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Tritrophic relations within the parasitoid guild of Halyomorpha halys (Hemiptera: Pentatomidae) and implications for biological control

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Abstract

Each year, invasive species cause significant ecological and economic damage globally, and their numbers are expected to increase due to the effects of climate changes and globalization. The brown marmorated stink bug, *Halyomorpha halys* (Stål, 1855) is a recent example of a serious invasive pest of worldwide importance. In the invaded area *H. halys* has caused severe damage to agricultural crops leading to an increase in the use of chemical insecticides and therefore a negative impact on the environment and human health. The low efficacy of insecticide treatments, combined with the lack of effective indigenous natural enemies, led to considering classical biological control a promising method for the long-term management of this pest in the invaded areas. Among the natural enemies of *H. halys*, the Hymenoptera egg parasitoids play a significant role.

This Ph.D. thesis focused on assessing the distribution of the egg parasitoid species in those invaded areas of Europe where *H. halys* is abundant, providing at a simple morphological diagnostic tool that could support the identification of species during field surveys (Chapter 1). The results of monitoring evidenced the wide distribution of two exotic egg parasitoids *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae), the main natural enemy of *H. halys* in the area of origin, and *Trissolcus mitsukurii* (Ashmead) (Hymenoptera: Scelionidae) as well the presence of the native *Anastatus bifasciatus* (Geoffroy) (Hymenoptera, Eupelmidae) and the exotic hyperparasitoid *Acroclisoides sinicus* (Huang & Liao) (Hymenoptera, Pteromalidae). The presence and the high impact of both *Trissolcus* species on *H. halys* egg masses led to consider these exotic species as valid candidates for the biological control of *H. halys* in the invaded area. For this reason analysis of risk assessment on non-target hosts (in Chapter 2) was conducted on both *Trissolcus* species through different multidisciplinary approaches. Although *no choice* tests have shown the polyphagous habitus of both species under laboratory conditions, tests that include the effects of the interactions between the pest and host plant, such as in the olfactometer bioassays, demonstrate the preference for the co-evolved host. The co-presence of the two *Trissolcus* species in the invaded area also suggest the need to investigate (in Chapter 3) how these species interact with each other and the way these species exploit the common resources under different competition scenarios. The behavioral responses and the different reproductive success of each species within the different cases provided information that could be useful to understand how the dynamic populations will evolve and how to improve the strategies in the biological control program against *H. halys*. The last step, following the discovery of the exotic parasitoid *A. sinicus*, frequently found associated with *H. halys* and its parasitoid guild, detailed studies were conducted on the reproductive and developmental biology of this species, until now completely unknown (in Chapter 4). The study of its biology confirmed a previous hypothesis of an obligate hyperparasitoid, in particular of Scelionidae species such as *T. japonicus* and

T.mitsukurii but also *Telenomus sp.*. Since obligate hyperparasitoids occupy the highest level in multitrophic systems *Ac. sinicus* may interfere with the population densities of the primary parasitoid, and, therefore, with the biological control program.

Overall, the results of this thesis provide an overview of the ecology and ethology of parasitoid species associated with *H.halys* in the invaded area and contribute to improving the management of this invasive species in the biocontrol programs.

Riassunto

Le specie invasive causano ogni anno danni ecologici ed economici ingenti a livello globale e il loro numero è destinato ad aumentare per effetto dei cambiamenti climatici e della globalizzazione. La cimice asiatica *Halyomorpha halys*, ormai diffusa in molte parti del mondo, ne costituisce un esempio. Nelle aree di nuova introduzione *H. halys* ha causato danni significativi alle coltivazioni agricole con conseguente aumento nell'utilizzo di insetticidi ed effetti a cascata sull'ambiente e la salute umana. La scarsa efficacia dei trattamenti unita al limitato impatto degli antagonisti naturali indigeni ha portato a considerare il controllo biologico classico il metodo più efficace per una soluzione a lungo termine. Tra i principali antagonisti naturali di *H. halys* gli imenotteri ooparassitoidi rivestono un ruolo rilevante.

Questa tesi di Dottorato ha avuto tra gli obiettivi quello di valutare la distribuzione delle specie di ooparassitoidi in quelle aree dell'Europa in cui *H. halys* è risultata essere più abbondante fornendo allo stesso tempo uno strumento di analisi morfologica utile ad una prima identificazione delle specie durante i monitoraggi in campo (Capitolo 1). Grazie al monitoraggio si è messo in evidenza la presenza di due specie esotiche di ooparassitoidi, *Trissolcus japonicus*, il principale antagonista naturale di *H. halys* e di *Trissolcus mitsukurii*, ma anche la diffusione del parassitoide indigeno *Anastatus bifasciatus*. La presenza delle due specie di *Trissolcus* e il loro elevato impatto sulle ovature di *H. halys*, hanno portato a considerare queste due specie come le migliori candidate per il controllo biologico di *H. halys*. Sono stati pertanto condotti studi di analisi del rischio (Capitolo 2) per valutare l'impatto potenziale che queste due specie possono provocare su ospiti indigeni. Sebbene da *no-choice* test si sia evidenziato l'aspetto polifago delle due specie, test che includono invece anche gli effetti delle interazioni ospite-pianta ospite, come quelli riprodotti grazie all'utilizzo dell'olfattometro, hanno mostrato la preferenza da parte del parassitoide verso l'ospite coevoluto.

