Host egg density is associated with a 68% increase in the odds of being parasitized. *Vice versa*, when the value of Host egg density is 0, a unit change in Plant cover is associated with a 33% increase in the odds of being parasitized. The interaction term shows that the odds ratio of Host egg density was estimated to increase by a multiplicative factor of 1.11 for each extra unit of Plant cover, with an increment of 88% in the odds of being parasitized.

From Fig. 8 one might suppose that the parasitization rate at high values of Host egg density was greater at higher levels of Plant cover than at lower levels for all plant species. Whilst it might be deduced that in correspondence with the minimum values of the Host egg density both at higher and lower levels of Plant cover, no differences in the odds of parasitization rate between different Plant species existed.

The pairwise comparisons show that the marginal means of the parasitization rate was higher on grapevine compared to the other two plant species, and in *Cornus* was higher than *Corylus* (Fig. 9).

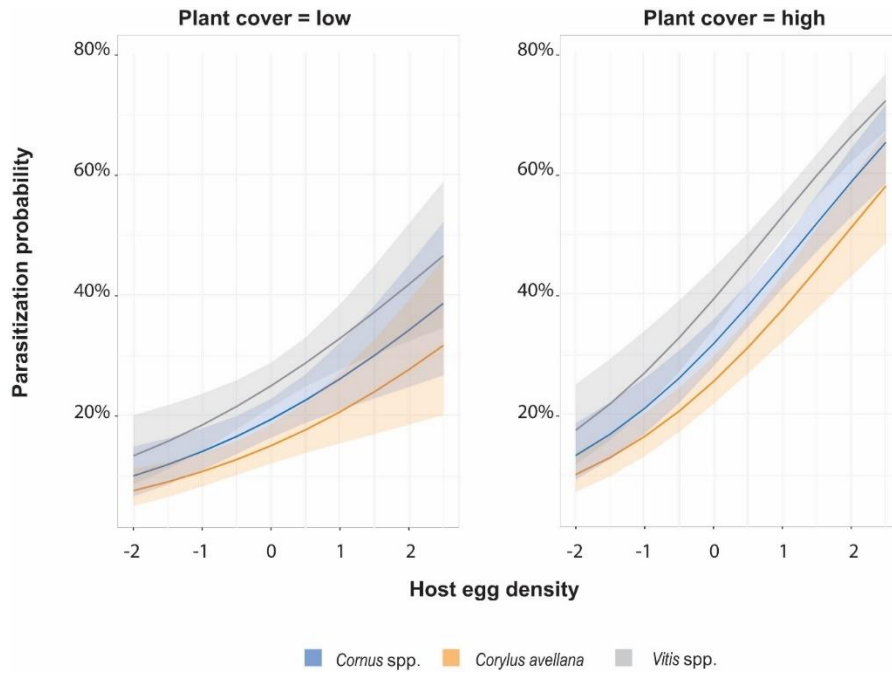


Figure 8. Predicted probability of egg parasitization for three Plant species, with respect to standardized Plant cover and standardized Host egg density (number of *M. pruinosa* eggs per Plant species in each site). The percentage of Plant cover was modeled as continuous variable. Two representative values of high and low level of the standardized distribution depicted by one standard deviation below and above mean value are reported in this graph. Lines represent the logistic fit, shading the 95% confidence interval. The probability of being parasitized increased with either the host density and the Plant cover. Model coefficients are provided in the supplementary material.

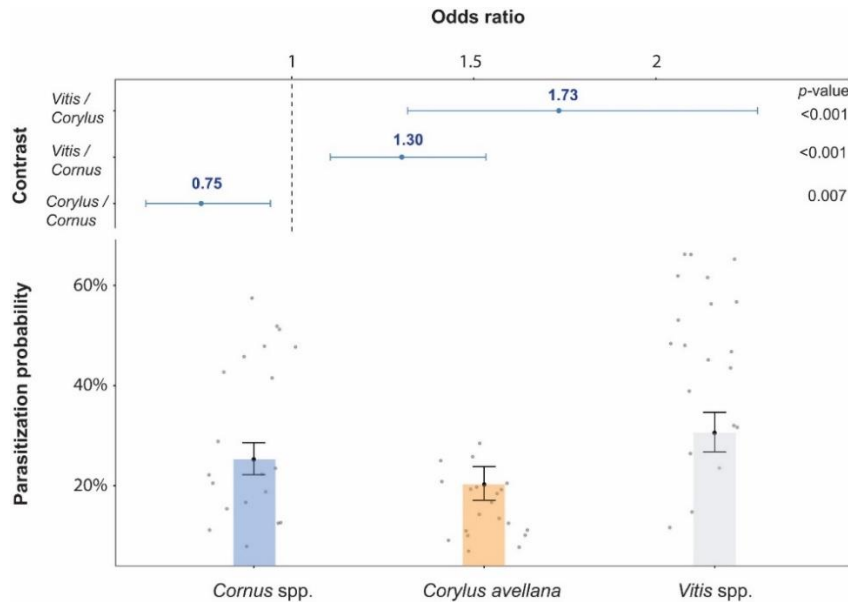


Figure 9. Top: the y-axis shows all pairwise comparisons among the three Plant species levels, while the x-axis represents the response which is the Odds ratio of the means of the two groups of Plant species in the comparison (p-values from the hypothesis tests are included on the right). Bottom: the estimated marginal means of the parasitization probability on the three plant species are represented. Data are presented on the back-logit scale with 95% confidence intervals (CI). The raw data are plotted as grey dots. Model coefficients are provided in the supplementary material.

Metcalfa pruinosa oviposition model

Concerning the model of *M. pruinosa* oviposition rate among plants, the final best-fit model included only the fixed factors Plant species and Plant cover whilst their interaction was not a significant predictor, and was dropped from the final selected model (see Tabs. S12, S13 in Supplementary material). When the factor Plant species referred to *Cornus* and the value is 0 for the factor Plant cover, the baseline rate of the oviposition is 8.1. It decreases by 31% when the Plant species is *Corylus* and increase by 32% when the Plant species is *Vitis*. All else being

equal, a unit change in Plant cover is associated with 25% increase in the rate of oviposition.

The pairwise comparisons show that the oviposition rate of *M. pruinosa* was greater on grapevine than the other plants, whilst in *Cornus* was higher than *Corylus* (Figs. 10, 11)

These models show that the parasitization depends on the number of eggs laid by *M. pruinosa*, which varies on different plant species.

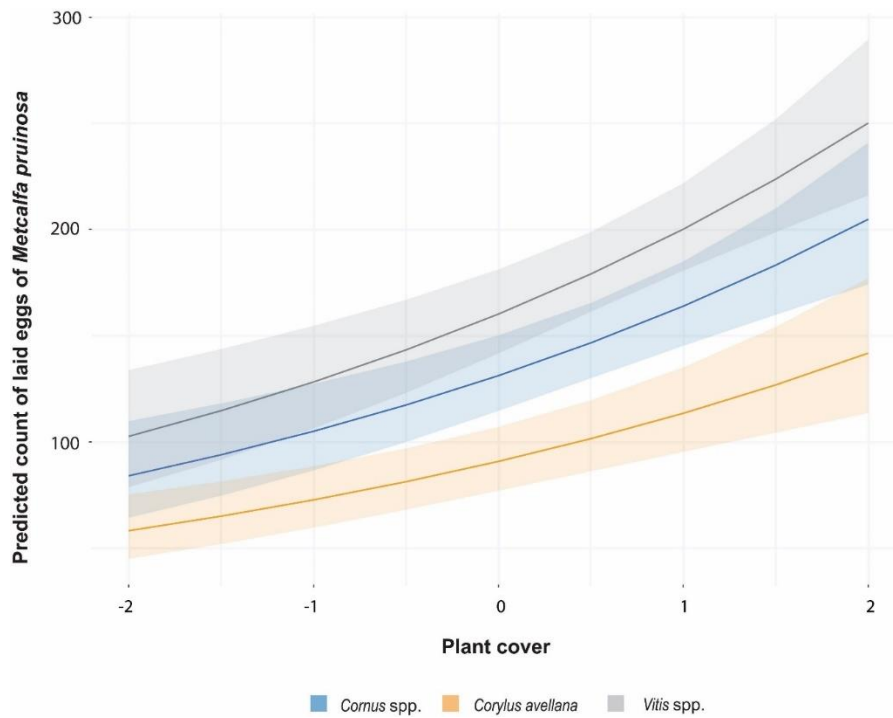


Figure 10. Curves of *Metcalfa pruinosa* oviposition on three Plant species as a function of the standardized variable Plant cover. Lines represent the Poisson negative binomial fit, shading represents the 95% confidence interval. The oviposition rate increased with Plant cover but differently according to the Plant species.

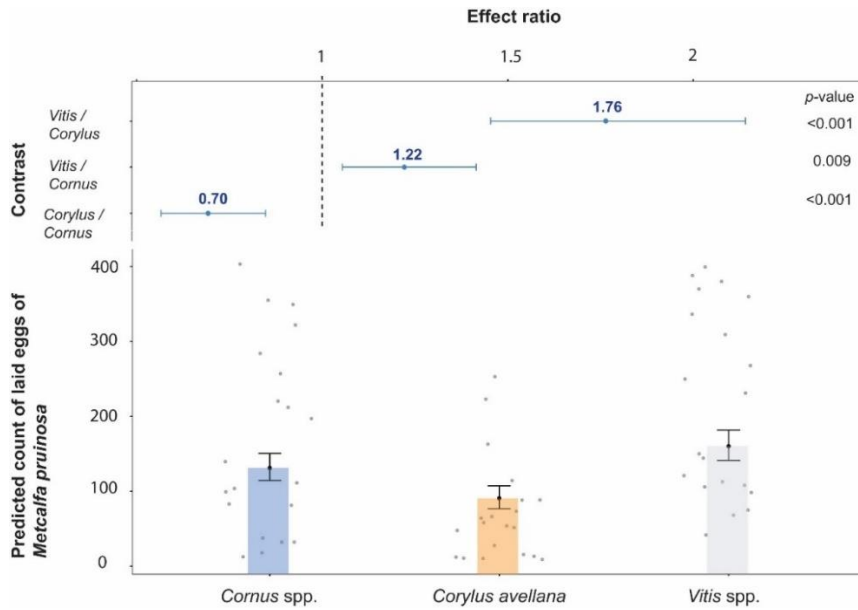


Figure 11. Top: the y-axis shows all pairwise comparisons among the three Plant species levels, while the x-axis represents the response which is the ratio of the means of the two groups of plant species in the comparison (p-values from the hypothesis tests are included on the right). Bottom: the estimated marginal means of *Metcalfa pruinosa* oviposition on the three Plant species. Data are presented on the back-Poisson negative binomial scale with 95% confidence intervals (CI). Model coefficients are provided in the supplementary material.

Voltinism and emergence curves

Concerning the *O. cf. collina* flight curves, in 2019 two peaks during the season were observed. The first specimen was collected on April 24th with the highest peak on May 15th and then ended June 10th. The second curve began July 19th with the highest peak on July 27th and ended on August 8th (see Fig. S7 in Supplementary material).

Comparison of the parasitisation rate of *Neodryinus typhlocybae* and *Oligosita cf collina* on *Metcalfa pruinosa*

The odds are significantly higher for *O. cf collina* than *N. typhlocybae* in seven sites out of eight (Figure 12). The parasitization rate of *N. typhlocybae* ranged from 18 % to 32 % (see Tab. S14 in Supplementary material). As regard the hypothesis of marginal independence of parasitoid species and *M. pruinosa* parasitization rate, the null hypothesis is refused using reasonable levels of significance ($\chi^2 = 168.7$ $df = 1$, p -value < 0.0001). The odds of parasitization *O. cf collina* are 2.01 (95% CI 1.81 - 2.23) times as high as *N. typhlocybae*. This relationship cannot be explained by the influence of the variable sites, in fact, parasitoid species and parasitization rate are not independent to the given sites (Mantel-Haenszel $\chi^2 = 160.75$ $df = 1$, p -value < 0.0001). Mantel-Haenszel estimate of the common odds ratio is 1.98 (95% CI 1.78, 2.21).

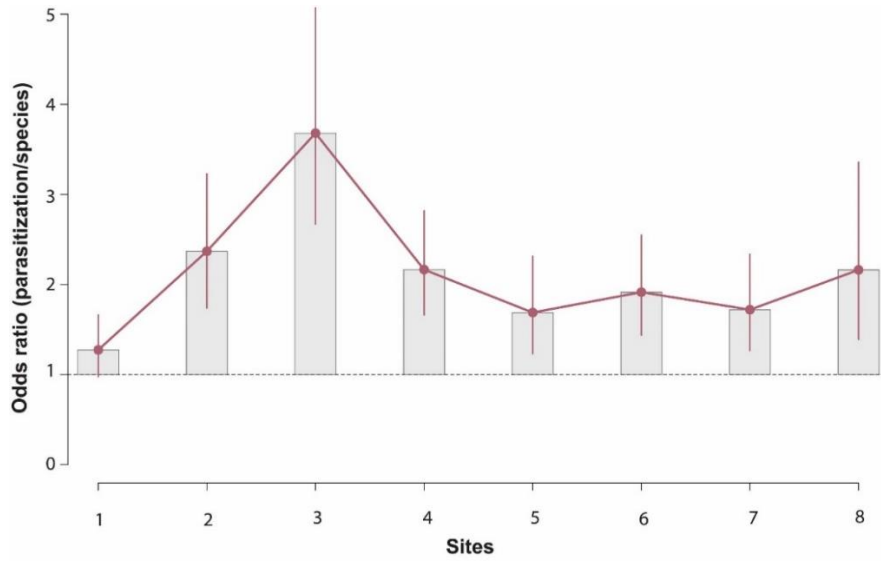


Figure 12. Odds ratios for parasitization and sites. Data are presented with 95% confidence intervals (CI). The highest value of odds ratio is found in site 3, where the odds of being parasitized by *Oligosita cf collina* are 3.68 times higher than *Neodryinus typhlocybae*. Only in site 1, where the odds of parasitization by the egg parasitoid increase of 27% compared to Dryinid, the comparison was not significant.

4.1.4. Discussion

Insect parasitoids spend most of their limited time in searching for a suitable host to reproduce. This activity is particularly arduous for egg-parasitoids since their immobile, and often hidden hosts, are hard to be located from a distance (Vinson 1998; Fatouros *et al.* 2008; Colazza *et al.* 2009). Egg-parasitoids have evolved the ability to detect host's volatile signals (Conti and Colazza 2012; Hilker and Fatouros 2015; Hilker and Meiners 2006), and usually show a wide host range (Chantarasa *et al.* 1984; Conti *et al.* 2004; Mansfield and Mills 2002). The presence of a new exotic potential host can lead to novel interactions and adaptation (Harvey 2015; Martorana *et al.* 2017).

Our results highlight that no egg parasitoids emerged from the rearing of *S. titanus* eggs as well as of *O. ishidae* eggs. Some Authors had sporadically observed egg parasitoids able to parasitize eggs of *S. titanus*, with a low parasitization rate (Malausa *et al.* 2003; Arzone and Alma 1994). The only two apparently parasitized eggs observed during this research, allow us to confirm those results and state that the *S. titanus* egg parasitization is uncommon. As far as *O. ishidae* is concerned, it is worthwhile to notice that fewer eggs of this leafhopper are found compared to the other two pests. Besides, it is known that *O. ishidae* lays eggs on grapevine in considerably lower number compared to other plants (Lessio *et al.* 2019), and the grapevine was the most abundant species plant in the vineyard agroecosystem. Therefore, we cannot state with certainty that no egg parasitoids were able to adapt on this leafhopper.

As regards *M. pruinosa*, at least two species of egg parasitoids have adapted to the Flatid, namely *O. cf. collina* and *C. livens*, with a clear predominance of the former. The latter was reported as *M. pruinosa* eggs parasitoid in a previous study without a specific parasitization rate (Raspi and Canovai 2003; Conti *et al.* 2004). The genus *Centrodora* Forester is considered cosmopolitan and includes primary and secondary parasitoids which develop on insects belonging to different orders (Viggiani 1994).

Many genera belonging to the sub-family Oligositinae are known to parasitize insects belonging to different orders including Diptera, Coleoptera, Orthoptera and Cicadellidae (Pinto and Viggiani 2004; Pinto 2006; Noyes 2019). The genus *Oligosita* Walker was redescribed by Pinto and Viggiani (2004). Lately, the systematic contribution in describing new *Oligosita* species has increased (Begum and Anis 2015; Bella *et al.* 2015; Ikram and Yousuf 2016; Liu and Li 2019); however, for *O. collina* group, as defined by Nowicki (1937) and Viggiani (Viggiani 1976), many species need to be identified yet, and their taxonomic position and associations with hosts found out. The association here described represents a new biocoenosis involving the exotic planthopper and *O. cf collina*. Indeed, several studies, indirectly concerning the possible adaptation of egg parasitoids to *M. pruinosa*, have never observed such a high parasitization rate (Conti and Bin 1999; Raspi and Canovai 2003).

The knowledge of biological traits as voltinism and parasitization rate of genera non-*Trichogramma* within the Trichogrammatidae is still limited (Nagarkatti and Nagaraja 1977; Noyes 2019). Maybe due to the different data collection methods, many other cases of egg parasitism among Oligositinae show different field parasitization rates. For instance, many species within Oligositinae were able to exert a high level parasitization rate on their hosts: the complex *Pseudoligosita aesopi* (Girault) and *Oligosita naias* Girault reached a maximum of 47% on *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae) (Watanabe 1992), and *Paracentrobia tapajosae* Viggiani exhibited a parasitization rate of about 45% on *Dalbulus maidis* (DeLong and Wolcott) (Moya-Raygoza *et al.* 2012; Moya-Raygoza *et al.* 2014). Other species of Oligositinae showed an interesting potential biological pest control (Logarzo *et al.* 2004; Sann *et al.* 2018; Bosco and Arzone 1991). In spite of these few examples, many Oligositinae species showed a negligible parasitization rate (Moya-Raygoza *et al.* 2012; Watanabe *et al.* 1992; Virla *et al.* 2019). The parasitization rate of *O. cf collina* on *M. pruinosa* (an average of more than 40 % in the survey areas and over 50 % in some sites) is similar to the parasitoid complex with the highest level of parasitization and,

being a new association, can be deemed remarkable. The parasitization rate was high in both years, but it is reasonable to expect a reduction of these values in consideration of the host-parasitoid dynamics trend of (Paparella *et al.* 2016; Hassell 2000).

The high parasitization level exhibited by *O. cf collina* allows us to affirm that the egg parasitoids spread in Piedmont and that a stable association with *M. pruinosa* in this region exists. Even if further studies are needed to confirm it over the years. From the results of the models, it can be deduced how *O. cf collina* is able to parasitize *M. pruinosa* eggs on plants of different species. The parasitization rate is mainly related to the density of host eggs and the abundance of plants that can be used as a substrate of oviposition.

Regarding the attraction due to the density of the host eggs, some studies on plant-leafhoppers have reported a different density-dependent relationship between host density and parasitization rate (Stiling 1980; Segoli 2016; Torres-Moreno and Moya-Raygoza 2020). However, the dynamics and the fitness factors behind this relation have not been proven yet. The preliminary results of this study reveal a positive effect of the host density on the parasitization rate. The host density calculated in this study refers to the density of eggs per plant species at a given site, while no multi-scale analysis was performed. Therefore, it is not comparable to a finer grain size like leaf as reported in some of the aforementioned studies. Moreover, Segoli (2016) observed a positive dependence at a grain size called field scale. Our results, referring to a site grain size, are in agreement with this conclusion. According to some Authors, if the density is evaluated at finer scales, the relationship with the parasitization tends to become zero or negative, since oviposition activity responds to other factors barely quantifiable and not fully correlated with fine-scale host density, and can increase with the observational grain size (Stiling 1987; Walde and Murdoch 1988). Nevertheless, in a multi-factor meta-analysis study, Gunton and Pöyry (2016) found limited and ambivalent support for the scale-specific foraging hypothesis. More in-depth

multi-scale studies are needed to clarify these aspects in this new biocoenosis on a finer grain size.

Concerning the reasons behind the positive dependence observed in this study, *O. cf collina* could be attracted by the exudates or honeydew produced by the *M. pruinosa* (Wilson and Lucchi 2007), as already known for other parasitoids (Desouhant *et al.* 2005). This secretion is another essential factor that can act as a contact-kairomone as reported for several insects, including plant-leafhoppers (Vinson 1978; Denno and Roderick 1990; Shaltiel and Ayal 1998). As reported in previous studies, egg parasitoids belonging to other genera of Trichogrammatidae respond to volatile compounds produced by or associated with host eggs, including scales from adult moths, sex pheromones, and compounds related to egg metabolic processes (Boo and Yang 2000; Jones *et al.* 1973; Morrison *et al.* 1980).

Mectalfa pruinosa is polyphagous and might feed and oviposit on the most prevalent plant species. Therefore, on these plants, the oviposition rate is likely to be higher as well as the consequent parasitization rate. In this research, the parasitization rate was greater on grapevine, both in case of lower and higher plant cover, than on the other plant species. It could be assumed that this Flatid behavior may be promoted by the characteristic bark of grapevine and by its growth habitus in the wood, even if this potential preference should be clarified and demonstrated. Nevertheless, in the absence of evidence pointing out the involvement of semiochemicals, we cannot exclude that the higher values of the parasitization rate on *Vitis* spp. is ascribable to synomones (Colazza *et al.* 2004; Chiappini *et al.* 2012; Lou *et al.* 2005; Moya-Raygoza 2020), as well as a major oviposition rate of *M. pruinosa* on the grapevine.

Another important factor in a biological control perspective concerns the number of generations that the parasitoid is able to perform on the host. Results on the voltinism trial reveal that the first adults emerge in the middle of April. During the adult emergence in April, no new host eggs were available while some *O. cf collina* specimens were collected at the time of the first host oviposition. This

observation led us to hypothesize a second generation on *M. pruinosa* wintering eggs like *Epiligosita vera* Viggiani, which accomplishes two generations on the overwintering eggs of *Lindbergina aurovittata* (Douglas) (Bosco and Arzone 1991). If this is true, *O. cf collina* would accomplish its second generation onto the same wintering eggs and benefit from not being forced to move and search for alternative hosts. We cannot exclude the following theories: (i) a second generation onto new eggs laid by plant-leafhoppers able to develop on other plants (e.g. some Typhlocybae species (Viggiani 1987; Triapitsyn *et al.* 2013)), as already hypothesized for some egg parasitoids (Chantarasa *et al.* 1984; Cerutti *et al.* 1991; Corbett and Rosenheim 1996; Moya-Raygoza and Becerra-Chiron 2014); (ii) the specimens emerged in the second half of July could belong to a quiescent part of the overwintering population as observed in other specimens belonging to the family Thrichogrammatidae (Gardner *et al.* 2013; Özder and Sağlam 2005; Rundle and Hoffmann 2003). These hypotheses need to be deepened with further and targeted trials.

Only one specimen was observed to emerge from each egg. Therefore, the phenomenon of superparasitism in this species, meaning two or more holes per egg, was not observed like in other Oligositinae species (Triapitsyn 2003; Virla *et al.* 2009) which would obviously have altered the parasitization rate. The females emerged in April, if males are present, begin to mate in a short time, on the contrary, if mating does not occur, immediately look for the host eggs into the bark to lay eggs. The oviposition behavior observed, is similar to other Trichogrammatidae species, showing antennal tapping on the host egg, before ovipositing, and their typical abdominal vibration (Al-Wahaibi *et al.* 2005; van Dijken and Waage 1987).

Concerning the preliminary comparison of the parasitization rate between *O. cf collina* and *N. typhlocybae*, the Dryinid parasitization rate detected ranged from 18 to 35% and was not too dissimilar to the values reported in other studies (Strauss 2009; Véték *et al.* 2019), while it was rather lower than those observed by other Authors (Girolami and Mazzon 1999; Malausa *et al.* 2006) who detected

a parasitization over 70%. Our results could be explained with the possible reduction in the *N. typhlocybae* population caused by hyperparasitoids (Guerrieri and Viggiani 2005; Noyes 2019; Viggiani *et al.* 2004). On the other hand, this could be attributed to the parasitization dynamics, which can oscillate over the years (Hassell 2000; Paparella *et al.* 2016). Nevertheless, it is well known that *N. typhlocybae* can perform a simultaneous predatory action in addition to the parasitization, which can have a significant impact on the flatid population (Girolami and Mazzon 1999; Strauss 2009). Thus, we consider the parasitization rate observed in some sites, as a confirmation of the success of the Dryinid settlement and its contribution to the control of the *M. pruinosa* population. However, the comparison of the parasitization rate performed by *O. cf collina* and *N. typhlocybae* pointed out a higher rate of the egg parasitoids.

The Palaearctic origin of *O. cf collina* must be confirmed as well as the establishment of this new association in other regions of the Palaearctic area. In this regard, further studies are ongoing to identify the species and investigate its biological traits like voltinism and overwintering behavior.

4.2. NEW HOST-PARASITOID ASSOCIATION INVOLVING A CUCKOO WASP AND TWO VECTORS OF THE FLAVESCENCE DORÉE PHYTOPLASMAS: *SCAPHOIDEUS TITANUS* AND *ORIENTUS ISHIDAE*

4.2.1. Introduction

Cuckoo wasps (Hymenoptera: Chrysididae) are ancestrally ectoparasitoids and secondarily cleptoparasites (Pennacchio and Strand 2006; Pauli *et al.* 2021). Ectoparasitoid cuckoo wasps develop and nourish from their hosts' larva by feeding externally on it, typically within the host's nest or puparium. In contrast, cleptoparasitic cuckoo wasps nourish from the food that the host provided for its own offspring (Cardinal *et al.* 2010; Wurdack *et al.* 2015). However, the specific oviposition strategy of many species is still unknown, and in recent years it has become evident that some cuckoo wasp species show features of parasitoids and of cleptoparasites during their development and may even act as endoparasitoids in their first instar stage (Carrillo and Caltagirone 1970; Veenendaal 2012; Winterhagen 2015). However, only very few cuckoo wasp species have been reported to behave as endoparasitoid in their early development.

Most of chrysidid species known so far use mason wasps and honey wasps (Vespidae: Eumeninae, Masarinae, Zethinae), apoid wasps (Crabronidae, Pemphredonidae, Psenidae, Sphecidae), and mason bees (Megachilidae) as hosts. However, species within the genus *Praestochrysis* are parasitoids of slug moths (Limacodidae). Members of the subfamily Cleptinae use sawflies (Tenthredinidae and Diprionidae) as host and finally, species of the subfamilies Amiseginae and Loboscelidiinae are parasitoids of walking sticks (Phasmatodea) (Kimsey and Bohart 1991). Scattered notes on the hosts of European cuckoo wasps (Hymenoptera, Chrysididae) were summarized by Pauli *et al.* (2019). The behaviour of some European genera in the tribe Elampini (e.g., *Holopyga*, *Omalus* and *Pseudomalus*) have only recently been described (Veenendaal 2011, 2012; Pärn *et al.* 2015; Winterhagen 2015). The oviposition strategy of species

in the genus *Elampus* has remained unknown, even if some species within this genus are known to be cleptoparasites of at least two genera of the apoid wasp family Psenidae: *Mimesa* Shuckard and *Mimumesa* Malloch whose species are known to be predators of both nymph and adult leafhoppers (Hemiptera: Cicadellidae) and planthoppers (Hemiptera: Delphacidae) (Mocsáry 1889; Spooner 1948; Rosenheim and Grace 1987).

Here we report first-time evidence for an endoparasitoid behavior of the cuckoo wasp *Elampus bidens* (Förster, 1853) (Hymenoptera: Chrysididae, Elampini) and describe the association of this species to two leafhoppers introduced to Europe and with major agricultural economic importance: *Scaphoideus titanus* and *Orientus ishidae*.

Biological control of *O. ishidae* and *S. titanus* has not been effective so far because of the low impact of entomophagous insects on their population development in their native range as well as in Europe (Schvester *et al.* 1962; Alma and Arzone, 1994; Arzone and Alma, 1994; Vidano, 1966; Nusillard *et al.* 2003; Chuche and Thiery, 2014; Bocca *et al.* 2020b). However, it is known that potential enemies of invasive species in the newly invaded areas need time to adapt to the new hosts or prey (Cornell and Hawkins 1993).

We here present our results i) examining the parasitization prevalence by *E. bidens* in nymphs of *O. ishidae* and *S. titanus* in multiple sampling plots in the Piedmontese areas over the course of five years. We (ii) describe the oviposition behavior in semi-field conditions and the larval development of *E. bidens*. And (iii) we present the results from our search for the indigenous host and the hypothesized predator of the leafhoppers that *E. bidens* apparently depends on to complete its lifecycle.

4.2.2. Materials and Methods

The field research was carried out over five years (2015-2019): The first three years were used to assess the possible Palaearctic parasitoids adaptation to *S. titanus* and the parasitization rate in Piedmont region. Based on these results, the following two years were used to sample additional leafhopper species in regions of Piedmont and Lombardy and to narrow down the range of possible leafhopper predators that *E. bidens* seemingly depends on for completing its life cycle. A preliminary study in semi-field conditions upon the larval development and the parasitic behaviour of *E. bidens* was conducted during the year 2020.

Scaphoideus titanus parasitization rate

The survey on the parasitization rate of *S. titanus* was conducted in Canavese and Monferrato Astigiano (Piedmont region, Northwestern Italy) from 2015 to 2017.

During 2015, a preliminary survey was carried out to investigate the possible adaptation of Palaearctic parasitoids to *S. titanus* in six sites located in the provinces of Turin and Asti. Based on these preliminary results, a new experimental design was set-up to study the variation in the parasitization rate between areas and over the years. This survey was conducted in three zones located in Piedmont region from 2016 to 2017. Two zones were in Canavese (zone 1 and zone 2) and one in Monferrato Astigiano (zone 3) (Fig.13). Monferrato zone is a rural region with high density of conventional vineyards with some patches of uncultivated zones or brushwood with wild grapevine. Vineyards cover 11% of the territory. Other main land uses include oak (*Quercus robur*), chestnut (*Castanea sativa*) and blacklocust (*Robinia pseudoacacia*) groves/forests (22%), hazel-nut orchard areas and other fruit crops (5%), arable lands (37%), grasslands and pastures (7%) and urban areas (7%). Canavese is a rural region characterized by a less intensive viticulture than Monferrato. Here vineyards cover only 2% of the territory. Other main land uses include oak (*Quercus robur*), chestnut (*Castanea sativa*) and blacklocust (*Robinia pseudoacacia*) groves/forests (37%), arable lands (31%), grasslands and pastures

(3%) and urban areas (7.5%). The climate of Astigiano and Monferrato belongs to type Cfa (temperate, without dry season and with hot summer), in terms of Köppen–Geiger's classification (Peel *et al.* 2007). In Astigiano, during the last five years, annual precipitation ranged from 426 to 803 mm with minimum values in July, December and January and with a maximum peak in May-June and October-November. Total annual rainfall averaged 648.5 mm, while the mean annual temperature was 12 °C (Asti climatic station, 123 m a.s.l.). In Canavese, during the last five years, annual precipitation ranged from 620 to 1017 mm with minimum values in December, January and September and with a maximum peak in April and October. Total annual rainfall averaged 875.3 mm, while the mean annual temperature was 12.6 °C (Caluso climatic station, 303 m a.s.l.). Zone 1 presented vineyards alternated between amateur and professional, while zone 2 has a prevalence of hobbyist vineyards.

Five sites per zone were chosen in uncultivated areas or woods where wild grapevine was present. For each site a surface of about 1500 - 2000 m² extent was sampled. Adult specimens of *S. titanus* were collected by sweep net every ten days, between the beginning of July (July 20) and the middle of October (October 20), for 3 minutes per plant per sampling site in each zone (N=10 plants per site). In order to assess the capability of the endoparasitoid larva to emerge from their host body, all the field collected specimens were reared into small cages (insect-proof polyethylene and nylon mesh 20 x 20 x 40 cm high) with a grapevine plant inside, according to the collection date, till their death and subsequently dissected by means of micro-pin-needles in the laboratory.

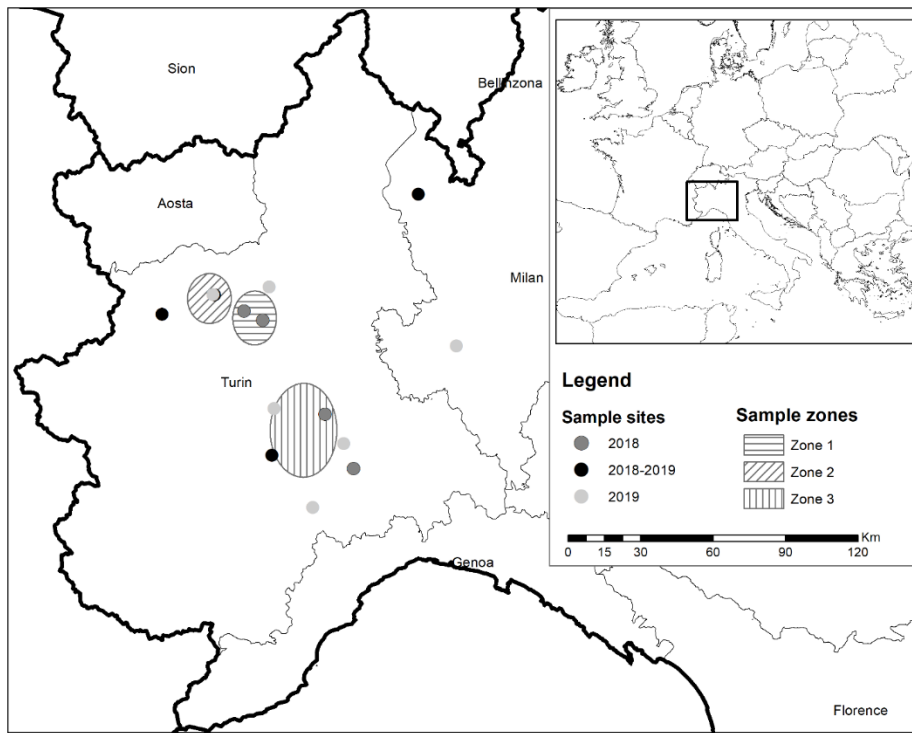


Figure 13. Study area and location of sample zones (line fill symbol areas) and sites (dots) sampled in 2018 (dark grey), 2019 (light grey), and both in 2018-2019 (black).

Survey on *Elampus bidens* association in the field

The study concerning our search for the indigenous host and the hypothesized predator wasps of the leafhoppers was carried out in different sites situated in some important Piedmontese vine-growing areas and some sites in Lombardy during 2018 and 2019 (see Tabs. S15, S16 in the supplementary material). These areas were characterized by agricultural lands, with high density of vineyards, uncultivated areas with wild grapevine and wood patches, mixed oak-forest, and riparian forest. Leafhopper nymphs and adults were collected by a sweep net in 15 days intervals covering a surface about 20 m in diameter, for 1 hour each location. The vegetation was sampled along its height till 3 m. Malaise traps were

also used to survey leafhopper predator wasps and *E. bidens* adults in 2018. Five traps were placed at four localities (Tab. S16), using 70 % ethanol as killing agent. The traps were checked every 15 days starting from the first of June until the end of October. We also collected wasps using a butterfly net in 2018 and 2019. Ten collecting spots, each one sampled for 30 minutes, were chosen in a linear transect at distance of 15 m from each other, per locality.

COI DNA barcoding of parasitoid larvae

We inferred the identity of parasitoid larvae found in *O. ishidae* and *S. titanus* with the COI DNA barcoding approach (Hebert *et al.* 2003). DNA was extracted from the tissues of parasitoid wasp larvae as described by Pauli *et al.* (2019). PCRs were performed as outlined by Pauli *et al.* (2019), applying the oligonucleotides LCO1490-JJ (5'-CHA CWA AYC ATA AAG ATA TYG G-3') and HCO2198-JJ (5'-AWA CTT CVG GRT GVC CAA ARA ATC A-3') (Astrin and Stüben 2008) or *Omalus*-F (equimolar mix of 5'-TTC GAC AAA CCA TAA GGA TAT TGG-3', 5'-TTC TAC AAA TCA TAA AGA TAT TGG-3', 5'-TTC AAC AAA TCA CAA GGA TAT TGG-3', 5'-TTC GAG TAA TCA CAA AGA TAT TGG-3', 5'-TTC AAC TAA TCA TAA AGA TAT TGG-3') and *Omalus*-R (equimolar mix of 5'-AGA AAT TAT TCC AAA ACC AG-3', 5'-CGA AAT TAT CCC AAA CCC AG-3', 5'-AGA AAT TAT TCC AAA TCC AG-3', 5'-AGA AAT TAT ACC AAA ACC AG-3') (Niehuis, present study) to prime the reactions. PCR products were cleaned with the Illustra ExoProStar Kit (GE Healthcare Life Sciences, Garching, Germany) and then sent for Sanger sequencing to Macrogen (Amsterdam, The Netherlands). Forward and reverse stands were assembled with the software Geneious version 10.2.3 (Kearse *et al.* 2012). The same software was used to compare the COI DNA sequences of the parasitoid larvae with COI DNA barcode sequences of various *Elampus* species (i.e., *E. bidens* [KY430834.1], *E. constrictus* [AJ514367.1; note that the sequence was submitted as referring to *E. panzeri*], *E. foveatus* [KY430833.1], *E. panzeri* [KY430835.1], and *E. spina* [transcriptome shotgun assembly

GBWX0000000.1] published by Niehuis and Wägele (2004) and Pauli *et al.* (2019). We sequence the DNA of in total 13 larvae. We additionally sequenced COI DNA barcodes of two adult *E. sanzii* to rule out the remote possibility that the barcoded parasitoid wasp larvae belong to this species (Tab. S17).