La co-presenza delle due specie di *Trissolcus* ha inoltre fornito la necessità di investigare i comportamenti messi in atto dalle due specie che utilizzano la stessa risorsa testando diversi possibili scenari di competizione diretta e indiretta. Le risposte comportamentali delle due specie e i differenti successi riproduttivi ottenuti nei diversi contesti hanno fornito informazioni utili a prevedere le dinamiche di popolazione delle due specie e a migliorare così i programmi di lotta biologica. Infine, dato il recente ritrovamento in Europa e negli USA di *Acroclisoides sinicus*, una terza specie esotica di parassitoide associata ad *H. halys*, studi di laboratorio sono stati condotti per esplorare la biologia di questa specie, fino ad oggi pressoché sconosciuta. I risultati hanno confermato l'ipotesi che si trattasse di un iperparassitoide obbligato, in particolare delle specie appartenenti alla famiglia Scelionidae, tra cui i due *Trissolcus* ma anche specie appartenenti al genere *Telenomus*, e quindi la

possibilità che *Ac. sinicus* possa interferire con le popolazioni degli ooparassitoidi e con i programmi di lotta biologica messi in atto.

Nel complesso, i risultati di questa tesi forniscono importanti informazioni sull'ecologia e dell'etologia delle specie di parassitoidi associate a *H. halys* nelle aree di nuova introduzione che saranno fondamentali per una corretta gestione di questa specie invasiva nei programmi di lotta biologica.

Introduction

Historical overview of biological control

Humans have quite a dominant position in many ecosystems depending on many other species for food and shelter and their influence is so pervasive throughout that find themselves competing with other organisms (Hajek and Eilenberg, 2018). When other organisms compete for resources with humans or negatively affect them in other ways, they are generally regarded as “pests” (Hill, 1997). Pests are taxonomically diverse ranging from microorganisms to plants and animals.

Humans have always needed to control pests affecting or competing with them directly and through the ages pest control practices have changed. The earliest known record of the use of naturally occurring compounds for pest control was around 2000 BC. in a Hindu book, the *Rig Veda*, written that makes mention of the use of compounds of poisonous plants for pest control (Hajek and Eilenberg, 2018). At the time of the pharaohs, the ancient Egyptian used compounds extracted from plants to help with insect control, for example, a popular recipe for pest control was to spray the house with Nitron water and firewood coal, mixed with ground “pipit ” plant. Around 1000 BC Homer the Greek mentioned using sulfur as a fumigant to control pests, and in 77 BC Pliny the Roman reported that arsenic was insecticidal (Hajek and Eilenberg, 2018).

Through time overriding methods for pest control were mostly cultural practices, such as rotating crops. For example, when soybean crops are rotated with corn (maize), the population of soil-dwelling nematodes that attack soybean roots declines when corn is growing, and after the corn crop has been harvested, soybeans can again be planted in the field. In general, through the use of cultural controls, growers were basically manipulating and augmenting the naturally occurring processes of pest suppression.

Several developments took place between World War I and II, setting the stage for major changes in pest control. Since that time, a cascade of different compounds belonging to an increasing number of chemical classes has been synthesized for pest control (Hajek and Eilenberg, 2018).

At the same time, for hundreds of years, humans have been introducing plants, animals, and other organisms around the world, in a relatively slow process of globalizing the Earth’s biota (DiCasti 1989). More recently, this process has significantly increased with modern trade, travel, and technology, so biological invasions have become a consequence of globalization. Globalization facilitates and intensifies the spread of invasive alien species— defined here as “alien species whose introduction does or is likely to, cause economic or environmental harm or harm to human health” (Executive Order 1999) – through intentional or accidental introductions: changes in land use, disturbance, destruction of natural areas but also climate change (Ziska et al. 2011) have often opened habitats for introduced species to become established.

To a large degree, the most serious insect and weed pests around the world are non-native, invasive species (Perrings et al. 2002). Many of these introduced species are not problematic in their areas of origin, where they are often controlled by a community of natural enemies (Hajek and Eilenberg, 2018).

Chemical pesticides have proved effective in many cases but in turn, these create even greater damage. Some of the problems associated with using pesticides include failure of pest control, contamination of the environment, and damage to human health. The development of pest resistance to insecticides is one of the main reasons why pest control failure occurs and since 1945, the number of insects, weeds and plant pathogens that have become resistant to pesticides has increased dramatically (Van Driesche and Bellows Jr, 2012).

Another way in which pesticide use can foster outbreaks of pest is the destruction of the target pest natural enemies which often are more sensitive to the pesticide than the pest itself (Wilson and Tisdell, 2001, Desneux et al. 2007). An indirect effect of pesticides is also creating new pests when natural enemies of ordinarily harmless species (or minor pest) are destroyed by chemicals (Hardin et al. 1995; Cloyd, 2012). In this way, the “secondary pests” reach higher densities than normal, begin to cause economic damage and become targets of pesticides themselves. Worldwide pesticide use has increased twelvefold since the early 1950s (Van Driesche and Bellows Jr, 2012).

Although synthetic chemical pesticides are still the most widely used method for pest control, for the reasons listed above, it is necessary to consider alternatives. Among these, biological control can be an extremely important alternative to pest management to preserve the local and global environments as well as human health (Lewis et al. 1997). In addition, one of the overriding benefits of biological control, when compared to any pest management approaches based on chemical pesticides, is that it can be permanent in nature and self-propagating. (Clercq et al. 2011; Van Driesche and Bellows, 2012; Hajek and Eilenberg, 2018). This approach is often thought of as a function of a healthy ecosystem and one that is beneficial for all living organisms, for this reason biological control can be considered one of the most important “ecosystem services” (Bengtsson, 2015, Hajek and Eilenberg, 2018).

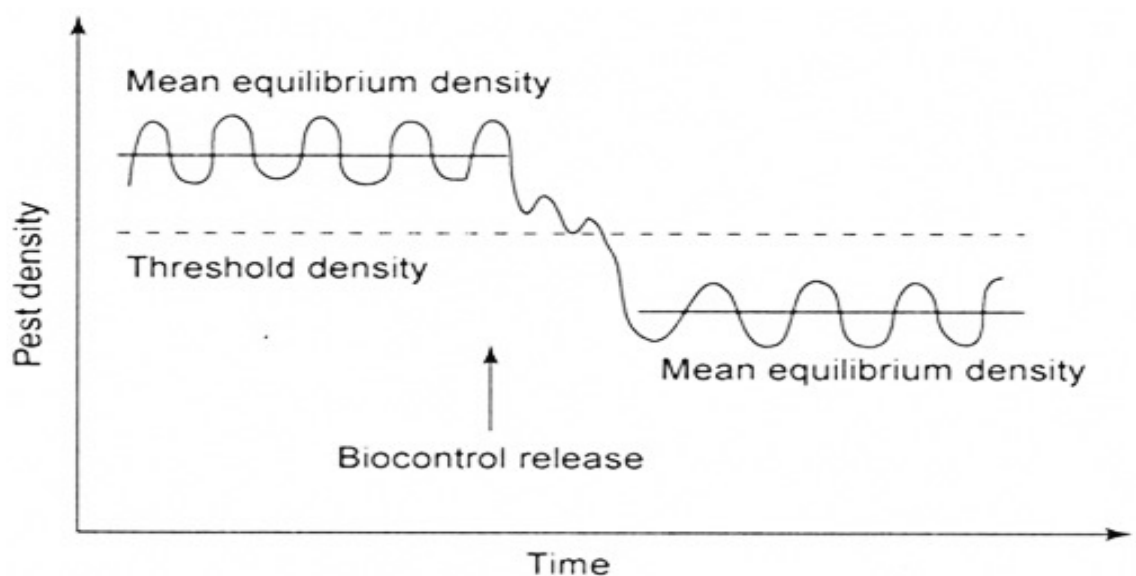
Populations of all living organisms are, to some degree, reduced by the natural actions of their predators, parasites, pathogens, and antagonists and these processes have been referred to as “natural control”. However, when organisms that are considered pests are controlled by natural enemies, it is often called “biological control”.(Hajek and Eilenberg, 2018). Biological control has been defined many times, but a commonly accepted definition is:

The use of living organisms to suppress the population of a specific pest organism, making it less abundant or less damaging than it would otherwise be. (Eilenberg et.al 2001)

The goal of several different biological control strategies is to reestablish the “balance of nature”, either by introducing exotic natural enemies for permanent establishment (classical biological control) or by altering the environment to conserve or enhance the activity of natural enemy populations (conservation biological control) (Ehrlich and Birch, 1967) (Fig. 1).

Chinese were the first to use natural enemies to control insect pests. Nests of the ant *Oecophylla smaragdina* were sold near Canton in the 3rd century for use in the control of citrus insect pests such as *Tessarotoma papillosa* (Lepidoptera). In 1776, in Europe, the control of *Cimex lectularius* was successfully carried out by the release of the predatory pentatomid *Picromerus bidens*. In Italy, the first use of classical biological control began in the 20th century with the inoculative release of natural enemies introduced to balance the severe epidemics of exotic pests (Nicoli, 1997). In this context, the first success was achieved by Antonio Berlese with the inoculative release in 1901 of the predator *Rodolia cardinalis* to control the cottony cushion scale of Australian origin, *Icerya purchasi* (Viggiani, 1994) and in 1906 of the parasitoid *Encarsia* (= *Prospaltella*) *berlesei* to control the white peach scale, from China, *Pseudaulacaspis pentagona* (Berlese, 1910).

Figure 1. Theoretical control of pest species using biocontrol agents, illustrating how pest densities might fluctuate over time before and after the introduction of a successful biocontrol agent. Before the introduction of the biocontrol agent, the pest density fluctuates around a mean equilibrium density that is above a threshold of economic or ecological impact. Following the introduction of the biocontrol agent, the pest densities stabilize at a new equilibrium level that is below the threshold of impact (Kilgore and Douth, 1967 adapted by Pearson and Callaway, 2003).