Larval development

During 2020, the interaction among leafhoppers and *E. bidens* was investigated using rearing cages (insect proof polyethylene and nylon mesh 40 × 40 × 100 cm high) in out-door conditions with one potted healthy wild grapevine plant (Kober 5BB) and one potted hazelnut tree (*Corylus avellana* L.) to feed the insects. Female cuckoo wasps (N=12) were collected in the first ten days of July from field in some localities where both leafhopper species were present. They were introduced individually into the cages. Third instar nymphs and adults of *S. titanus* and of *O. ishidae* (N=15 nymphs, and N=10 adults for each species) were put into each cage. A diet consisting of honey and pollen was given the cuckoo wasps in addition to a solution of water-honey daily sprayed onto the leaves. Parasitization behaviour was monitored daily. In order to assess differences in larva development between the two leafhopper species and a preference in the parasitization by *E. bidens*, records of successful host detection were kept. A host detection was recorded as successful when the searching female approached and examined the leafhopper, then attacks it by clamping the host like a ball for at least 2-3 seconds. The successful parasitization can be easily detected after dissection by finding an *E. bidens* egg. After parasitization, parasitized leafhoppers were individually stored in cylindrical plexiglass cages (diameter 15 cm, height 25 cm) with a leaf of grapevine or hazelnut for feeding. To determine the larval development, three nymphs of each species (N=6 for each parasitization cohort) were dissected every two days starting 24 hr after parasitization until the death of the last specimen (about 30 days). Leafhoppers were killed with ethyl acetate in the lab before dissection. The specimens were dissected into a phosphate buffered saline (PBS) solution onto a microscope slide (25 × 75 × 1

mm). Larvae presence were assessed by dissecting each leafhopper specimen under a stereomicroscope. The abdomen was separated from the rest of the body using a couple of needles.

Statistical analysis

SCAPHOIDEUS TITANUS PARASITIZATION RATE

Scaphoideus titanus parasitization rate was modelled using Generalized Linear Mixed Models (GLMM) using the package glmmTMB (version 1.0.2.1; Brooks *et al.* 2017) publicly available for the statistical software R version 4.0.3 (R Core Team 2020). Specifically, we used a logit-link and assumed a logistic distribution for the error of the latent variable. The response, originally recorded as parasitization success or failure, was linearly modelled using a log odds transformation. The fixed effects included in this model were *zone* and *year* and with their interaction. *Year* (two levels), *zone* (three levels) were modelled as categorical. *Year* was considered as a fixed factor as there are insufficient levels of the factor in the data on which to base an estimate of the variance of the population of effects (Crawley 2002; Bolker *et al.* 2009). GLMM was fitted using *site* as random effects. Given the data collected, we decided to check if the variable “data” (time interval in which the leafhopper samples were collected) could be included as fixed effects. To evaluate this, we rely on the comparison with the BIC index for the two models (an index who takes in to account the log-likelihood of the model and a penalization term for the number of parameters used, see Tab. S18 in Supplementary material), from which we choose the smaller one. Moreover, also from the diagnosis of the model residuals (Figs. S9, S10) the model chosen seems preferable.

For the selected model chosen by BIC, the residuals were examined using the function `testUniformity()` of the DHARMA package (version 0.3.3.0; Hartig 2020) (Fig. S9) in the R statistical software. Marginal means and contrast were additionally estimated using the emmeans package (version 1.5.3; Lenth 2020).

When comparing the parasitization rate between *S. titanus* and *O. ishidae*, three variables were taken into account: the *number of leafhoppers* parasitized and not parasitized; *leafhopper species* (two levels) and *site* (six levels). The analysis was conducted separately for each year. Two hypotheses were tested: marginal independence, namely whether or not *parasitization rate* and *leafhopper species* are independent; conditional independence: whether the relationship between *parasitization rate* and *leafhopper species* can be explained by the *site*, specifically if, given the variable *site*, the parasitization rate is different for different leafhopper species (specifically, given the variable *site*, if the independence between *parasitization rate* and *leafhopper species* exists). The hypothesis of marginal independence between *species* and *parasitization rate* variables ignoring the *site* variable was verified using the chi-square (χ^2) test. The Cochran-Mantel-Haenszel test for six partial 2 by 2 contingency tables controlling for *site* was performed to verify the independence of the *parasitization rate* and *Species* variables given by the *Site* variable (conditional independence model).

To compare the proportion of parasitized *S. titanus* with the proportion of parasitized *O. ishidae* reared in semi-natural conditions was used the chi-square (χ^2) test.

4.2.3. Results

Scaphoideus titanus parasitization rate

During the preliminary survey carried out in 2015, a total of 364 *S. titanus* specimens were collected, 19% of which carried an endoparasitoid larva inside. The larvae were identified as *E. bidens* (Hymenoptera, Chrysididae) by means of a DNA comparison between the adults and the larvae. The data obtained during the first-year survey were not taken into account to model the parasitization rate.

During 2016, a total of 1,168 *S. titanus* specimens were collected and the parasitization rates recorded were: $23.2\% \pm 5.5$ (raw mean \pm standard deviation) in *zone 1*, $27\% \pm 6$ in *zone 2*, and $8\% \pm 14$ in *zone 3*. In *zone 1*, the parasitization rate peak was $28.7\% \pm 20$ (September 20), in *zone 2* it was $36.8\% \pm 12$ (August 10), and in *zone 3* it was $11.3\% \pm 14$ (July 20). In *zone 1* and *zone 2*, the endoparasitoid larvae were found till the beginning of October, in *zone 3* till the end of September.

During 2017, a total of 1,323 *S. titanus* were collected. The parasitization rates were: $3.7\% \pm 3.9$ in *zone 1*, $24\% \pm 5.3$ in *zone 2*, and $6.3\% \pm 9$ in *zone 3*. In *zone 1*, the parasitization rate peak was $7.85\% \pm 11$ (September 10), in *zone 2* it was $36\% \pm 14$ (July 20), and in *zone 3* it was $8.48\% \pm 14$ (September 30). As in 2016, the larvae were found till the beginning of October both in *zone 1* and in *zone 3* and till the end of September in *zone 2*.

Based on the experiment design we choose to rely on generalized mixed linear models, where the sites are treated as random effects. Therefore, the variables “years” and “zone” and their interaction are considered as fixed effects, meanwhile the variability in sites is considered as random factor. In particular, the data generating process is assumed as follow: “*Log odds of being parasitized* \sim *year*zone+(I|site)*”. The selected model included the predictors *year* and *zone* and their interaction. The results indicate that when the model referred to *year* 2016 and *zone 1*, the baseline odds of the parasitization is 0.44. The baseline odds

decreased by 86% during 2017 and by 96% in *zone 3* and decreased by 18% in *zone 2*, compared to *zone 1* (Tab. 6). The interaction terms show that the odds ratio of year 2017 was estimated to increase by a multiplicative factor of 3.70 in the *zone 3*, and by a multiplicative factor of 6.47 in the *zone 2* in the same year. Therefore, for *zone 3*, the year 2017 is associated with a 48% decrease in the odds of being parasitized and for the *zone 2* the same year is associated with a 0.1% decrease in the odds of being parasitized.

There was significant effect of the factor *zone* on the parasitization rate, and this effect was dependent on the factor *year* (Fig. 14). Multiple comparisons of the estimated marginal means revealed that, during 2016, the parasitization rate was higher in *zone 1* than in *zone 3*, and in *zone 2* than in *zone 3* but there were no significant differences among the two zones in Canavese (*zones 1 and 2*) (Fig. 14A; GLMM contrast, see Tab. S19 in the supplementary material).

During 2017, despite a drastic decrease in the parasitization estimated marginal mean in *zone 2*, no differences were found between this zone and *zone 1* due to the high variability of the parasitization rate in the latter. Therefore, in 2017 the only significant difference was between *zone 1* and *zone 3* (Fig. 14A; Tab. S19). Concerning the comparison of the parasitization rate between *years* in each *zone*, there was a significant difference in *zone 2*, but there were no differences between the *year* 2016 and 2017 in the remaining *zones* (Fig. 14B; GLMM contrast, see Tab. S20 in the supplementary material).

As regard the rearing of *S. titanus* parasitized specimens, during the two surveyed years, about 30 % of the specimens were able to survive more than 30 days. However, no larva completed its cycle and emerged from the leafhoppers.

Table 6. Results of the select generalized linear mixed effects model (GLMM) testing the effects of Zone, Year and their interaction on parasitization rate.

Parameter	Estimate	SE	z	Pr(> z)
(Intercept)	-0.8146	0.6017	-1.354	0.17577
Year 2017	-1.9722	0.3979	-4.957	***
zone 3	-3.2887	0.9589	-3.430	***
zone 2	-0.2029	0.8441	-0.240	0.8101
year 2017 :zone 3	1.3074	0.4849	2.696	0.007
year 2017:zone 2	1.8669	0.4242	4.401	***

SE standard error of parameter estimates. z z-score testing whether the parameter estimate is significantly different from zero. Pr(|z|) probability of the observed z-score being greater than the critical value. Significant at ≤ 0.001 (***).

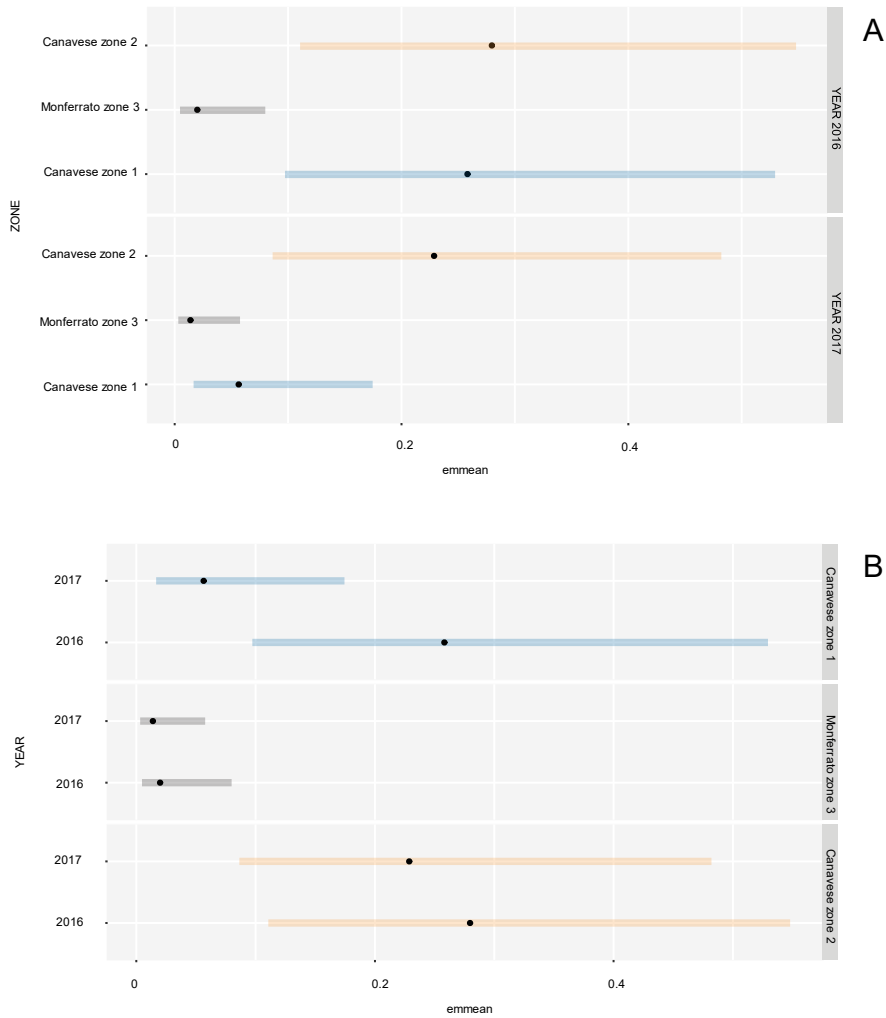


Figure 14. Estimated marginal means of the parasitization probability among the three investigated zones in the years 2016 and 2017 (A). Estimated marginal means of the parasitization probability between the two years levels separately for each zone (B). Data are presented on the back-logit scale with 95% confidence intervals (CI). Pairwise comparisons are provided in the supplementary material.

Survey on *Elampus bidens* association in the field

LEAFHOPPER HOSTS

A total of 4,662 planthoppers and leafhoppers belonging to 13 species (Tab. S15) were collected during 2018 and 2019 to detect the potential hosts of *E. bidens*. The endoparasitoid larvae were found only in *S. titanus* and *O. ishidae* (Fig. 15), both in nymphs from 3rd instar and adults.

In 2018, a total of 920 *S. titanus* and 850 *O. ishidae* were collected by sweep net, and 217 larvae were found inside *S. titanus* and 224 were found in *O. ishidae* (Tab. S15). Whereas in 2019, a total of 413 *S. titanus* and 949 *O. ishidae* were collected, and 72 endoparasitoid larvae were found inside *S. titanus* specimens and 245 larvae in *O. ishidae*.

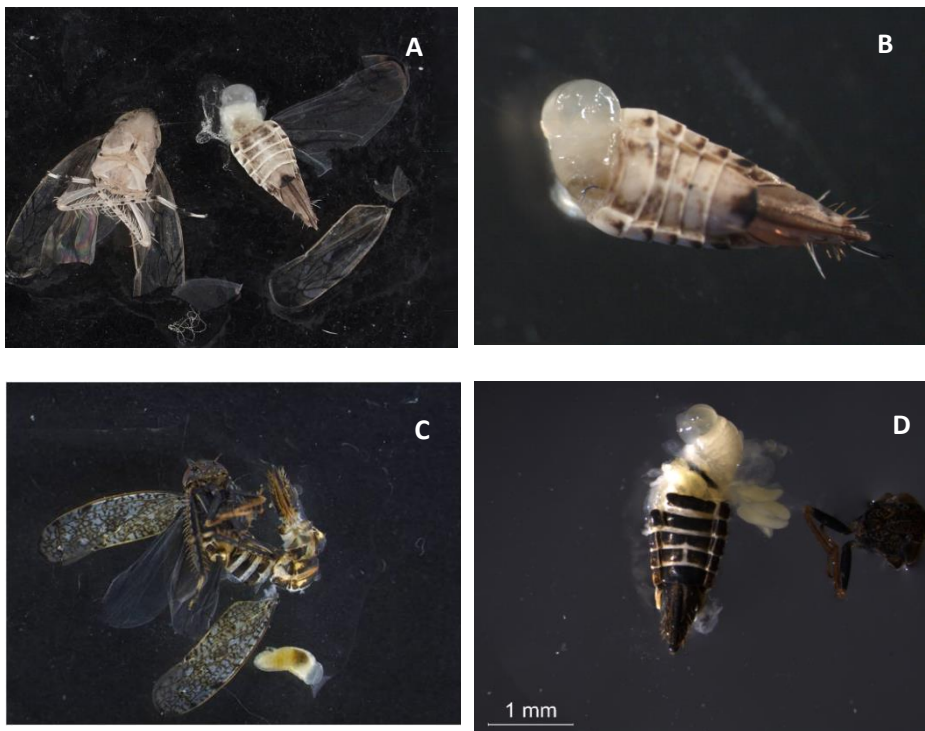


Figure 15. Larvae of *Elampus bidens* inside specimens of *Scaphoideus titanus* (A, B) and *Orientus ishidae* (C, D).

Looking at both years combined, at five localities (sites: 2, 7, 11, 12, 13), no parasitized leafhoppers were found, while at other five sites (sites: 1, 3, 6, 10, 15), parasitized samples of *S. titanus* and *O. ishidae* were contemporarily found (Tab. S15). A Chi-square test was performed to investigate which host species is statistically more susceptible to be parasitized. This test compares the empirical distribution of the parasitized nymphs of the two species and evaluates how far it deviates from the null hypothesis of marginal independence (same parasitization rate).

At site 3 *S. titanus* shows a significantly higher log odds of being parasitized compared to *O. ishidae*, while at site 10 *O. ishidae* shows a higher log odds than *S. titanus* (Fig. 16). In regard to the hypothesis of marginal independence of leafhoppers species and *E. bidens* parasitization rate, the null hypothesis cannot be rejected using reasonable levels of significance ($\chi^2 = 0.40$, $df = 1$, p -value = 0.52). Therefore, a host preference by *E. bidens* would not emerge as there is not a clear association between the parasitization rate and the leafhopper species. Furthermore, leafhoppers species and parasitization rate are conditional independent from the given sites, meaning that between sites there is not a prevalence of parasitization of one species on the other (Mantel-Haenszel $\chi^2 = 0.1$ $df = 1$, p -value = 0.75).

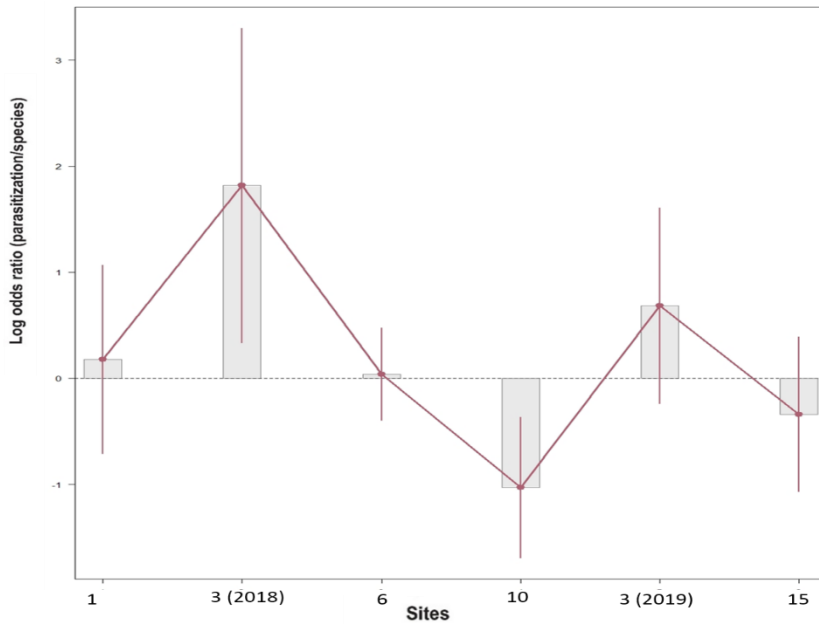


Figure 16. Log Odds ratios of being parasitized between the two leafhopper species for each site in which both of them were found parasitized. In only two sites there is a significant prevalence of a species than the other one: *Scaphoideus titanus* on *Orientus ishidae* in site 3 (2018) and *Orientus ishidae* on *Scaphoideus titanus* in site 10. Data are presented with 95% confidence intervals (CI).

PREDATOR WASPS AND ELAMPUS BIDENS ADULTS

As far as the survey on predator wasps and *E. bidens* adults is concerned, based on the literature (Mocsáry 1889; Spooner 1948; Rosenheim and Grace 1987), the study on predator wasps was limited on those species known to be able to predate leafhoppers.

In 2018, the survey was carried out both by means Malaise traps and butterfly net. A total of three Hymenoptera specimens belonging to the family Bembicidae and nine belonging to Psenidae were found (*sensu* Sann *et al.* 2018), whilst no *Elampus* specimen was found by means of Malaise traps. Among the

Bembicidae, all specimens were collected at the beginning of July. Two of them were identified as *Gorytes quadrifasciatus* (Fabricius), and one specimen was identified as species of the genus *Argogorytes* Ashmead. Concerning Psenidae, all specimens were identified as *Psen ater* (Oliver). A total of nine adult specimens of *E. bidens* were collected at the end of June and the first ten days of July, and 14 living *P. ater* specimens were collected in the second half of July until the end of September by means butterfly net. *Psen ater* collection occurred in 80 % of the sites where larvae or adults of *E. bidens* were previously found by us (Tab. S16).

In 2019, 23 adult specimens of *E. bidens* were found at six localities, the collections exclusively took place in the first week of July similarly to the previous year. Two specimens of *Gorytes quadrifasciatus* (Fabricius) were collected in two localities, whilst 19 specimens of *P. ater* were captured in six localities in the equivalent period of the previous year. Psenid wasps were found in 70 % of sampled sites where larvae or adults of *E. bidens* were previously collected (Tab. S16).