Insights of natural enemies

The natural enemies used to control invertebrates are taxonomically as well as functionally diverse. Early models (Mason et al. 2008; Stiling and Cornelissen, 2005) suggested the following general attributes as characterizing successful biological control agents: (1) host specificity, (2) synchrony with the pest, (3) high rate of increase, (4) ability to survive periods with few to no prey, and (5) good searching ability.

When natural enemies control populations of prey or hosts, this has been called population regulation. Populations are generally controlled by some combination of extrinsic factors, such as the effects of natural enemies, or climate conditions and intrinsic factors such as genetic changes in a population or intraspecific and interspecific competition (Ehrlich and Birch, 1967).

The behavior of natural enemies and pests such as the responses of natural enemies to pest densities, and actions of natural enemies and hosts on a spatial scale are central issues in developing theories regarding how natural enemies persist and coexist with their prey or host (Hajek and Eilenberg, 2018). Most natural enemies have hosts that they preferentially attack but many are also able to use a secondary set of hosts when the most common preferred hosts are unavailable. The use of a secondary host or prey species is sometimes referred to as a spillover event, however, the specificity of natural enemies, as cited above, is an aspect that must be always considered for the biological control of pests (Colautti et al. 2004, Hajek and Eilenberg, 2018). Since classical biological control foresees the introduction of exotic natural enemies in a new environment, close attention has always been paid prior to the use and release of biological control agents. In general, for classical biological control, introducing a specialized exotic natural enemy to control a pest from the area of origin (an “old association”) is considered much safer than introducing an exotic natural enemy to control a pest that the natural enemy did not evolve with (a “new association”) (Hajek and Eilenberg, 2018) although some studies show that this is not always true (Hokkanen & Pimentel 1989).

Both native and non-native biological control agents may in theory cause undesired side effects (Goettel and Inglis 2006; Hopper et al. 2006; van Lenteren et al. 2003) but in most cases, the use of an exotic natural enemy is required for effective suppression of an exotic pest. There have been huge economic and ecological benefits from the use of exotic biological control agents. The economic benefits are not regularly assessed but cost benefit analyses—offering a systematic way of determining if the use of biological control results in a net gain—have been made for several arthropod pests (De Clercq et al. 2011).

Potential risks of biological control agents can be considered in three categories: risks to human and animal health, plant health risks, and environmental risks. In the case of invertebrate biological control agents, potential risks to the environment are generally considered to be the most important

(De Clercq et al. 2011). Environmental risks are those whereby introductions of a biological control agent lead to non-target impacts which include changes in the abundance or distribution of native species, intraguild interactions or competitive displacement that interferes with the efficacy of native natural enemies of pest species, vectoring harmful pathogens to native species, genetic dilution of native species via hybridization with closely related exotics, and any major change in the balance of native species up to perturbation of ecosystem functions such as pollination (van Lenteren et al. 2006a). Obviously, non-target effects can range from very small effects, e.g. 2% parasitization of a parasitoid biological control agent on a non-target insect on a local level, to massive effects on a large scale (Driesche and Hoddle, 2017).

A new species that establish into an ecosystem is bound to impact in some way and there are examples of polyphagous parasitoids or generalist predators which have been very damaging to non-target organisms. A difficult but hotly debated topic is that exotic biological control agents may cause unpredictable and potentially significant indirect effects for native species. Obviously, polyphagous agents especially have the potential to do so but even rather host specific ones may have individual impacts as well. For this reason, the evaluation of the host range, defined as the group of potential hosts that, for example, a parasitoid species can attack successfully (Shaw, 1994), is one of the most important steps prior to considering the release of biological control agent (Santos and Quicke, 2011). The range of hosts utilized by a natural enemy can be ranging from monophagous to oligophagous to polyphagous. Generally, control programs aim to use natural enemies that are monophagous, those species that use only one host or prey species (Sheehan, 1986). However, efficient, strictly monophagous natural enemies are rarely available and/or not easy to culture successfully.

More generally, species that usually exploit only one host or prey species occasionally switch and use different host/prey species, for this, these species are considered the best choice for biological control programs. Instead, polyphagous species benefit from numerous hosts so they are carefully considered for the introduction of exotic species in biological control programs (Hajek and Eilenberg, 2018).

International laws and agreements together with a growing interest in the import and release of exotic biological control agents require harmonized and appropriate regulation. However, the provisions of this legislation vary considerably from one country to another. In Europe, a commission for the International Organization for Biological Control IOBC/WPRS was established in 2003 and a meeting of scientists, together with industry and regulators of biological control, led to A Document Providing Detailed Guidance on Regulatory Procedures for Exotic and Indigenous Biological Control Agents (Bigler et al., 2005). More recently, the European Commission has published a call for proposals for the development of a balanced regulatory system for organic products (micro-organisms and macroorganisms). It is expected that within a few years EU Member States and other European

countries will be able to regulate the agents of biological control of invertebrates on the basis of uniform principles (Bigler et al. 2006).