COI DNA barcoding of parasitoid larvae

We inferred the identity of 13 larvae collected at six different locations and collected in 2015 and 2018 by COI DNA barcoding. The obtained COI DNA barcode sequences were identical among each other and with a reference sequence of *E. bidens* (Hymenoptera: Chrysididae: Chrysidinae: Elampini) published by Pauli *et al.* (2019) (GenBank acc. no.: MT876911- MT876924, see Tab. S17). At the same time, the COI DNA barcode sequences differed consistently with those of other species of the genus *Elampus* that are known to or that could theoretically occur at the study sites (i.e., *E. constrictus* (Förster, 1853), *E. foveatus* Mocsáry, 1914, *E. panzer* (Fabricius, 1804), *E. sanzii* Gogorza, 1887, *E. spina* (Lepelletier, 1806)).

Larval development

During 2020, eight females of *E. bidens* were able to survive for an average of 24.25 ± 8.9 days under rearing conditions, while all the others died relatively soon. After a few days inside the cages, females began to scour the plants for the nymphs. The seeking behaviour was mainly focused on the lower part of the leaves where the nymphs usually feed on, and mainly seems to occur through visual stimuli at short distance. Indeed, it was often observed that the host was not immediately detected even if close to the parasitoid. The parasitization phase was always preceded by a short, unusual prey examination period. During this phase, the female remains almost immobile, with antennae straight backwards, contrarily to all previously reported parasitization observations (Carrillo and Caltagirone 1970; Veenendal 2012; Winterhagen 2015) and approaches the prey displaying small quick movements. Subsequently, the females attack their preys clamping and releasing them at the end of the oviposition (Fig. 17). Cuckoo wasp eggs or larvae were found inside the third and following nymphal instars. As for the adult prey, the female showed an excessive hesitation during the examination phase, allowing the leafhoppers to flee. Only one adult of *S. titanus* was successfully parasitized, all other attempts failed before *E. bidens* female could launch the attack.

A total of 87 (46 *O. ishidae* and 41 *S. titanus*) leafhoppers were parasitized during this experimental survey and no statistical differences were found between the parasitized proportion of leafhopper species ($\chi^2 = 0.134$, $P = 0.417$, $df = 1$). All the parasitized nymphs were able to moult and reach the adult stage.

The egg of *E. bidens* found in *S. titanus* is oval and hyaline and measures 470-540 μm . Inside parasitized *S. titanus* and *O. ishidae* we observed different development phases of the first larval stage. The first instar emerged from the egg two days after the oviposition and measured 560-750 μm . It is typically hymenopteriform and semi-transparent, with 13 segments and unsclerotized head. Larval morphology shows no differences in body shape through different instars, except the size ranging from about 0.5 mm to nearly 3.0 mm in length

(Fig. 18 B-D) and coloration became darker internally for food and waste accumulated during the development. The larva remains at this stage in a sort of quiescence: in fact, even 25 days after the parasitization it was possible to find it alive but static and reached the last instar inside the leafhopper in an average of 11.33 ± 4.16 (mean \pm standard deviation) days in *S. titanus* and 8.67 ± 3.05 days in *O. ishidae*. All the leafhopper specimens survived for more than twenty days, maintaining the ability to fly and move, but less reactive and more static than the others not parasitized. The larvae of the second stage were only observed on three specimens collected from the field. They are similar to the larvae of the previous stage with sickle-shaped mandibles.

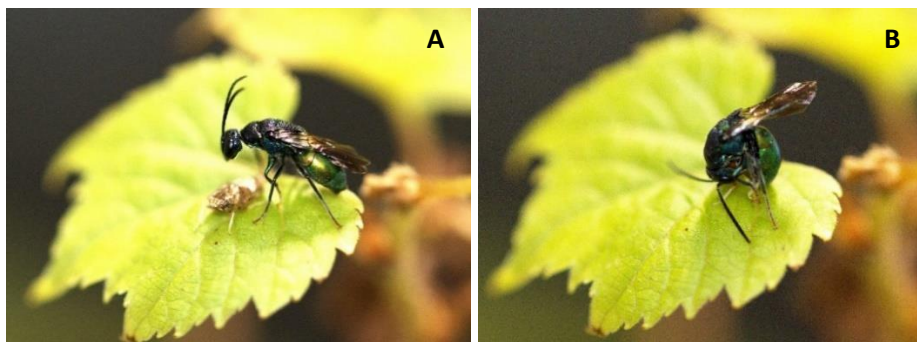


Figure 17. Parasitization of a *Orientus ishidae* nymph by *Elampus bidens*: host examination phase (A); attack phase with the prey clamping (B).

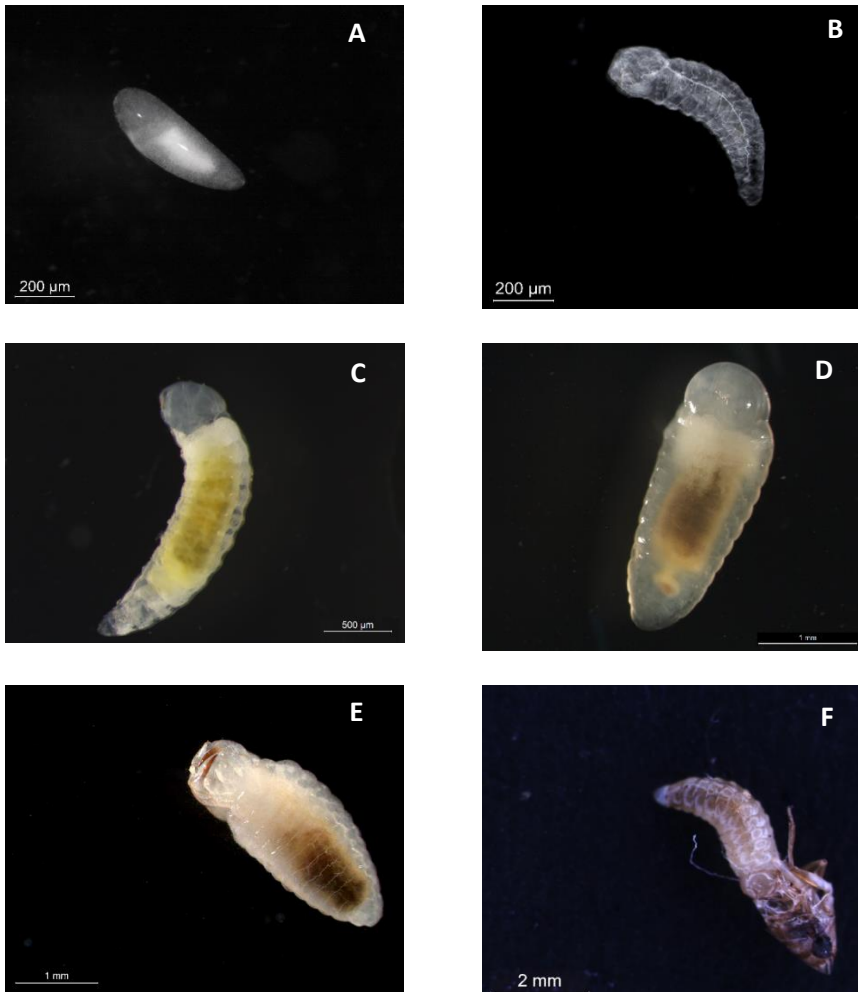


Figure 18. *Elampus bidens* larval development from oviposition to fourth-instar larva: Egg (A). First-instar larva (B). Second instar larva (C). Third instar larva (D). Fourth instar larva (E). Fourth instar larva inside *Scaphoideus titanus* body (F).

4.2.4. Discussion

Trojan horse strategy

During this research we documented for the first time an association that involves two exotic leafhoppers *S. titanus* and *O. ishidae* and the chrysidid *E. bidens*. So far, the *E. bidens* cleptoparasitic strategy and his behaviour resulted undocumented, and according to what arised fom this study they do not strictly fit within the endoparasitoid and ectoparasitoid dichotomy. This cuckoo wasp acts like an endoparasitoid during the first larval instars by feeding on non-viable tissues and keeping the leafhopper host alive until its purpose (entering the wasp nest) is achieved. Subsequently, it becomes an idiobiont, feeding on the preys accumulated by the predator wasp in its nest to accomplish its development. This kind of associations are already known as “Trojan horse” strategy (Strohm and Liebig 2008); namely, the chrysidid wasp female lays an egg into the body of a leafhopper host-prey that is subsequently captured and carried into the brood cell by a predator wasp. This parasitization behaviour is adopted to elude the defence strategies used by the host wasps to prevent the parasitoid from entering its nest (Strohm and Liebig 2008; Wurdak *et al.* 2015).

Previously, in the literature, Winterhagen (2015) and Paukkunen *et al.* (2015) described the behaviour of other members within different Elampini genera (*Omalus* Panzer, 1801 and *Pseudomalus* Ashmead, 1902). Those species lay their eggs onto the body of the host prey that are then carried into the host nest. No information on the larval development inside the predated aphid used as vector was mentioned, whereas a somehow similar endoparasitoid behaviour was previously described for the Nearctic chrysidid *Pseudolopyga taylori* (Bodenstein, 1939) and for the Palaearctic *Holopyga generosa* (Förster, 1853) (Carrillo and Caltagirone 1970; Veenendaal 2012). *Pseudolopyga taylori* ovoposits its eggs in 1st and 2nd nymph instars of a lygaeid bug of the genus *Nysius* (Heteroptera: Lygaeidae), later predated by a crabronid wasps in the genus *Solierella* Spinola, 1851, whereas *Holopyga generosa* (Förster, 1853), ovoposits

its eggs in true bug nymphs of the genus *Palomena* Mulsant and Rey, 1866 (Heteroptera: Pentatomidae), later predated by astatid wasps of the genus *Astata* Latreille, 1796.

The endoparasitoid habit provides an evolutionary advantage to the parasitoid wasp, avoiding the risk that the egg is detected and removed by the predator. Species of the genus *Elampus* are known to be cleptoparasites of at least two genera of the apoid wasp family Psenidae: *Mimesa* Shuckard, 1837 and *Mimumesa* Malloch, 1933 (Mocsáry 1889; Spooner 1948; Rosenheim and Grace 1987), both known to be predators of nymphal and adult cicadellids and delphacids. Nevertheless, these field observations were based on the finding of *Elampus* adults in psenid wasps nest, without any note on the predating strategy used by the chrysidid wasps.

Indigenous and exotic leafhopper hosts

From the current study emerged that *S. titanus* and *O. ishidae* are the host's prey parasitized in their nymphal stage by *E. bidens*. No other leafhopper species were found parasitized. This result might be explained by the major presence of the two FDP vectors in the sample sites compared to other leafhoppers. Indeed, a rapid spread of the two exotic cicadellids, followed by their population increase, has occurred in the vineyard agroecosystem in these years since their introduction (Lessio *et al.* 2016, 2019; Alma *et al.* 2019). Despite no other parasitized leafhoppers were found in this survey, it is reasonable to assume the existence of a previous association between an indigenous host and *E. bidens*. It is possible that the chrysidid wasp has been shifting to the most abundant hosts present in its habitat. Jones *et al.* (2015) stated that the parasitoid can rapidly adapt to a new host, increasing its fitness and over generations subsequently abandoning the original host. This behaviour might explain the absence of Chrysidid larva inside native leafhopper species.

The exotic origin of the leafhoppers beside the Palaeartic origin of the chrysidid wasp highlights the recent origin of this association. No studies carried out on the

biology and biological control of *S. titanus*, since its first record in Europe, have reported any possible presence of an endoparasitoid (Vidano 1964; Cravedi 1993; Alma and Arzone 1994; Nusillard *et al.* 2003), but they only reported ectoparasitoids belonging to the family Dryinidae.

When a parasitoid colonizes a new habitat or when it shifts to a new host, the parasitization rate can initially exhibit a high value on several potential hosts and then rapidly decrease if the hosts are not adequate, due to the time needed for an effective adaptation (Cornell and Hawkins 1993). In this research, we found parasitized leafhoppers over five years in most of the surveyed areas, suggesting that this might be a stable association rather than a consequence of an occasional interaction. Monitoring and data collection are still ongoing to verify this hypothesis through time.

Importance of parasitization of exotic pest

In sampled areas where the parasitoid was collected, the average annual parasitization rate has reached in some cases a value of 30 %. This is the first report in which such a high average annual parasitization rate on *S. titanus* is documented. In fact, biological control of *S. titanus* has not been effective so far because of the low impact of entomophagous insects on their population development in their native range as well as in Europe (Schvester *et al.* 1962; Vidano 1966; Nusillard *et al.* 2003; Chuche and Thiery 2014; Bocca *et al.* 2020b). In France tests on biological control of *S. titanus* by releasing natural enemies from the Nearctic zone or increasing local populations of natural enemies have shown a low percentage of parasitized individuals (<1 %) (Nuisillard *et al.* 2003). Similarly, in Italy it has been demonstrated that two species of Palearctic Dryinidae were able to parasitize *S. titanus*: *Gonatopus sepsoides* Westwood and *Gonatopus lunatus* Klug with a modest percentage of parasitization (< 1 %) (Arzone and Alma 1994).

Our results on the parasitization rate showed that the parasitism estimates were always high throughout the season and in both years in zone 2, whereas it

significantly dropped in the second year in the zone 1, and it was significantly lower than the other two zones in the zone 3. Moreover, the parasitization rate reduction in the zone 1 coincided with a wild grapevine uprooting. That action also involved other plant species compromising the entire vegetation structure, leading us to consider the two events very closely related. In fact, the modification of the habitat could have destroyed the predator wasp nests or altered in other ways the biocoenosis. The zone 3 showed the lowest parasitization rate, probably because the environment in Asti province might not be really suitable since *E. bidens* is known to prefer more humid landscapes (Rosa 2003, pers. data), and has a more intensive farming management. Although these data need more targeted surveys, we can assume that the simplification of agricultural landscapes, dominated by highly disturbed crop habitats, can threaten natural *E. bidens* populations whilst a biodiversity abundance and habitat management techniques might play a decisive role in the current *E. bidens* spread.

Endoparasitic development

From the rearing of the parasitized leafhoppers came to light that many specimens were able to survive for about a month but the larvae never emerged from them, and died along with the hosts. Furthermore, we observed that *E. bidens* larvae primarily feed on the leafhoppers' gonads. In fact, none of the parasitized males had intact testis and none of the parasitized females had intact ovaries. It has been reported that the larvae of other koinobiont parasitoids are able to survive into their host's body by feeding on non-viable tissues (Harvey and Strand 2002; Harvey *et al.* 2011). The larvae found in the leafhopper nymphs collected in field had a smaller size than the larvae observed in the adults (data not shown). In addition, only one leafhopper adult was parasitized in the trial under semi-natural conditions. Therefore, we assume that the parasitization primarily or exclusively occurs upon the juvenile instars. Indeed, even if host quality is correlated to host size or stage of parasitism (Harvey and Strand 2002), host immunity of larger hosts may compromise the larval development (Harvey 2005). With regard to larval development, very little information is available in the literature. Carrillo

and Caltagirone (1970) and Veenendaal (2012) estimated a time of 15 days from egg-laying to the pupa and our results could fall within these timelines. The study described by Carrillo and Caltagirone (1970) is quite similar, since they assert that the second larval stage emerges from the parasitized nymph after having perceived an external stimulus, continuing its development outwardly. Moreover, in our case we cannot exclude that an eventual paralysis of the host provoked by the predator sting can induce a more rapid larval development.

Final host

As regards the survey on predatory wasps and *E. bidens* adults, 47 specimens belonging to two families, Bembicidae and Psenidae, were collected during the two years of investigation with a clear prevalence of the species *P. ater*.

We have reasons to assume that the most likely leafhopper predator could be *P. ater*. This species was frequently observed and collected in the same site where *E. bidens* was detected. In particular, it was identified in 60% of sampled sites where the chrysidid wasp was found in 2018 and in 70% of the sites screened in 2019. When comparing the parasitization rate between *S. titanus* and *O. ishidae* at sites, where both leafhoppers were found, the rate moves in favour to the most abundant species at a given sampling site. Furthermore, a prevalence of parasitization between the two species has not been verified both in the field and in the experiment under rearing conditions. Being the Psenidae species generalist predators (Rosenheim and Grace 1987), it is likely that they tend to hunt for the most abundant species close to the nests. However, it is likely that more than one psenid or bembicid species are able to prey the leafhoppers. If that was true, *E. bidens* would be an unspecialized brood parasitoid. The absence of *E. bidens* larvae at some sites, despite the high population density of the two leafhoppers host, are consistent with the idea that the foraging strategy of the host predators does not necessarily depend only on the leafhoppers, but additionally on the spatial structure of the environmental and abiotic factors that can influence their patches and habitats (Hassell and Southwood 1978).

As far as the *E. bidens* cleptoparasitic habit is concerned, we assume that the larvae need to perceive the venom of the final host to activate their own metamorphosis, in agreement with ideas put further by Carrillo and Caltagirone (1970). Indeed, when trying to rear *E. bidens* in semi-natural conditions, no larvae were able to complete their cycle, emerge from the leafhoppers and feed on other stored leafhoppers. According to this assumption, the larva behaves as an endoparasitoid at the beginning of its larval development inside the leafhopper hosts, and afterwards as an ectoparasitoid to the detriment of the psenid preys to complete its development. Furthermore, the results highlight that there is temporal asynchrony in the field between *E. bidens* and *P. ater* adults, confirming that the larva inside the leafhoppers must survive until it is captured by the final host.

In koinobiont endoparasitism larval development can be affected by predation: koinobionts often induce changes in many typical host behaviours to reduce their odds of dying (Brodeur and McNeil 1992; Adamo 1997). In contrast, in this newly observed association the ultimate goal of the *E. bidens* larva is the leafhopper vector capture by the predator. In regard to this, we observed that parasitized cicadellids are visibly more stressed than healthy ones and this could facilitate the capture of leafhoppers.

Although a temporal asynchrony between *E. bidens* and *P. ater* adults was observed in the field, we cannot exclude that the leafhoppers are predated not long after their parasitization, namely when the cuckoo wasp is still at the egg stage, as asserted by Winterhagen (2015) about the association between *Omalus biaccinctus* and *Passaloecus* spp. This could reduce the risk of *E. bidens* being detected by the final host due to the weight increase of the parasitized leafhopper. Moreover, the synchronicity would allow the chrysid to select the nymphs to parasitize directly in the hunting territories of the final host, simply by following its movements (as in the case observed by Winterhagen 2015), thus reducing the risks of dispersion and loss of progeny, concentrating them in restricted arenas.

In any case, the question remains as to which is or are the final hosts of the cuckoo wasp in order to evaluate correctly not only the parasitization rate of the chrysidid wasp on the two invasive leafhoppers but, above all, the predation rate of the final host and therefore, the real incidence of predation and parasitization on the populations of *S. titanus* and *O. ishidae*.

5. FINAL CONSIDERATIONS

This research showed as *S. titanus* biological parameters have been largely underestimated so far, and sheds light on some unknown biological aspects of *O. ishidae* nymphs. Moreover, this work has highlighted some unpublished associations involving the three leafhoppers.

The knowledge of some biological characteristics, such as longevity and reproductive potential, are of fundamental relevance in the pest management. As evidenced by this study, the reproductive and longevity potential of *S. titanus* is considerably higher than estimated in the literature. Moreover, the presence of the vector was detected until late season in the vineyards. Some females with mature eggs were collected until October 30th, whilst in seminatural condition few individuals were able to lay eggs until the end October - early November. A serious implication of the high longevity is the extension of the adult inoculation period (Galletto *et al.* 2016), and a temporal expansion of the risk of infecting the vineyards no longer protected by insecticide treatments. This is all the more serious if we consider that *S. titanus* is able to acquire and transmit FDp at the adult stage as well, as recently demonstrated, reducing the latency acquiring period (Alma *et al.* 2018). Based on this research, *S. titanus* spread over the years in the vineyard territory and the epidemic development of FDp can be partially explained. It would be appropriate to reconsider the phytosanitary management of the vineyards after harvesting, in order not to frustrate the insecticides treatments performed in summer. This work raised questions about the role of some biotic and abiotic environmental variables on *S. titanus* biological traits. For instance, the effect of different temperature during the oviposition period, and the possible role of different cultivar of *Vitis vinifera*. Further researches are needed to clarify also the real potential fertility of this vector, related to the progeny able to hatch from the overwintering eggs laid at different times during the oviposition period, and the possible sperm supply depletion in the spermatheca before the end of the oviposition activity.

As regard *O. ishidae*, its capability of laying eggs on grapevine is confirmed as well as its presence in wooded areas on the edge of the vineyards. So, the danger level of this pest is even more relevant if combined with the recent evidence of some of its host plant (e.g. willow, hazelnut) tested positive for 16SrV phytoplasmas in Switzerland (Casati *et al.* 2017).

As far as the study of new associations involving the three exotic species is concerned, important results were obtained regarding both the egg parasitoids and the koinobiont parasitoids. No egg parasitoids emerged from the incubation of the eggs of *S. titanus* and *O. ishidae*, whilst a new association between the oophagous *O. cf collina* and the Flatid *M. pruinosa* was described. During the two-year study and in the two investigated areas, we found out a high parasitization rate never detected before on *M. pruinosa*. Finally, the preliminary study concerning the comparison between the parasitization rate of *O. cf collina* and *N. typhlocybae* showed that in most of the surveyed sites the oophagous is prevalent on the Dryinid. Following these results, an important question arises: has the *M. pruinosa* population been decreasing during the last decades because of the dryinid *N. typhlocybae* alone? On top of that, is the Dryinid parasitization activity currently higher or more effective than the egg parasitoid? The aim of this research was to emphasize the egg parasitoid role in the Flatid population control rather than comparing the efficiency in controlling *M. pruinosa* or diminish the results obtained with the Dryinid. Therefore, further in-depth studies are needed to quantify a possible synergic effect. Over the past 10 years, in some geographical areas *M. pruinosa* has been reported as a pest causing considerable damage to crops, forest and ornamental trees (Kim *et al.* 2011; Lee and Wilson 2010; Byeon *et al.* 2018; Kim and Kil 2014). Moreover, new information emerged on its ability to be a vector of 16SrI-B subgroup phytoplasma, highlighting the importance of this pest for agriculture and the need to control its spread (Mergenthaler *et al.* 2020). The discovery of an oophagus able to adapt to this Flatid may help to deepen knowledge on the planthopper containment

effectiveness in some European countries and open up new perspectives on its biological control.

Regarding the study of associations involving koinobiont parasitoids, we reported for the first time the biology of *E. bidens* and describe an unpublished association between this cuckoo wasp and two invasive leafhoppers: *S. titanus* and *O. ishidae*. This association is characterized by the “Trojan horse” strategy. This strategy raises questions about the mechanism of parasitization and the predation of the leafhoppers: do *E. bidens* females randomly parasitize leafhoppers in order to permit some of their larvae to be captured by the psenid and Bembicinae wasps? Are parasitized leafhoppers more likely to be preyed on than non-parasitized ones (e.g., because the cuckoo wasp causes the leafhopper to emit semiochemicals, or because parasitized leafhoppers are more stressed and weaker than the healthy leafhoppers)? In both cases, *E. bidens* eggs and larvae should not be recognized by the predator. Some cleptoparasitic cuckoo wasps are known to mimic the chemical profile of their host in order to avoid chemical detection (Strohm *et al.* 2008; Wurdack *et al.* 2015). While *E. bidens* does not enter the host nest, it is imaginable that the cuckoo wasp nonetheless applies some form of camouflage on the leafhopper cuticle. Specifically, the CHC profile of the host prey could be mimicked not to leave any trace behind, which could advise the host that the prey is parasitized. Cuckoo wasps could also reduce the amount of their CHC profile to leave behind only compounds that occur in all insects (Lenoir *et al.* 2001; Kroiss *et al.* 2009; Bagnères and Lorenzi 2010). The findings of the present work shed light on a new association involving multiple insect species with possible economic implications. In fact, the presence of a cuckoo wasp able to parasitize two FDP leafhoppers vectors, showing in some areas a high parasitization level and one or more final hosts species, draw attention to the uncultivated areas. These ones, can be considered not as a mere issue but as a resource in those portions of the vineyard agroecosystem whereby the control of leafhoppers is difficult by other means. Despite this, the preliminary results on leafhoppers parasitization rate so far presented, do not allow us to assert that the

combined action of the final host (psenid wasp) and the chrysidid wasps could control the population of *S. titanus* or *O. ishidae* in uncultivated environments yet. However, taking into consideration the consequences deriving from the increase in the use of insecticides (Biondi *et al.* 2013b; Begg *et al.* 2017) and a concurrent increase in the public demand for sustainable viticulture, not to mention the absence of effective control strategies against FDP-vectors, the discovery of new indigenous natural enemies that live in the uncultivated areas, adjacent to the vineyards, opens up new perspectives for the integrated pest management.

Spontaneous vegetation that constitutes linear elements of separation between the cultivated fields, is an integral part of the vineyard agroecosystem, representing a fundamental component of the Piedmontese viticultural landscape. The scientific literature is full of examples of experiments documenting how the diversification of cultivation systems and landscape with high proportions of semi-natural habitat often leads to a reduction in the phytophagous population (Altieri 2004; Bianchi *et al.* 2006; Burgio *et al.* 2006; Barberi *et al.* 2010; Jonsson *et al.* 2010; González-Chang *et al.* 2019; Martin *et al.* 2019). According to Rabb (1978), an agroecosystem should be conceived as an area large enough to include those uncultivated surfaces that influence crops through exchanges of organisms, matter and energy between different communities. Many Authors (Landis *et al.* 2000; Bianchi *et al.* 2006; Tschardtke *et al.* 2007; Letourneau *et al.* 2011; Tschardtke *et al.* 2012; Holland *et al.* 2017) argued about complex landscapes, suggesting that a more effective biological control can be achieved when a higher amount of semi-natural habitat and less intensively managed farmland are present.

Despite this mass of experimental evidence, according to some studies surrounding non-crop habitat does not consistently improve the pest management, meaning habitat conservation may bolster production in some systems and depress yields in others (Tschardtke *et al.* 2016; Karp *et al.* 2018).

We should address further studies upon the native parasitoid community to investigate how a rational habitat management within the vineyard agroecosystem can enhance the presence of the leafhopper predators and parasitoids, and limit the negative effect of exotic pests evaluating the effectiveness of these areas in containing leafhopper re-infestation flows.

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NEW INSIGHTS ON *SCAPHOIDEUS TITANUS* BIOLOGY AND THEIR IMPLICATIONS FOR INTEGRATED PEST MANAGEMENT

A particular thanks to dott. Enrico Caprio (Università di Torino, DBIOS) and dott. Alex Cucco (Imperial college London) for the statistical revision.

PALAEARCTIC EGG PARASITOID INTERACTION TO THREE GRAPEVINE EXOTIC PESTS IN NORTH WESTERN ITALY: A NEW ASSOCIATION INVOLVING *METCALFA PRUINOSA*

Particular thanks to dott. Alex Cucco and dott. Nicola Pronello (Università di Torino, Dipartimento di Economia e Statistica) for the statistical revision; Ilaria Salvetti and Fausto Tessitore for their support during the filed activity.

NEW HOST-PARASITOID ASSOCIATION INVOLVING A CUCKOO WASP AND TWO VECTORS OF THE FLAVESCENCE DORÉE: *SCAPHOIDEUS TITANUS* AND *ORIENTUS ISHIDAE*

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8. SUPPLEMENTARY MATERIALS

8.1. NEW INSIGHTS ON *SCAPHOIDEUS TITANUS* BIOLOGY AND THEIR IMPLICATIONS FOR INTEGRATED PEST MANAGEMENT

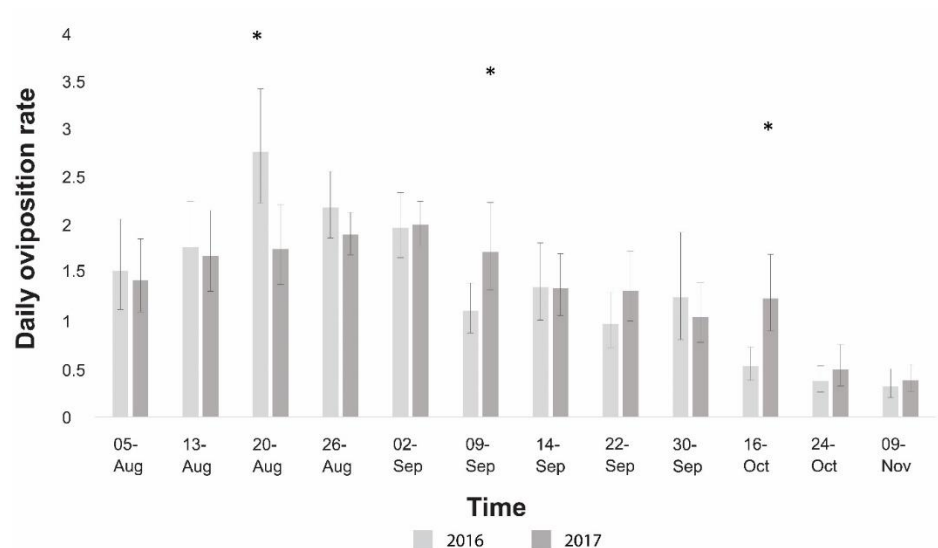


Figure S1. Comparison of *Scaphoideus titanus* estimated marginal means of the daily oviposition rate between the two years through all the season. Bars with (*) show significant differences within each date (Tukey tests, $P < 0.05$). Predicted values are back-transformed from the log scale and are presented with 95% confidence intervals (CI).

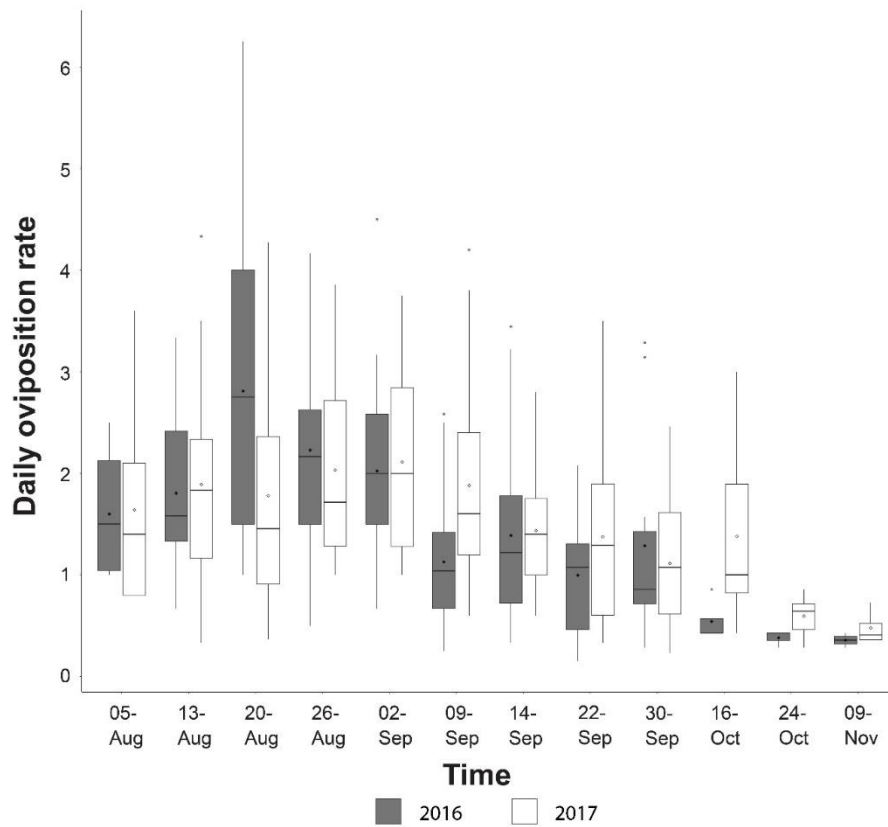


Figure S2. Comparison of the *Scaphoideus titanus* daily oviposition rate between the two years through all the season. Boxplots showing upper whisker (maximum data point), interquartile range box (top line = 75% of the data; middle line = median; lower line = 25% of the data) and lower whisker (minimum data point). The rumble inside each box shows the raw mean.

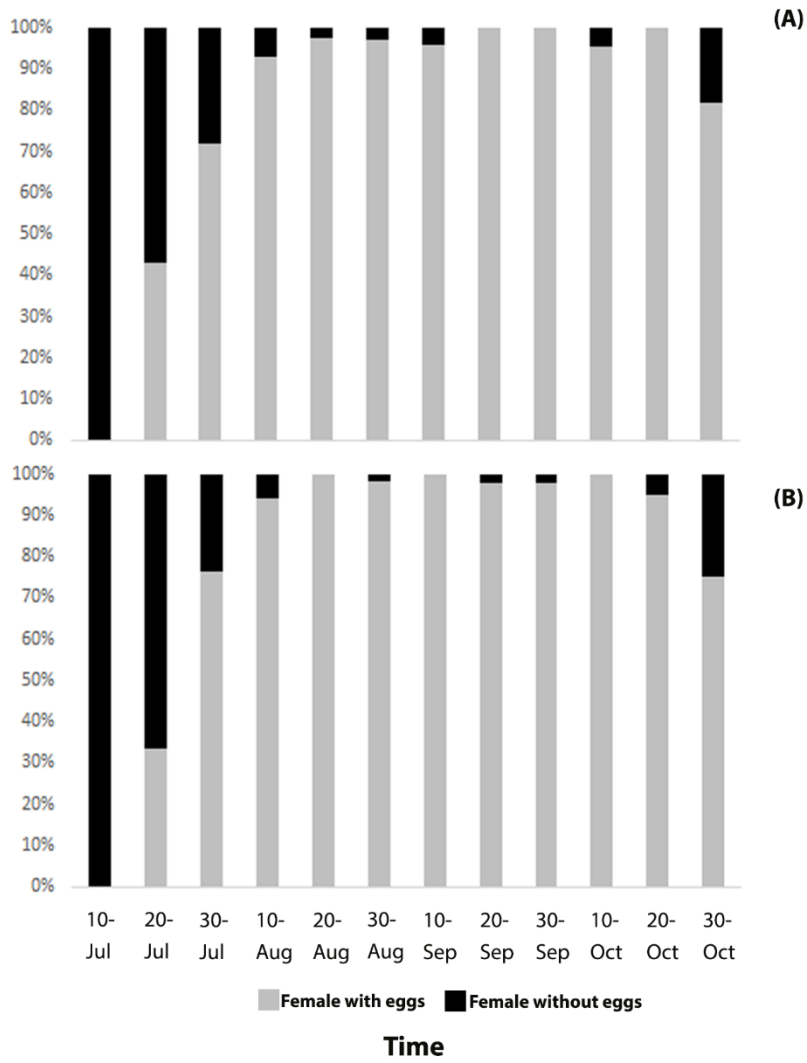


Figure S3. Proportion of females carrying mature eggs at different times of the season between the two surveyed areas. (A): Canavese; (B) Asti Monferrato.

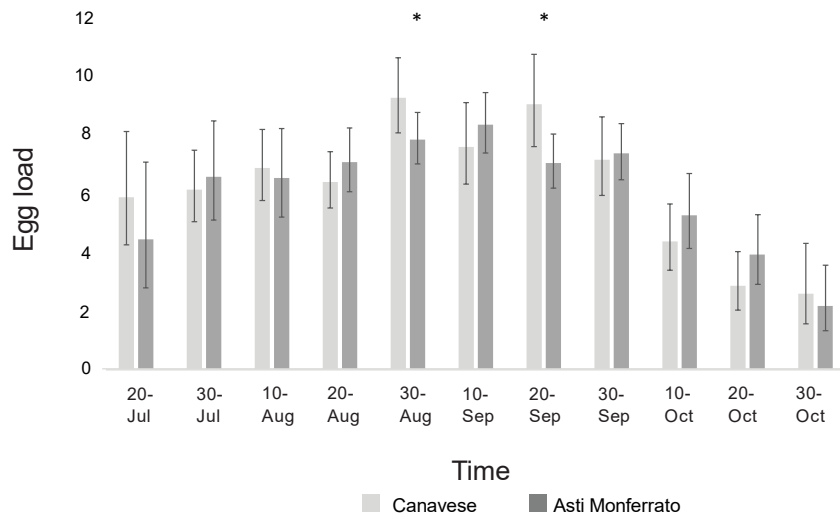


Figure S4. Comparison of *Scaphoideus titanus* estimated marginal means of the egg load trend among the two surveyed areas through all the season. Bars with (*) show significant differences within each date (Tukey tests, $P < 0.05$). Predicted values are back-transformed from the log scale and are presented with 95% confidence intervals (CI).

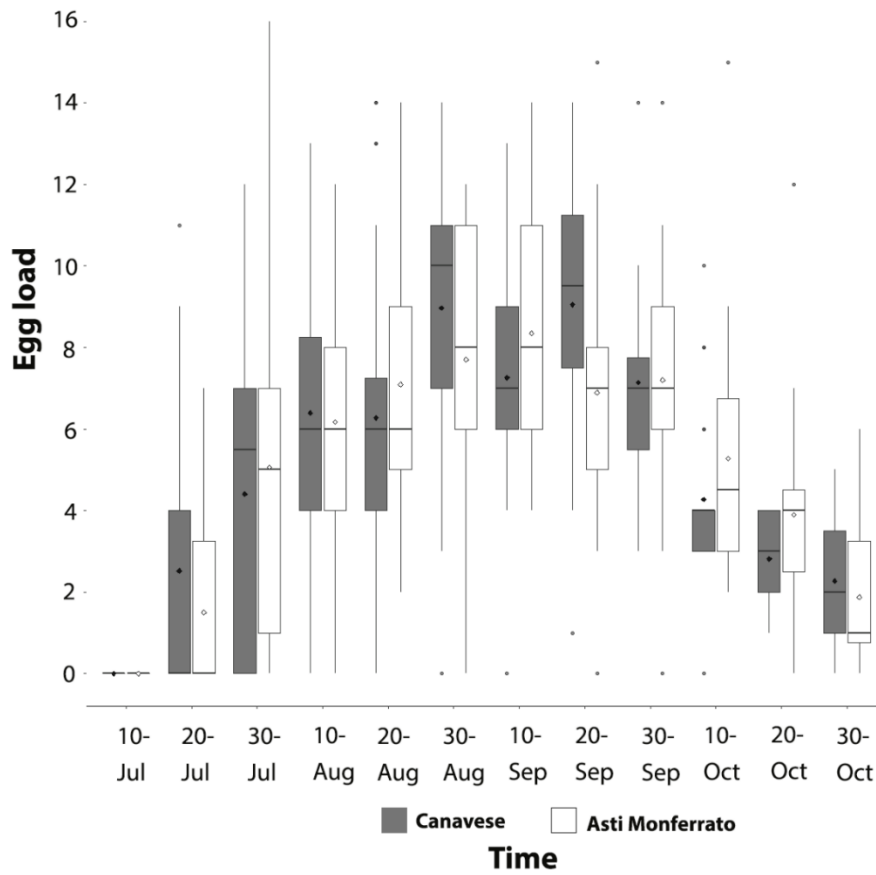


Figure S5. Comparison of the *Scaphoideus titanus* egg load trend between the two surveyed areas through all the season. Boxplots showing upper whisker (maximum data point), interquartile range box (top line = 75% of the data; middle line = median; lower line = 25% of the data) and lower whisker (minimum data point). The rumble inside each box shows the raw mean.