Parasitoids have been used extensively and have been central to the growth of biological control of arthropods (see next chapter). The high degree of host specificity, a common characteristic of many parasitoid species, makes these natural enemies the first choices for classical biological control introductions (Waage and Hassell, 1982, Godfray, 1994, Hajek and Eilenberg, 2018). In an estimation made in 2016, a total of 1512 species of parasitoids had been introduced for classical biological control worldwide (Hajek and Eilenberg, 2018).

Insect parasitoids are important subjects of behavioral and population studies because they are remarkably common in nature, are frequently easy to raise and handle and, more importantly, are key species for the biological control of many insect pests (Waage and Hassell, 1982; Godfray, 1994). The majority of these were parasitic wasps.

The Parasitoids

General Introduction

The term “parasitoids” was introduced by Reuter in 1913 with the following description:

Adult female parasitoids are free-living, feed on nectar, pollen or as predators and forage actively for their arthropod hosts on plants and other substrates. Usually, on locating a host, the female lays one or more eggs on or in it, and the ensuing larvae consume the host tissue, killing the host in the process.

It is only in the last twenty years that this term has become universally accepted.

In many ways, parasitoids are intermediate between predators and parasites: like predators, they always kill the host they attack; like many parasites, they require just a single host on which to mature. A parasitoid is defined by the feeding habit of its larva. In fact, the larva feeds exclusively on the body of another arthropod, its host, eventually killing it. Only a single host is required for the parasitoid to complete development, and often a number of parasitoids develop gregariously on the same host (Eggleton and Belshaw 1992, Godfray, 1994; Harvey, 2004a).

Parasitoids are abundant components of nearly all terrestrial ecosystems, both in terms of number of species and number of individuals. They are extremely common among wasps (Order Hymenoptera), less common among flies (Order Diptera), and although found in the beetles (Order Coleoptera),

moths and butterflies (order Lepidoptera), and lacewing order (Neuroptera), this lifestyle is rare in these last groups (Godfray, 1994).

Assuming that parasitoids constitute 20%-25% of the 8 million species of insects, these estimates of around 1.6-2 million on the number of species of parasitoids on earth.

The hosts of parasitoids are almost exclusively insects themselves, although spiders and even centipedes are occasionally parasitized (Godfray, 1994; Quicke 1997; Fei et al. 2022). All parasitoids belong to the holo-metabolous insect orders so, their life cycle can be divided into four stages—egg, larva, pupa, and adult—and for this, they (Godfray, 1994; Harvey, 2005).

Hosts are usually located by the adult parasitoid female who lays her eggs either directly on the host or in its immediate vicinity (Vinson, 1976; Godfray, 1994; Harvey, 2005). Other adult parasitoid females, such as those belonging to the Hymenoptera order, have highly specialized ovipositors which are used both to access and assess hosts, lay eggs and inject various secretions on or into them. These venom secretions may cause temporary or permanent paralysis, or modify the host's immune system and/or its metabolic functions (Gauld and Bolton 1988, Godfray 1994; Quicke 1997). Some parasitoids, like those to attack “concealed” hosts, developed long ovipositors that when not in use, either extend beyond the end of the abdomen enclosed between a protective valve or are coiled inside the abdomen of the female (Godfray, 1994, Quicke et al. 1999, Vilhelmsen, 2003).

The juvenile stages of insects are more frequently attacked but few groups attack also adult insects. The parasitoids can be classified by the stage of development they attack; *egg parasitoids*, *larval parasitoids*, *pupal parasitoids*, or *adult parasitoids* (Mills, 1992; Godfray, 1994; Brodeur and Boivin, 2004). However, some parasitoids lay eggs in one host stage but their progeny do not kill the host until it has entered a later stage, for example, *egg-larval* and *larval-pupal parasitoids* (Godfray, 1994).

Parasitoids can be divided into two classes by the feeding behavior of their larvae. Some parasitoids develop within the body of their host, feeding from the inside, these are known as *endoparasitoids*, someone else, the *ectoparasitoids*, live externally. Most fall into one of these groups though there are a minority of species that spend their first few instars as *ectoparasitoids* before burrowing into their hosts to become endoparasitoids, or vice-versa.

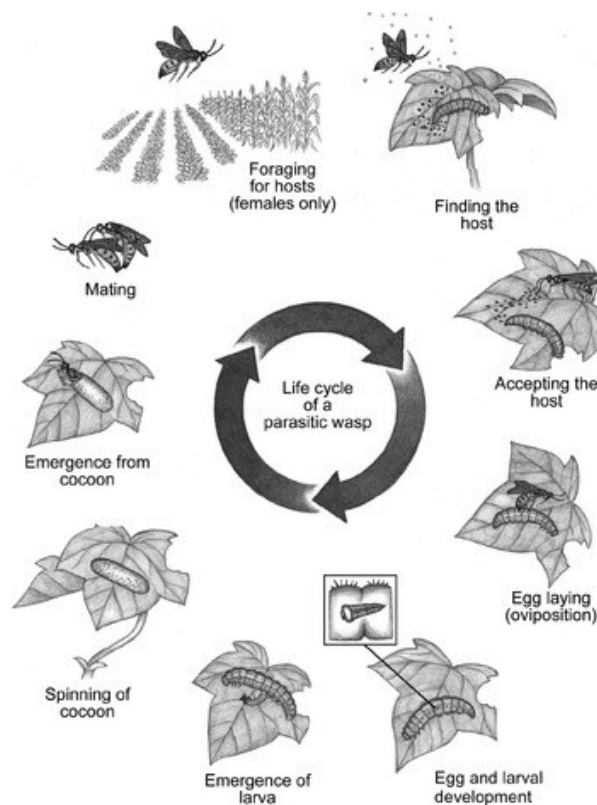
There are different types of parasitization; if further eggs are deposited on the host by the same species of parasitoids, *superparasitism* occurs. If a second female of a different species lays her eggs on the host, and if the larvae of this second female compete with the resident larvae for host resource, *multiparasitism* occurs. Instead, if the larvae of the second species feed, non on the host, but on the parasitoid larvae already present, *hyperparasitism* occurs. Hyperparasitism in turn is generally of two kinds: *facultative hyperparasitoids*, where the individual is able to attack unparasitized host individuals and only develop as hyperparasitoids when eggs are laid on a previously parasitized host,

in contrast, *obligate hyperparasitoids* are only able to develop as parasitoids of parasitoids (Godfray, 1994; Brodeur and Boivin, 2004). Parasitoids that allow hosts to continue to grow in size after parasitism are called koinobionts as opposed to idiobionts, where the parasitoid are known to only attack and develop in non- growing host stages such as eggs or pupae (Haeselbarth 1979; Askew and Shaw 1986; Harvey, 2005).

Life strategy

Although some unusual variants exist, the majority of parasitoids have broadly similar life histories and face many similar evolutionary challenges (Harvey, 2005). Insect parasitoids include the smallest living insects (species of Mymaridae, some <0.2 mm long) as well as the impressive ichneumonids of the genus *Megarhyssa*, which may be 150 mm long, including a 100-mm ovipositor (Quicke, 2015). They exploit one of the most varied biological resources on Earth (other insects and some other arthropods), and do so in an impressive variety of ways (Gauld & Bolton, 1988; Godfray, 1994; Quicke, 1997; Jervis and Ferns, 2011). However, the complexity of the environments inhabited by most parasitoids, finding a suitable host appears a critical step. Salt (1935) was the first to divide host location and attack into “ecological” and “behavioral” components, in fact, the process of locating the “correct” hosts can be thought of in terms of a progression of several generalized steps: locating the host habitat, host location, and then evaluating the host acceptance and host suitability (Fig.2).

Figure 2. Example of the life cycle of a parasitic wasp. (drawn by Patricia J. Wynne; Tumlinson et al. 1993)



-Locating the host habitat and finding the host

Long-distance habitat location is difficult to study and replicate under laboratory conditions but may involve a combination of visual, olfactory, and sometimes auditory cues. The cues originating from the host itself might be difficult to detect over long distances, more likely, cues from the host's microhabitat can attract parasitoids in the absence of the host itself such as volatile profiles emitted by the plants host damaged by the herbivorous. The most likely scenario right now is the complexity of the interaction between host plant, host, and parasitoid and the complexity of *semiochemical* associated (*allelochemicals and pheromones*). In addition, the parasitoid can obtain useful information about the distribution of hosts both from its emergence site and also from its experiences while searching for hosts (Godfray and Waage 1988, Mueller, 1983, McAuslane et al. 1990a).

-Accepting host

Parasitoids use a variety of cues to assess the quality of their hosts. One of the first attempts to understand the proximate mechanisms involved in host acceptance was Salt's study (1935). Many species, especially those attacking immobile hosts such as egg, pupae or scale insect, spend a great time externally examining the host by tapping or drumming with their antennae over its surface. Chemical cues perceived through receptors in the antennae and tarsi are undoubtedly of great importance in host acceptance and chemical compounds present on the surface of the host may be the final stimuli for oviposition (Vinson, 1985). Chemical cues also may act in conjunction with shape, size, and texture. If accepted, at this stage, the female attempt to probe the host with its ovipositor.

-Egg laying (oviposition)

Parasitoids frequently insert their ovipositor into a host but do not go on to lay an egg. The ovipositor is normally covered in sensillae (Quicke et al. 1999) and it seems likely that the insect is rejecting the host after perceiving that it is unsuitable for oviposition (Quicke et al. 1999, Vilhelmsen, 2003).

Parasitoids have developed specialized methods for successfully ovipositing in and on acceptable hosts and ovipositors differ in length and morphology (Vilhelmsen, 2000; Vilhelmsen, 2003). Ectoparasitoids attacking concealed hosts often inject a permanent toxic when laying eggs that paralyzes the host to prevent host movement (Gauld and Bolton, 1988, Godfray, 1994; Quicke, 1997). In contrast, endoparasitoids must contend with the host immune system because they are living within a host (Gauld and Bolton, 1988, Godfray, 1994).