Table S1. Selection of predictors in order to obtain the most parsimonious model through Likelihood Ratio Test (LRT) and Wald chi-square tests.

	Df	AIC	LRT	Pr(>Chi)
<none>		1382.6		
Sex	1	1393.8	13.22	0.0003
Year	1	1381.5	0.89	0.34

Table S2. Estimated regression coefficients (β), standard errors (SE), 95% confidence intervals (CI), hazard ratios (e^β), Z-scores, and *P* values for Cox proportional hazards models with Sex and Year covariates, and interaction between Sex and Year.

Covariates	β	e^β	SE	CI	<i>z</i>	<i>P</i>
Sex: Male	0.63	1.87	0.24	1.17 to 2.99	2.62	0.008
Year: 2017	-0.12	0.88	0.21	0.58 to 1.33	-0.59	0.55
Interaction Effects	-0.06	0.93	0.32	0.50 to 1.74	-0.21	0.83
Sex: Male						

Overall significance of the model: Likelihood ratio test= 14.03, *P* =0.003;
Wald $\chi^2=14.62$; *P* = 0.002

Table S3. Results of the most parsimonious generalized linear mixed effects model (GLMM) testing the effects of Date, Year and their interaction on daily oviposition rate.

Parameter	Estimate	SE	z	Pr(> z)
(Intercept)	0.40	0.14	2.95	**
Year 2017	-0.06	0.18	-0.36	0.72
Second date	0.15	0.15	0.17	0.31
Third date	0.60	0.16	3.83	***
Fourth date	0.36	0.14	2.50	*
Fifth date	0.26	0.15	1.73	0.08
Six date	-0.32	0.17	-1.91	0.06
Seventh date	-0.12	0.18	-0.63	0.531
Eighth date	-0.45	0.18	-2.41	*
Ninth date	-0.19	0.23	-0.85	0.40
Tenth date	-1.05	0.19	-5.43	***
Eleventh date	-1.41	0.21	-6.70	***
Twelve date	-1.57	0.25	-6.40	***
Year 2017: Second date	0.01	0.20	0.06	0.95
Year 2017:Third date	-0.40	0.21	-1.82	0.07
Year 2017:Fourth date	-0.07	0.19	-0.40	0.69
Year 2017:Fifth date	0.08	0.20	0.41	0.68

Year 2017:Six date	0.50	0.23	2.17	*
Year 2017:Seventh date	0.06	0.24	0.23	0.81
Year 2017:Eighth date	0.36	0.25	1.47	0.14
Year 2017:Ninth date	-0.11	0.29	-0.38	0.70
Year 2017:Tenth date	0.91	0.26	3.42	***
Year 2017:Eleventh date	0.35	0.30	1.15	0.25
Year 2017:Twelve date	0.25	0.31	0.80	0.43
<hr/>				
Dispersion model				
(Intercept)	-0.07	0.82	-0.09	0.90
Year 2017	-0.13	1.06	-0.12	0.77
Second date	0.29	1.03	0.28	0.24
Third date	1.03	0.88	1.17	0.82
Fourth date	0.21	0.92	0.23	0.73
Fifth date	0.30	0.89	0.34	0.14
Six date	1.28	0.87	1.45	0.08
Seventh date	1.54	0.89	1.72	0.17
Eighth date	1.28	0.92	1.38	0.26
Ninth date	1.12	0.99	1.13	0.19
Tenth date	-3.07	2.33	-1.32	0.63
Eleventh date	-5.53	11.63	-0.47	0.18
Twelve date	-3.75	2.80	-1.34	0.83

Year 2017:Second date	0.27	1.28	-1.34	0.53
Year 2017:Third date	0.74	1.16	0.63	0.21
Year 2017:Fourth date	-4.91	3.89	-1.26	0.12
Year 2017:Fifth date	-3.15	2.05	-1.54	0.87
Year 2017:Six date	-0.19	1.17	-0.17	0.32
Year 2017:Seventh date	-1.21	1.21	-1.00	0.70
Year 2017:Eighth date	0.47	1.20	0.39	0.84
Year 2017:Ninth date	0.25	1.28	0.20	0.15
Year 2017:Tenth date	3.60	2.51	1.44	0.67
Year 2017:Eleventh date	4.99	11.67	0.43	0.80
Year 2017:Twelve date	1.03	4.14	0.25	0.90

SE standard error of parameter estimates. z z-score testing whether the parameter estimate is significantly different from zero. Pr(|z|) probability of the observed z-score being greater than the critical value. Significant at ≤ 0.05 (*). ≤ 0.01 (**). ≤ 0.001 (***). not significant (NS).

Table S4. Results from a generalized linear mixed effects model (GLMM) testing the effect of the dates and the different cohorts on the daily oviposition rate.

Parameter	Estimate	SE	z	Pr(> z)
Year 2016				
(Intercept)	0.64	0.11	5.85	***
Second cohort	-0.07	0.12	-0.58	0.56
Third cohort	-0.15	0.10	-1.50	0.13
Fourth cohort	-0.14	0.11	-1.27	0.20
Second date	0.27	0.11	2.10	*
Third date	0.21	0.12	1.81	0.07
Fourth date	-0.15	0.14	-1.06	0.29
Fifth date	-0.22	0.15	-1.52	0.12
Sixth date	-0.57	0.15	-3.90	***
Seventh date	-0.54	0.15	-3.19	**
Eighth date	-0.91	0.19	-4.72	***
Ninth date	-1.16	0.25	-4.73	***
Tenth date	-1.53	0.35	-4.35	***
Year 2017				
(Intercept)	0.29	0.10	2.76	**
Second cohort	-0.10	0.11	-1.00	0.34

Third cohort	-0.14	0.10	-1.30	0.19
Fourth cohort	-0.22	0.14	-1.56	0.12
Second date	0.33	0.10	3.13	**
Third date	0.38	0.12	3.23	**
Fourth date	0.41	0.13	3.30	***
Fifth date	0.26	0.13	2.08	*
Sixth date	-0.10	0.14	-0.75	0.45
Seventh date	-0.24	0.17	-1.41	0.16
Eighth date	-0.1330	0.1665	-0.798	0.42
Ninth date	-0.76	0.21	-3.70	***
Tenth date	-1.16	0.38	-3.07	**

SE standard error of parameter estimates. z z-score testing whether the parameter estimate is significantly different from zero. Pr(|z|) probability of the observed z-score being greater than the critical value. Significant at ≤ 0.05 (*), ≤ 0.01 (**), ≤ 0.001 (***), not significant (NS).

Table S5. Results from a generalized linear mixed effects model (GLMM) testing the effect of the dates on the egg load trend.

Parameter	Estimate	SE	z	Pr(> z)
Conditional model				
(Intercept)	1.77	0.14	12.24	***
Second date	0.04	0.17	0.26	0.79
Third date	0.16	0.16	0.96	0.34
Fourth date	0.09	0.16	0.54	0.59
Fifth date	0.46	0.16	2.91	**
Sixth date	0.26	0.16	1.55	0.12
Seventh date	0.43	0.16	2.64	**
Eighth date	0.19	0.17	1.18	0.23
Ninth date	-0.29	0.18	-1.61	0.11
Tenth date	-0.72	0.21	-3.43	***
Eleventh date	-0.82	0.27	-3.05	**
Asti Monferrato Area	-0.28	0.25	-1.11	0.27
Second date : Asti Monferrato Area	0.35	0.29	1.20	0.23
Third date : Asti Monferrato Area	0.23	0.28	0.81	0.42
Fourth date : Asti Monferrato Area	0.38	0.27	1.40	0.16

Fifth date : Asti Monferrato Area	0.11	0.26	0.43	0.67
Sixth date : Asti Monferrato Area	0.37	0.27	1.38	0.16
Seventh date : Asti Monferrato Area	0.03	0.27	0.11	0.91
Eighth date : Asti Monferrato Area	0.31	0.27	1.14	0.25
Ninth date : Asti Monferrato Area	0.47	0.29	1.56	0.12
Tenth date : Asti Monferrato Area	0.60	0.32	1.84	0.06
Eleventh date : Asti Monferrato Area	0.09	0.39	0.25	0.80
<hr/>				
Zero-inflated model				
(Intercept)	0.45	0.33	1.37	0.17
Second date	-1.48	0.46	-3.19	**
Third date	-3.12	0.69	-4.48	***
Fourth date	-4.90	1.24	-3.95	***
Fifth date	-4.24	0.79	-5.32	***
Sixth date	-4.67	1.10	-4.23	***
Seventh date	-4.72	1.14	-4.14	***
Eighth date	-4.70	1.12	-4.20	***
Ninth date	-5.07	2.74	-1.85	0.06

Tenth date	-5.72	6.20	-0.92	0.36
Eleventh date	-2.40	0.96	-2.50	*

SE standard error of parameter estimates. z z-score testing whether the parameter estimate is significantly different from zero. Pr(|z|) probability of the observed z-score being greater than the critical value. Significant at ≤ 0.05 (*). ≤ 0.01 (**). ≤ 0.001 (***). not significant (NS).

- 1 **Table S6.** Full, best and null models with their AICc according to a maximum error risk of 0.5. Full model: model with all factors; Selected model: model
- 2 selected based on the lower AICc and the Log-likelihood-ratio test; Null model: model including only the intercept and the random effect.

Response variable	Models	Distribution	Variables	AICc	Delta AICc selected model to null model
Oviposition rate	Full model	Generalized Poisson	Year * Dates + Temperature + ar1(Dates + 0 Subject) + offset(log(rate)). ziformula = ~ 0. dispformula = ~ Year * Dates	3075.2	
	Selected model	Generalized Poisson	Year * Dates + ar1(Dates + 0 Subject) + offset(log(rate)). ziformula = ~ 0. dispformula = ~ Year * Dates	3066.1	121.0
	Null model	Generalized Poisson	1	3198.7	
Oviposition rate among cohorts (year2016)	Full model	Generalized Poisson	Cohorts + Dates + ar1(Dates + 0 Subject) + offset(log(rate)). ziformula = ~ 0. dispformula = ~ Dates	1528.9	
	Selected model:	Generalized Poisson	1	1516.7	-16.8
	Null model				

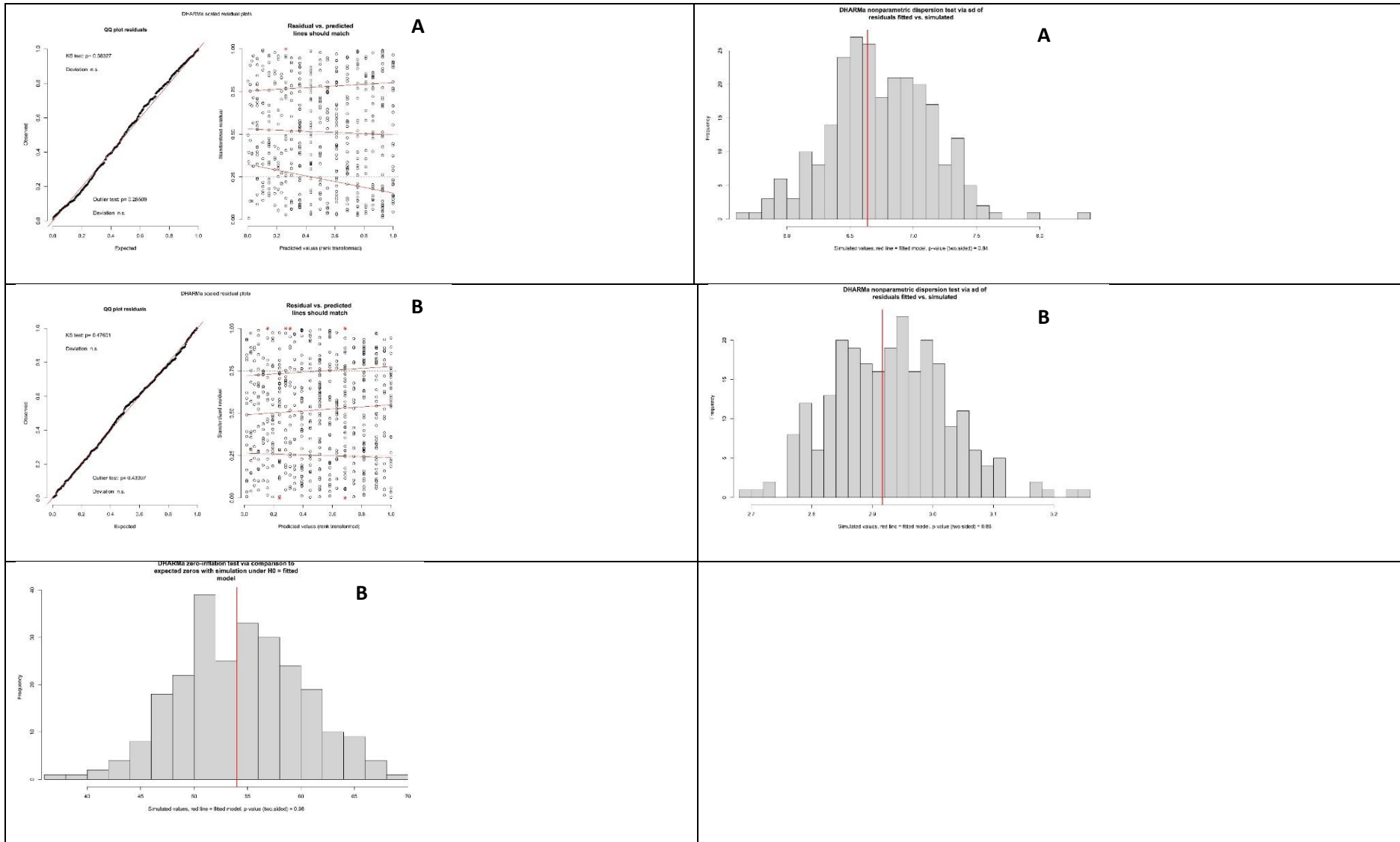
Response variable	Models	Distribution	Variables	AICc	Delta AICc selected model to null model
Oviposition rate among cohorts (year2017)	Full model	Generalized Poisson	Cohorts + Dates + ar1(Dates + 0 Subject) + offset(log(rate)). ziformula = ~ 0. dispformula = ~ Dates	1578.6	
	Selected model:	Generalized Poisson	1	1563.59	-19.5
	Null model				
Egg load trend	Full model 1	Zero-Inflated Generalized Poisson	Dates * Area + Temperature * Year + (1 area : sito). ziformula = ~ Area * Year + Dates	2788.1	
	Full model 2	Zero-Inflated Generalized Poisson	Dates * Area + Dates : Year + (1 area : sito). ziformula = ~ Dates	2789.1	

Response variable	Models	Distribution	Variables	AICc	Delta AICc selected model to null model
	Selected model	Zero-Inflated Generalized Poisson	Dates * Area + (1 area : sito). ziformula = ~ Dates	2780.2	397.5
	Null model	Zero-Inflated Generalized Poisson	1	3177.6	

3

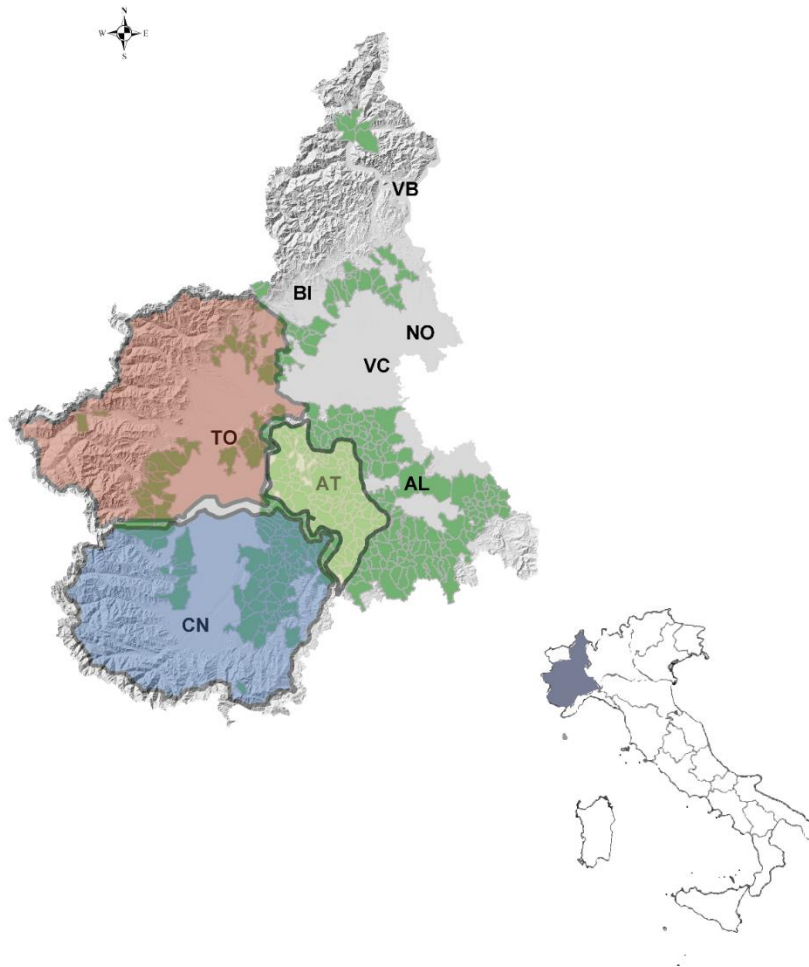
4

5 **Table S7:** Graphs of the residual diagnostic for the selected models concerning the Oviposition rate (A) and the Egg load trend (B).



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7 8.2. PALAEARCTIC EGG PARASITIDS INTERACTION TO THREE GRAPEVINE
8 EXOTIC PESTS IN NORTH WESTERN ITALY: A NEW ASSOCIATION INVOLVING
9 *METCALFA PRUINOSA*



10

11 **Figure S6.** Map of the surveyed areas in Piedmont. Blue surface (Cuneo province), and yellow
12 surface (Asti province) referred to the study on the parasitization rate of *Oligosita cf collina*.
13 Red surface (Turin province) refers to the comparison between the parasitization rate of
14 *Neodryinus typhlocybae* and *Oligosita cf collina* group on *Metcalfa pruinosa*. The green plots on
15 the map represent the wine-growing municipalities in Piedmont region.

16

17 **Table S8.** Study on the parasitization rate of *Oligoista cf collina* during 2017 and 2018: coordinates of the collection sites.

Area	Year	Site	Coordinates
Asti Province	2017	1	44°49'15.4"N 8°18'04.5"E
		2	44°47'32.5"N 8°17'44.9"E
		3	45°03'07.2"N 8°01'59.4"E
		4	44°45'39.4"N 8°24'31.8"E
		5	44°52'04.4"N 8°18'51.6"E
	2018	6	44°57'43.5"N 8°15'28.6"E

7 44°59'36.6"N 8°09'44.3"E

8 44°57'42.9"N 8°17'09.5"E

9 45°02'11.1"N 8°04'57.2"E

10 44°49'17.3"N 8°01'08.7"E

Cuneo Province

2017

1 44°36'37.9"N 8°12'24.7"E

2 44°36'50.6"N 8°11'51.4"E

3 44°36'37.9"N 8°12'24.7"E

4 44°48'26.6"N 7°58'55.1"E

5 44°47'30.4"N 7°58'01.4"E

2018

6 44°30'42.9"N 7°56'47.6"E

7 44°32'53.2"N 7°59'10.1"E

8 44°41'31.8"N 8°14'35.2"E

9 44°48'28.2"N 7°57'41.6"E

10 44°42'29.4"N 8°19'22.6"E

19 **Table S9.** Survey on the comparison between *Oligosita cf collina* and *Neodryinus typhlocybae*: Coordinates for the collection sites relative to the
20 comparison.

Area	Year	Site	Coordinates
Turin Province	2018/2019		
		1	45°23'48.3"N 7°40'03.1"E
		2	45°18'41.6"N 7°54'16.5"E
		3	45°20'30.8"N 7°49'16.1"E
		4	45°17'27.5"N 7°56'30.1"E
		5	45°20'00.1"N 7°49'53.8"E
		6	45°19'34.4"N 7°51'18.2"E
		7	45°16'43.3"N 7°37'56.9"E
		8	45°25'59.4"N 7°57'22.4"E

21

22

23 **Table S10.** Results of the selected generalized linear mixed effects model (GLMM) testing the effects on the parasitization rate of Plant species,
 24 Host egg density, Plant coverage and the interaction between Host egg density and Plant cover.

Parameter	Estimate	SE	z	P
(Intercept)	-1.08589	0.08678	-12.512	***
<i>Corylus</i> sp.	-0.28625	0.09458	-3.026	**
<i>Vitis</i> sp.	0.26396	0.06839	3.859	***
Host eggs density	0.52363	0.06935	7.550	***
Plant cover	0.28382	0.05300	5.355	***
Host eggs density: Plant cover	0.10732	0.05420	1.980	0.047704

25 SE standard error of parameter estimates. z z-score testing whether the parameter estimate is significantly different from zero. $\Pr(|z|)$
 26 probability of the observed z-score being greater than the critical value. Significant at ≤ 0.05 (*). ≤ 0.01 (**). ≤ 0.001 (***). not significant (NS).

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29 **Table S11.** Candidate GLMM predicting the *Metcalfa pruinosa* egg parasitization (dependent variable correspond to success or failure of
 30 parasitization). Best subset selection approach was used; results shown only the top 10 candidate models. Group Site nested within Area was
 31 included as random effect in all models to take into accounts the dependences among measures in each Area (1 | area/site)".

Response variable: number of eggs ejected from communal nest			Overall model significance	
Fixed effects	AIC _i	ΔAIC _i	Wald's X ²	P
[1.] ~plant species+plant cover+plant species:host eggs density+host eggs density:plant cover	410.626317189967	0.000000	725.3	<0.0001
[2.] ~plant species+host eggs density+plant cover+plant species:host eggs density+host eggs density:plant cover	410.626320914904	0.000004	725.3	<0.0001
[3.] ~plant species+plant cover+plant species:host eggs density	411.497259738122	0.870943	722.43	<0.0001
[4.] ~plant species+host eggs density+plant cover+plant species:host eggs density	411.497259870215	0.870943	722.43	<0.0001
[5.] ~plant species+host eggs density+plant cover+host eggs density:plant cover	411.695475559671	1.069158	720.23	<0.0001

[6.]	~plant species+host eggs density+plant cover+plant species:host eggs density+plant cover:plant species+host eggs density:plant cover	411.720921207443	1.094604	728.21	<0.0001
[7.]	~plant species+plant cover+plant species:host eggs density+plant cover:plant species+host eggs density:plant cover	411.720921876676	1.094605	728.21	<0.0001
[8.]	~plant species+host eggs density+plant species:host eggs density+plant cover:plant species+host eggs density:plant cover	411.720922147976	1.094605	728.21	<0.0001
[9.]	~plant species+plant species:host eggs density+plant cover:plant species+host eggs density:plant cover	411.720923594172	1.094606	728.21	<0.0001
[10.]	~plant species+plant cover+plant species:host eggs density+plant cover:plant species	412.065634384171	1.439317	725.86	<0.0001

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35 **Table S12.** Results of the selected generalized linear mixed effects model (GLMM) testing the effects on the oviposition rate of Plant species and
 36 Plant coverage.

Parameter	Estimate	SE	z	P
(Intercept)	2.20649	0.07570	29.147	***
<i>Corylus</i> sp.	-0.36801	0.10752	-3.423	***
<i>Vitis</i> sp.	0.27576	0.10049	2.744	**
Plant cover	0.22721	0.04606	4.933	***

37 SE standard error of parameter estimates. z z-score testing whether the parameter estimate is significantly different from zero. Pr(|z|)
 38 probability of the observed z-score being greater than the critical value. Significant at ≤ 0.05 (*). ≤ 0.01 (**). ≤ 0.001 (***). not significant (NS).

39

40 **Table S13.** Candidate GLMM predicting the *Metcalfa pruinosa* oviposition (dependent variable corresponds to the count of laid eggs of *Metcalfa*
 41 *pruinosa*). Best subset selection approach was used; results shown only the top 10 candidate models. Group Site nested within Area was
 42 included as random effect in all models to take into accounts the dependences among measures in each Area (1 | area/site)".

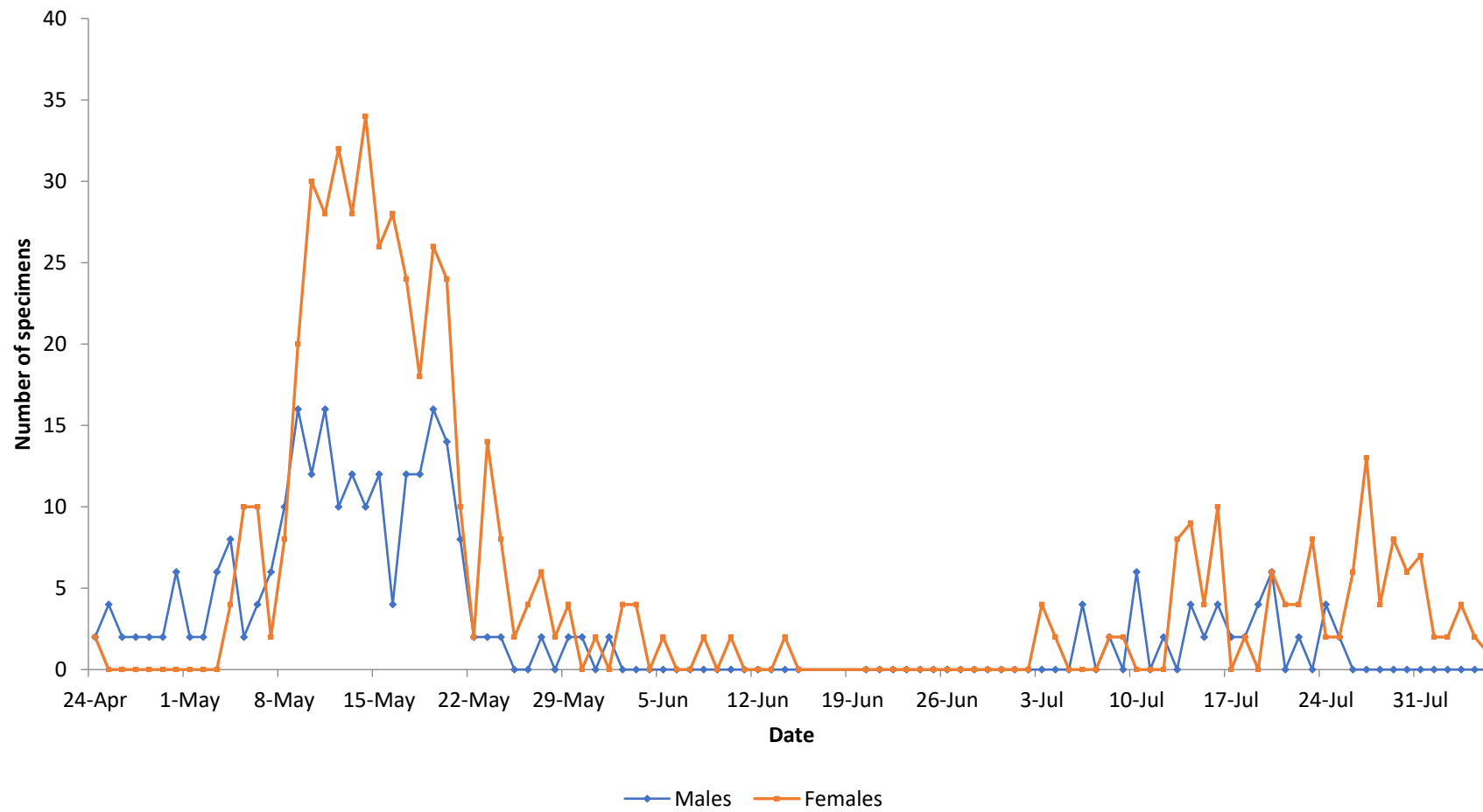
Response variable: number of eggs ejected from communal nest				Overall model significance	
Fixed effects	AIC _i	ΔAIC _i	Wald's X ²	P	
[1.] ~plant species+plant cover+ offset(log(piante))	605.161772115018	0.000000	591.16	<0.0001	
[2.] ~plant species+plant cover:plant species+ offset(log(piante))	605.794000229603	0.632228	587.79	<0.0001	
[3.] ~plant species+plant cover+plant cover:plant species+ offset(log(piante))+(1 area/site)"	605.794000232042	0.632228	587.79	<0.0001	
[4.] ~plant species+plant cover+year+ offset(log(piante))	606.818019074198	1.656247	590.82	<0.0001	
[5.] ~plant species+year+plant cover:plant species+ offset(log(piante))	607.543683592333	2.381911	587.54	<0.0001	
[6.] ~plant species+plant cover+year+plant cover:plant species+ offset(log(piante))	607.543683601835	2.381911	587.54	<0.0001	
[7.] ~plant cover+plant species:year+ offset(log(piante))	607.924954709873	2.763183	587.92	<0.0001	
[8.] ~plant cover+year+plant species:year+ offset(log(piante))	607.924954710409	2.763183	587.92	<0.0001	
[9.] ~plant species+plant cover+plant species:year+ offset(log(piante))	607.924954713611	2.763183	587.92	<0.0001	

[10.]	~plant species+plant cover+year+plant species:year+ offset(log(piante))	607.924954714603	2.763183	587.92	<0.0001
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47 **Figure S7.** Daily emergence of *Oligosita cf. collina* from field collected parasitized eggs.

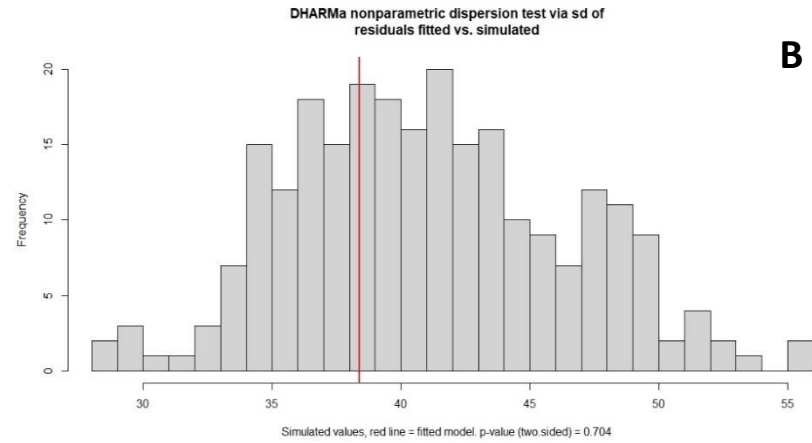
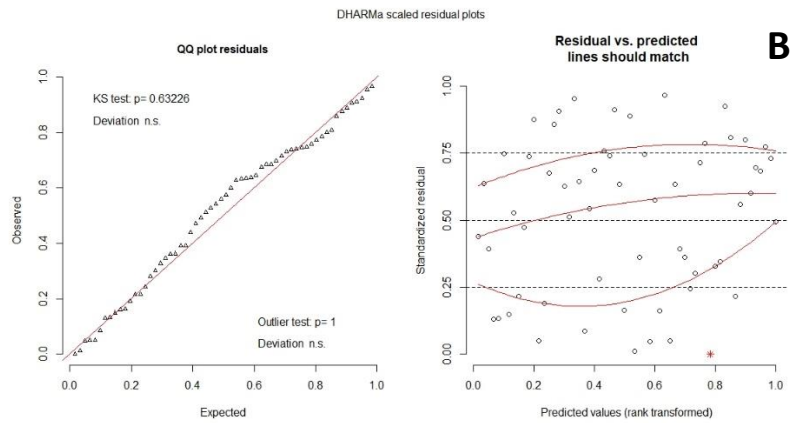
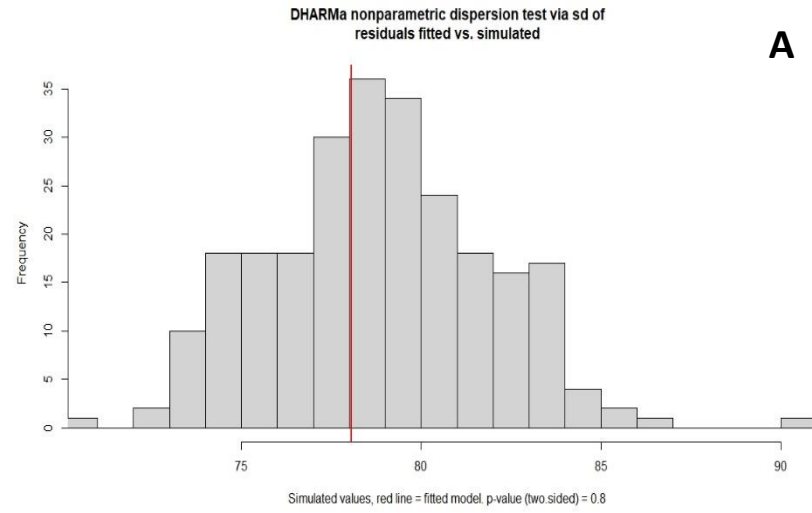
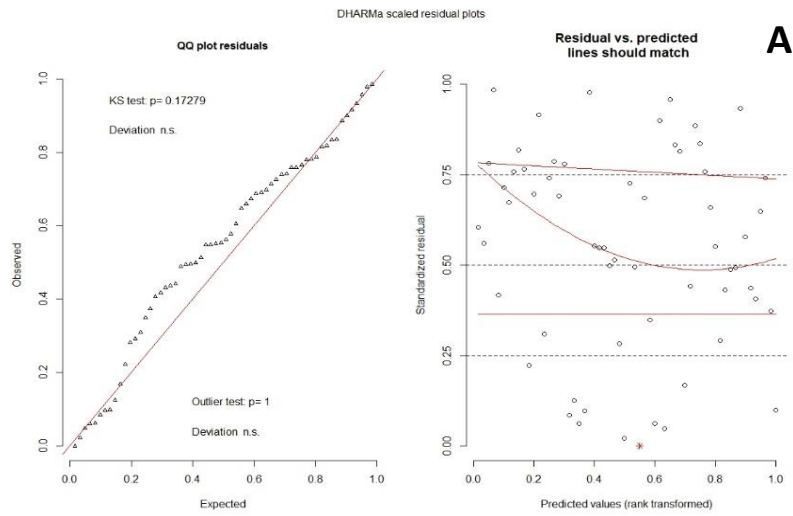
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49 **Table S14.** Parasitization rate of *Oligosita cf collina* and *Neodryinus typhlocybae* in each site.

Site	Parasitization rate of <i>Oligosita cf collina</i>	Parasitization rate of <i>Neodryinus typhlocybae</i>
1	0.34	0.30
2	0.44	0.25
3	0.47	0.18
4	0.36	0.20
5	0.45	0.32
6	0.41	0.26
7	0.39	0.29
8	0.46	0.27

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53 **Figure S8.** Graphs of the residual diagnostic for the selected models concerning the probability of parasitization **(A)**, and *Metcalfa pruinos*
 54 oviposition rate **(B)**.

8.3. NEW HOST-PARASITOID ASSOCIATION INVOLVING A CUCKOO WASP AND TWO VECTORS OF THE FLAVESCENCE DORÉE PHYTOPLASMAS: *SCAPHOIDEUS TITANUS* AND *ORIENTUS ISHIDAE*

Table S15. Number of leafhoppers collected during 2018 and 2019 explorations in North-west Italy in different geographic locations.