-Host marking

A number of parasitoids are known to leave chemical marks in the environment that are recognized by other parasitoids but in general four explanations have been put forward to explain the marking. Marking may: (1) improve the efficacy of the parasitoid population by increasing the evenness of the host attack (Field, 1990; Godfray, 1994, Nufio and Papaj, 2001). (2) allow the female to avoid a host she has just attacked. (Nufio and Papaj, 2001; Brodeur and Boivin, 2004) (3) be an altruistic act to

help related females forage efficiently (Haynes and Yeargan, 1999). (4) alert other females that the host has already been attacked (Roitberg and Mangel, 1988; Nufio and Papaj, 2001; Brodeur and Boivin, 2004). A number of parasitoid species are able to detect that a host has been attacked by a different species and refrain from further parasitism (Mackauer, 1990). It may be harder for a parasitoid to evolve the ability to detect chemical marks left by a different species-marking may evolve to prevent self-superparasitism and so the recognition of conspecific marks is preadaptation. Any system of communication incurs costs for both the sender and the receiver of signals (Bradbury and Vehrencamp, 1998). These costs include the biological ‘machinery’ involved in production, release, and reception of the signal (Bradbury and Vehrencamp, 2000; Nufio and Papaj, 2001). When it comes to invasive host species parasitoids may thus need to evolve phenological, behavioral or ecological specializations before they can colonize the new hosts and the need for such adjustments also may reduce the percentage of predation or parasitization (Cornell and Hawkins 1993).

Mechanisms of competitions.

Competition among organisms for resources such as food (Belovsky, 1997, Denno and McClure, 1995, Kaplan and Denno, 2007), living space (Lawton and Strong, 1981), refugees from extreme weather conditions or natural enemies (Berryman and Hawkins, 2006), and mates (Jarrige et al. 2016) has long been a major focus of ecological research (Ode et al. 2021). Competition among insects, as with all life forms, plays a major role in influencing life-history evolution, trait expression, niche size, and ultimately the structure and function of ecological communities (Kaplan and Denno, 2007).

Parasitoid-parasitoid interactions can frequently be viewed as a case of mutual predation when one individual kills and most often eats the other one (Brodeur and Rosenheim, 2000). Interactions between parasitoids involve protagonists from the same species or from different species that have evolved similar strategies to exploit a common resource, in the same habitat (Brodeur and Boivin, 2006). Furthermore, suitable hosts for parasitoid development are often scarce or distributed very heterogeneously in nature, thereby imposing strong selection pressure on adult females to locate hosts (Ode et al. 2021). It is quite frequent for several parasitoids to be found searching a patch simultaneously.

The competition is density-dependent and may be important in stabilizing the population dynamic issues (Hassell and Varley, 1969; Brodeur and Boivin, 2006). Parasitoids are susceptible to competition for hosts first of all because most of them are highly specialized, attacking only one or a few species of hosts in nature (Godfray, 1994).

In general, competition among parasitoids involves a mixture of both extrinsic and intrinsic competition (Harvey et al. 2013) that influence population dynamics by providing also important selective forces that shape behaviors related to the research and exploitation of resources (Boivin and

Brodeur, 2006). In turn, the competition can be “direct” or “indirect”. Direct extrinsic competition occurs when females of the same (intra-specific) or different (inter-specific) species simultaneously attack the same host egg mass, or their juvenile stages are both present in the host egg (direct intrinsic competition) (Boivin and Brodeur, 2006; Cusumano et al. 2012).

Instead, the indirect competition occurs when a female encounters a host egg mass already parasitized by a different species and reacts by adapting its foraging behavior (e.g., deciding to multiparasitize, or not). In direct competition, females may ignore each other and continue searching, suspend searching to interact with each other, or one or both parasitoids may leave the patch. The resulting interactions may be negative for both competitors, by wasting searching time, loss of energy, and risk of severe injury (Hokyo and Kiritani, 1966; Field et al. 1998).

Parasitoid adult females often use aggressive behavioral strategies to monopolize hosts (Mills, 1991; Boivin and Brodeur, 2006). For instance, some female parasitoids may guard host patches by aggressively chasing other females away or, when this fails, through physical combat. Such phenomenon has also been observed in hyperparasitoids.

Extrinsic competition and aggression among female parasitoids may generate outcomes where the owner (first arrived) female wins or where she is driven off by the intruder. The intruder female parasitoids thus can monopolize or kill hosts previously parasitized by con- or heterospecific females and this can be done by laying eggs on previously parasitized host to kill eggs or larvae inside of them, a process known as ovicide or larvicide (Ito and Yamada 2014, Leveque et al. 1993, Pérez-Lachaud et al. 2004, Strand and Godfray 1989, Takasu et al. 1997). In this contest of direct intrinsic competition, the first instar is generally the fighting instar and it often presents morphological adaptations, it is mobile (with a caudal appendage or setae) and is mandibulated (van Baaren *et al.* 1997); Morphologically, the following instars are often unable to fight and can be killed by the first instar when second oviposition occurs soon after the first one (Boivin and Brodeur, 2006).

Most parasitoids parasitize many individual hosts in a small period of time and in particular when parasitoid densities are high, host patches can be rapidly depleted of unparasitized hosts, resulting in an ever-increasing risk of females re-encountering previously parasitized hosts. Under these conditions, the parasitoids face two choices: (a) to remain on a patch where the encounter rate of unparasitized hosts is steadily declining or leave in search of higher-quality patches elsewhere and, if they stay, (b) to accept (i.e., engage in superparasitism or multiparasitism leading to intrinsic competition) or reject previously parasitized hosts. These foraging options are based on a complex interplay of ecological (e.g., host density and spatial distribution) and physiological and behavioral (e.g., female age, egg load, handling time, physical condition, previous experience) parameters.