Site	Year	Location	Habitat	Leafhoppers species	Number of leafhoppers collected	Number of <i>Elampus bidens</i> larvae found				
1	2018	Portacomaro (AT) 44°57'43.9"N 8°15'29.6"E	Brushwood with grapevine	<i>Scaphoideus titanus</i>	152	31				
				<i>Orientus ishidae</i>	41	7				
				<i>Anoplotettix</i> spp.	45	0				
				<i>Cicadella viridis</i>	93	0				
				<i>Aphrophora alni</i>	12	0				
				<i>Aphrophora salicina</i>	21	0				
				<i>Aphrodes makarovi</i>	8	0				
				<i>Metcalfa pruinosa</i>	61	0				
				2		Mombaruzzo (AT) 44°45'38.7"N 8°24'32.9"E	Wood with wild grapevine	<i>Orientus ishidae</i>	93	0
								<i>Scaphoideus titanus</i>	68	0
<i>Issus</i> spp.	3	0								
<i>Metcalfa pruinosa</i>	26	0								
<i>Cicadella viridis</i>	32	0								
3		Borgiallo (TO) 45°23'48.2"N 7°40'02.4"E	Abandoned vineyard with a <i>Salix caprera</i> and <i>Castanea sativa</i> wood					<i>Scaphoideus titanus</i>	95	45

				<i>Orientus ishidae</i>	26	2
				<i>Cicadella viridis</i>	13	0
4	Ceres (TO) 45°20'03.6"N 7°23'10.8"E	Mixed oak- wood		<i>Orientus ishidae</i>	106	41
5	Tonengo (TO) 44°57'43.9"N 8°15'29.6"E	Brushwood with grapevine		<i>Scaphoideus titanus</i>	245	78
				<i>Philenus spumarius</i>	45	0
				<i>Neophilenus spp.</i>	53	0
				<i>Anoplotettix fuscovenosus</i>	25	0
6	San Giorgio Canavese (TO) 45°20'30.5"N 7°49'19.4"E	Abandoned vineyard with mixed oak-wood		<i>Orientus ishidae</i>	268	83
				<i>Scaphoideus titanus</i>	121	39
				<i>Anoplotettix fuscovenosus</i>	61	0
7	Montà d'Alba (CN) 44°48'23.4"N 7°58'55.7"E	Brushwood with grapevine		<i>Scaphoideus titanus</i>	163	0
				<i>Orientus ishidae</i>	81	0
				<i>Japananus hyalinus</i>	32	0
8	Bodio Lomnago (VA) 45°46'35.3"N 8°45'09.9"E	Mixed oak- wood		<i>Orientus ishidae</i>	235	91
				<i>Anoplotettix spp.</i>	45	0
				<i>Cicadella viridis</i>	87	0

				<i>Aphrophora</i> spp.	21	0
				<i>Metcalfa</i> <i>pruinosa</i>	68	0
9		Mazzè (TO) 45°18'32.2"N 7°55'27.4"E	Mixed oak- wood with wild grapevine	<i>Scaphoideus</i> <i>titanus</i>	76	24
				<i>Metcalfa</i> <i>pruinosa</i>	89	0
				<i>Philenus</i> <i>spumarius</i>	47	0
				<i>Cicadella</i> <i>viridis</i>	31	0
1	2019	Portacomaro (AT) 44°57'43.9"N 8°15'29.6"E	Brushwood with grapevine	<i>Scaphoideus</i> <i>titanus</i>	42	11
				<i>Orientus</i> <i>ishidae</i>	21	0
				<i>Anoplotettix</i> spp.	6	0
				<i>Cicadella</i> <i>viridis</i>	64	0
				<i>Aphrodes</i> <i>makarovi</i>	12	0
				<i>Metcalfa</i> <i>pruinosa</i>	43	0
10		Cuornè (TO) 45°24'06.5"N 7°39'47.7"E	Mixed oak- wood with wild grapevine	<i>Orientus</i> <i>ishidae</i>	187	91
				<i>Scaphoideus</i> <i>titanus</i>	69	12
11		Montafia (AT) 44°58'49.5"N 7°59'26.9"E	Brushwood with grapevine	<i>Orientus</i> <i>ishidae</i>	94	0
				<i>Anoplotettix</i> spp.	24	0
				<i>Aphrodes</i> <i>makarovi</i>	8	0
				<i>Cicadella</i> <i>viridis</i>	36	0

8	Bodio Lomnago (VA) 45°46'35.3"N 8°45'09.9"E	Wet woodland with a prevalence of willows, alders and hazelnuts	<i>Orientus ishidae</i>	265	83
			<i>Anoplotettix</i> spp.	45	0
			<i>Cicadella viridis</i>	87	0
			<i>Anoplotettix</i> spp.	38	0
4	Ceres (TO) 45°20'03.6"N 7°23'10.8"E	Mixed oak-wood	<i>Orientus ishidae</i>	85	34
3	Borgiallo (TO) 45°23'48.2"N 7°40'02.4"E	Abandoned vineyard with mixed willows and chestnut-wood	<i>Scaphoideus titanus</i>	72	37
			<i>Orientus ishidae</i>	27	7
			<i>Cicadella viridis</i>	13	0
12	Perletto (CN) 44°36'50.7"N 8°11'51.5"E	Brushwood with grapevine	<i>Scaphoideus titanus</i>	38	0
			<i>Metcalfa pruinosa</i>	56	0
			<i>Japananus hyalinus</i>	4	0
13	Albiano (TO) 45°25'60.0"N 7°57'21.5"E	Abandoned vineyard	<i>Scaphoideus titanus</i>	43	0
			<i>Orientus isidae</i>	26	0
			<i>Cicadella viridis</i>	13	0
7	Montà (CN)	d'Alba Abandoned vineyard	<i>Scaphoideus titanus</i>	91	0

	44°48'23.4"N 7°58'55.7"E	and mixed oak-wood	<i>Orientus ishidae</i>	93	0
14	Garlasco (PV) 45°13'06.6"N 8°56'49.9"E	Riparian forest	<i>Orientus ishidae</i>	34	3
15	Rocchetta Tanaro (AT) 44°51'08.9"N 8°21'24.9"E	Riparian forest and mixed oak- wood with wild grapevine	<i>Orientus ishidae</i> <i>Scaphoideus titanus</i> <i>Anoplotettix</i> spp. <i>Aphrophora alni</i> <i>Cicadella viridis</i>	117 58 49 38 76	34 12 0 0 0

Table S16. Number of predator wasps and *Elampus bidens* adults collected during 2018 and 2019 explorations in North-west Italy using different collections methods (Malaise traps, butterfly nets) in different geographic locations.

Site	Year	Location	Habitat	Collection Method	Psenid species	Number of apoid wasps and <i>elampus bidens</i> adults
1	2018	Portacomaro (AT) 44°57'43.9"N 8°15'29.6"E	Brushwood with grapevine	Malaise trap	<i>Gorytes quadrifasciatus</i>	2
					<i>Psen ater</i>	8
				Butterfly net	<i>Argogorytes</i> spp.	1
6		San Giorgio Canavese (TO) 45°20'30.5"N 7°49'19.4"E	Mixed oak-wood	Butterfly net	<i>Psen ater</i>	8
					<i>Gorytes quadrifasciatus</i>	1
2		Mombaruzzo (AT) 44°45'38.7"N 8°24'32.9"E	Wood with wild grapevine	Butterfly net	0	0
4		Ceres (TO) 45°20'03.6"N 7°23'10.8"E	Mixed oak-wood	Butterfly net	<i>Psen ater</i>	2
					<i>Elampus bidens</i>	4
7		Montà d'Alba (CN) 44°48'23.4"N 7°58'55.7"E	Brushwood with grapevine	Malaise trap	0	0
				Butterfly net	0	0
16		Caluso (TO) 45°18'08.4"N 7°54'56.0"E	Vineyard	Malaise trap	0	0
				Butterfly net	0	0

8		Bodio (VA) 45°46'35.3"N 8°45'09.9"E	Lomnago	Mixed oak-wood	Butterfly net	<i>Psen ater</i>	2
						<i>Elampus bidens</i>	5
3		Borgiallo (TO) 45°23'48.2"N 7°40'02.4"E		vineyard	Malaise trap	<i>Psen ater</i>	1
					Butterfly net	0	
1	2019	Portacomaro (AT) 44°57'43.9"N 8°15'29.6"E		Brushwood with grapevine	Butterfly net	<i>Psen ater</i>	2
6		San Canavese (TO) 45°20'30.5"N 7°49'19.4"E	Giorgio	Mixed oak-wood	Butterfly net	<i>Psen ater</i>	8
						<i>Gorytes quadrifasciatus</i>	1
4		Ceres (TO) 45°20'03.6"N 7°23'10.8"E		Mixed oak-wood	Butterfly net	<i>Psen ater</i>	2
						<i>Elampus bidens</i>	4
7		Montà d'Alba (CN) 44°48'23.4"N 7°58'55.7"E		Brushwood with grapevine	Butterfly net	0	
16		Caluso (TO) 45°18'08.4"N 7°54'56.0"E		Vineyard	Butterfly net	0	
8		Bodio (VA) 45°46'35.3"N 8°45'09.9"E	Lomnago	Mixed oak-wood	Butterfly net	<i>Psen ater</i>	4
						<i>Elampus bidens</i>	11

					<i>Gorytes quadrifasciatus</i>	1
3	Borgiallo 45°23'48.2"N 7°40'02.4"E	(TO)	vineyard	Butterfly net	<i>Elampus bidens</i>	3
					<i>Psen ater</i>	1
10	Cuornè (TO) 45°20'03.6"N 7°23'10.8"E		Mixed oak- wood with wild grapevine	Butterfly net	<i>Psen ater</i>	2
					<i>Elampus bidens</i>	5

Table S17. DNA sample and GenBank accession numbers.

Sam ple ID	COI Barcode GenBank Acc. No.	Order	Family	Subfamily	Tribe	Genus	Species	Sex	Det.	Country	Locality	Coordinates	Date	Leg.
ON1 1260	MT876911	Hymenoptera	Chrysididae	Chrysidinae	Elampini	<i>Elampus</i>	<i>bidens</i>	NA	Oliver Niehuis	Italy	Bodio Lomnago (VA)	45°46'35.3"N 8°45'09.9"E	19/07/2018	Federico Marco Bocca
ON1 1261	MT876912	Hymenoptera	Chrysididae	Chrysidinae	Elampini	<i>Elampus</i>	<i>bidens</i>	NA	Oliver Niehuis	Italy	Bodio Lomnago (VA)	45°46'35.3"N 8°45'09.9"E	19/07/2018	Federico Marco Bocca
ON1 1262	MT876913	Hymenoptera	Chrysididae	Chrysidinae	Elampini	<i>Elampus</i>	<i>bidens</i>	NA	Oliver Niehuis	Italy	Ceres (TO)	45°20'03.6"N 7°23'10.8"E	01/08/2018	Federico Marco Bocca
ON1 1263	MT876914	Hymenoptera	Chrysididae	Chrysidinae	Elampini	<i>Elampus</i>	<i>bidens</i>	NA	Oliver Niehuis	Italy	Ceres (TO)	45°20'03.6"N 7°23'10.8"E	03/08/2018	Federico Marco Bocca
ON1 1264	MT876915	Hymenoptera	Chrysididae	Chrysidinae	Elampini	<i>Elampus</i>	<i>bidens</i>	NA	Oliver Niehuis	Italy	Bodio Lomnago (VA)	45°46'35.3"N 8°45'09.9"E	19/07/2018	Federico Marco Bocca
ON1 1265	MT876916	Hymenoptera	Chrysididae	Chrysidinae	Elampini	<i>Elampus</i>	<i>bidens</i>	NA	Oliver Niehuis	Italy	S. Giorgio Canavese (TO)	45°20'30.5"N 7°49'19.4"E	September 2015	Federico Marco Bocca
ON1 1266	MT876917	Hymenoptera	Chrysididae	Chrysidinae	Elampini	<i>Elampus</i>	<i>bidens</i>	NA	Oliver Niehuis	Italy	Borgiallo (TO)	45°23'48.2"N 7°40'02.4"E	September 2015	Federico Marco Bocca
ON1 1267	MT876918	Hymenoptera	Chrysididae	Chrysidinae	Elampini	<i>Elampus</i>	<i>bidens</i>	NA	Oliver Niehuis	Italy	Portacomaro (AT)	44°57'43.9"N 8°15'29.6"E	September 2015	Federico Marco Bocca
ON1 1268	MT876919	Hymenoptera	Chrysididae	Chrysidinae	Elampini	<i>Elampus</i>	<i>bidens</i>	NA	Oliver Niehuis	Italy	Portacomaro (AT)	44°57'43.9"N 8°15'29.6"E	August 2015	Federico Marco Bocca
ON1 1269	MT876920	Hymenoptera	Chrysididae	Chrysidinae	Elampini	<i>Elampus</i>	<i>bidens</i>	NA	Oliver Niehuis	Italy	Portacomaro (AT)	44°57'43.9"N 8°15'29.6"E	August 2015	Federico Marco Bocca
ON1 1270	MT876921	Hymenoptera	Chrysididae	Chrysidinae	Elampini	<i>Elampus</i>	<i>bidens</i>	NA	Oliver Niehuis	Italy	Portacomaro (AT)	44°57'43.9"N 8°15'29.6"E	15/09/15	Federico Marco Bocca
ON1 1271	MT876922	Hymenoptera	Chrysididae	Chrysidinae	Elampini	<i>Elampus</i>	<i>bidens</i>	NA	Oliver Niehuis	Italy	Mazzè (TO)	45°18'32.2"N 7°55'27.4"E	11/10/15	Federico Marco Bocca
ON1 0555	MT876923	Hymenoptera	Chrysididae	Chrysidinae	Elampini	<i>Elampus</i>	<i>sanzii</i>	male	Paolo Rosa	Italy	Pondel (AO)	45°40'56.1"N 7°13'14.6"E	19/06/2012	Paolo Rosa
ON1 0556	MT876924	Hymenoptera	Chrysididae	Chrysidinae	Elampini	<i>Elampus</i>	<i>sanzii</i>	male	Paolo Rosa	Italy	Vaglio Serra (AT),	44°47'39.9"N 8°20'49.0"E	10- 25/08/2017	Paolo Rosa

1

2 **Table S18.** Selected and alternative models with their AIC and BIC values.

Models:

Selected model: `cbind(parasitized, healthy) ~ year * zone + (1 | site), zi=~0, disp=~1`

Alternative model: `cbind(parasitized, healthy) ~ year * zone + data + (1 | site), zi=~0, disp=~1`

	Df	AIC	BIC	logLik	deviance	Chisq	Chi Df	Pr(>Chisq)	
Selected model	7	571.50	595.51	-278.75	557.50				
Alternative model	15	561.09	612.53	-265.55	531.09	26.414	8	0.000892	***

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5 **Table S19.** The graph shows all pairwise comparisons among the three zone levels separately for each year.
 6 Post-hoc analysis using sidak adjustment was conducted.

Contrast	Odds ratio	SE	CI	Pr(> z)
Year 2016				
Canavese zone 1 / Monferrato zone 3	26.81	25.71	2.80 to 257.62	0.002
Canavese zone 1 / Canavese zone 2	1.22	1.03	0.17 to 8.98	0.99
Monferrato zone 3 / Canavese zone 2	0.04	0.04	0.005 to 0.43	0.004
Year 2017				
Canavese zone 1 / Monferrato zone 3	7.25	7.19	0.70 to 75.34	0.13
Canavese zone 1 / Canavese zone 2	0.19	0.17	0.02 to 1.51	0.17
Monferrato zone 3 / Canavese zone 2	0.03	0.02	0.003 to 0.25	0.0006

7 SE standard error of parameter estimates. CI confident interval. Pr(|z|) probability of the observed Odds ratio
 8 being greater than the critical value.

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10

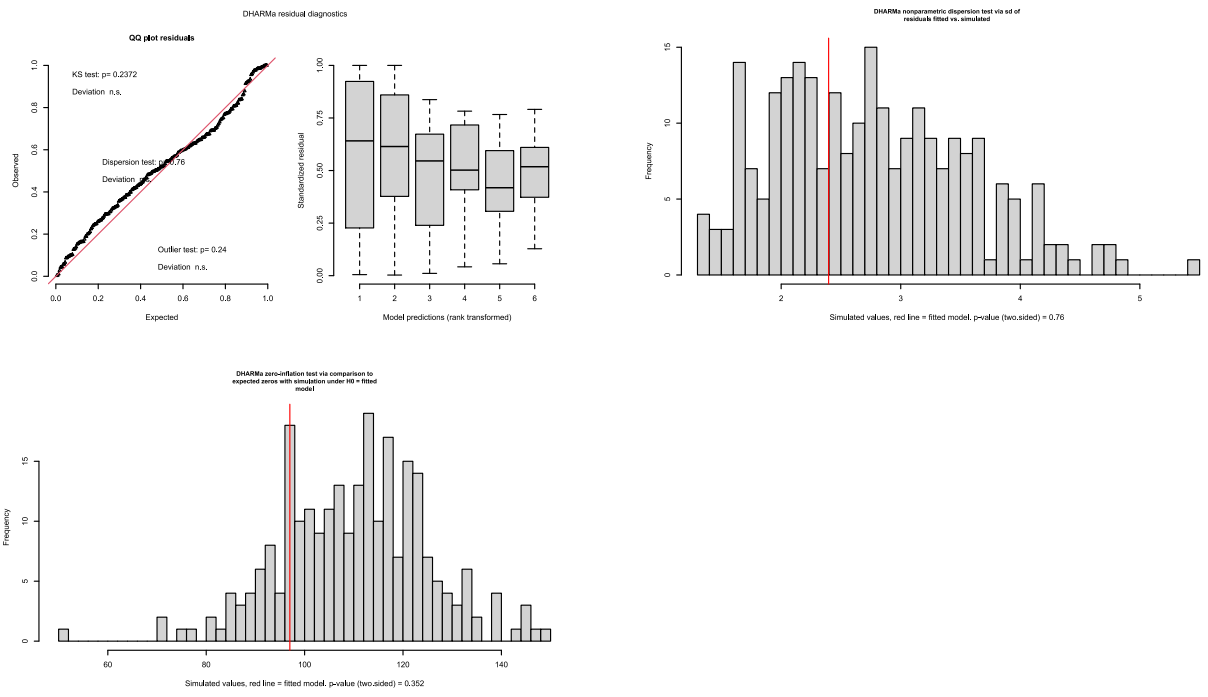
11 **Table S20.** The graph shows all pairwise comparisons between the two years levels separately for each zone.
 12 Post-hoc analysis using sidak adjustment was conducted.

Contrast	Odds ratio	SE	CI	Pr(> z)
Canavese zone 1				
Year 2016 / Year 2017	7.19	2.86	3028 to 15.74	<.0001
Canavese zone 2				
Year 2016 / Year 2017	1.11	0.16	0.83 to 1.49	0.47
Monferrato zone 3				
Year 2016 / Year 2017	1.94	0.54	1.12 to 3.36	0.01

13 SE standard error of parameter estimates. CI confident interval. Pr(|z|) probability of the observed Odds ratio
 14 being greater than the critical value.

15

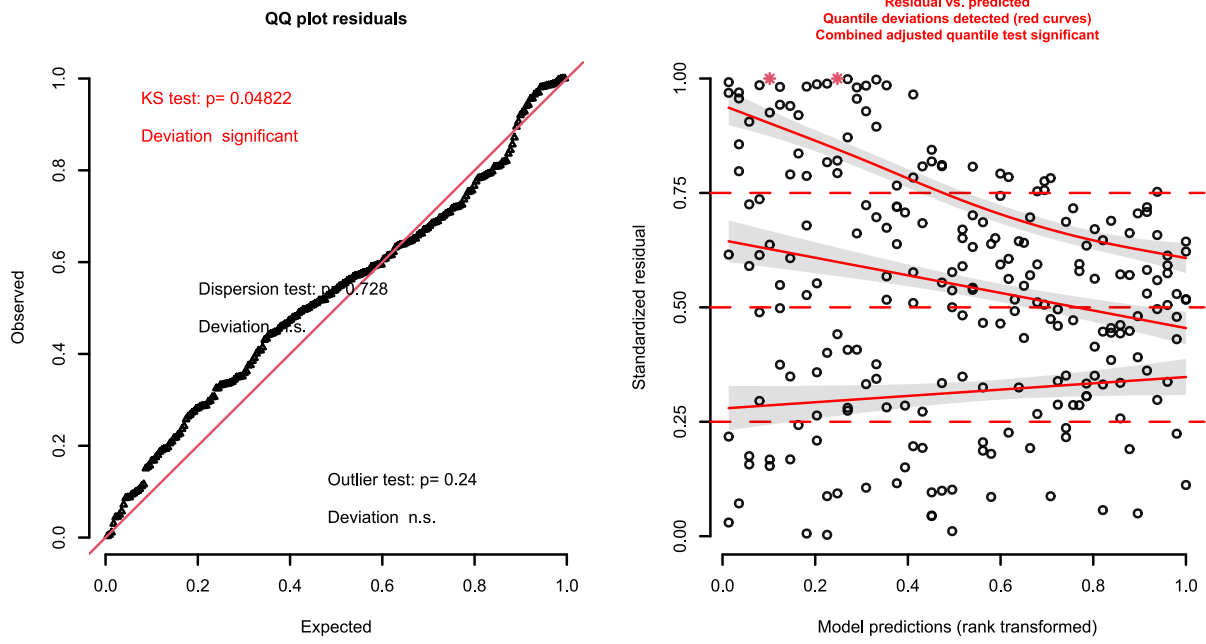
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17 **Figure S9.** Graphs of the residual diagnostic for the selected models concerning the probability of
 18 parasitization.

19

DHARMA residual diagnostics



20

21 **Figure S10.** Graphs of the residual diagnostic for the alternative model concerning the probability
22 of parasitization.

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