Adult parasitoids with more efficient foraging behaviors are expected to be stronger extrinsic competitors. Foraging efficiency is determined by the time spent locating hosts (search time), the

duration of the oviposition sequence (handling time), and the quality of hosts accepted (Godfray, 1994, Harvey, 2005).

Search time is correlated with a parasitoid's ability to use host-associated semiochemicals (e.g., kairomones, synomones), with faster search times being associated with higher sensitivity to cues indicative of host presence (Colazza et al. 1999, De Moraes, 1999, Peri et al. 2014).

Superior host-finding abilities can also arise from a parasitoid's ability to use a greater diversity of host-related cues (Ode et al. 2021). Furthermore, associative learning of chemical cues is a significant element of parasitoid foraging behavior that can determine the outcome of competitive interactions among adults (Cusumano et al. 2014). Parasitoids can reduce competition by attacking hosts at different ontogenetic stages.

Even though host size is often positively correlated with adult parasitoid size and fitness (Visser, 1994), parasitoids can mitigate competition by attacking smaller hosts (Briggs, 1993). Aggression may in turn be a species- or individual-specific trait, or it may vary with the age, egg load, and physiological condition of the parasitoids foraging within a patch (Wajnberg et al. 2006). Trophic and competitive interactions among different natural enemies of *H. halys* likely to influence biological control outcomes. Relevant intraguild interactions could include those (1) between parasitoids and predators (including native predatory pentatomids), (2) among indigenous parasitoids, and (3) between indigenous and exotic parasitoids.

Case Study: The invasive species *Halyomorpha halys* and its natural enemies

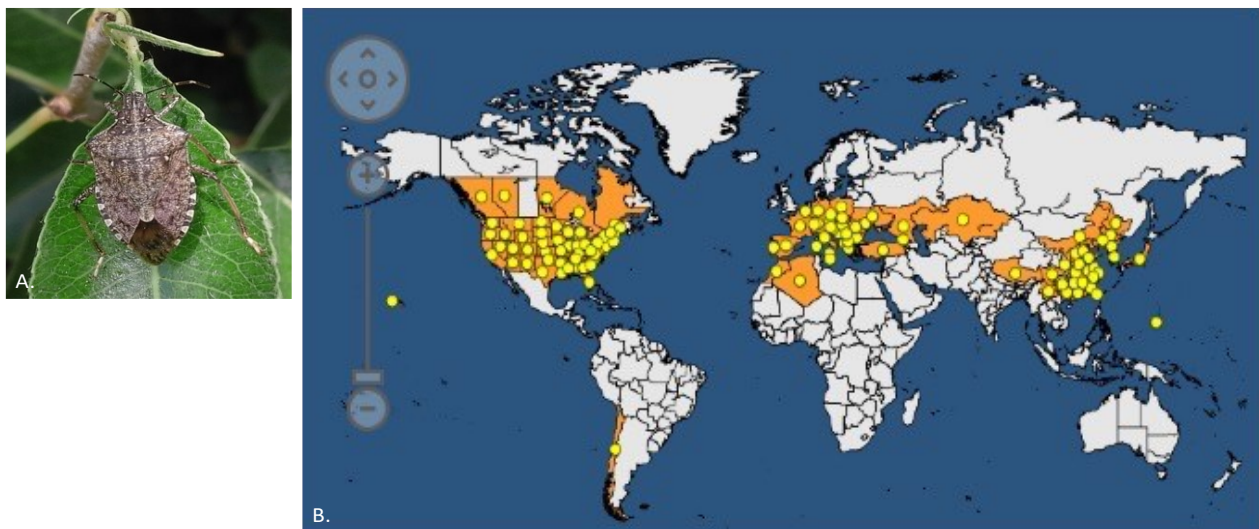
The Brown marmorated stink bug *Halyomorpha halys* (Stål) is an invasive pentatomid, native to East Asia, first found outside its range in 1996 in the US (Hoebeke and Carter, 2003; Haye et al. 2014a; Wermelinger et al. 2007). This pest, as most of invasive species, is known to spread rapidly to new areas as a result of human activity and the movement of goods (Levine and D'Antonio, 2003; Haye et al. 2014a).

Current and potential geographic distribution

The native range of *Halyomorpha halys* comprises China, Japan, and Korea (Hoebeke and Carter, 2003). In China, *H. halys* is widely distributed throughout the temperate and subtropical regions of Eastern China. In Japan it occurs mainly on the islands (Kobayashi et al. 1972), in South Korea is further found all across the country.

The first discovery of *H. halys* established populations outside its range dates back to 1996 in the city of Allentown in Eastern Pennsylvania (Hoebeke and Carter ,2003) and since that time it has spread throughout the United States and Canada. The first established population of *H. halys* in Europe was detected in Zurich, Switzerland in 2007 (Wermelinger et al 2008) but the numerous contemporaneously findings suggest that it had already been present for much longer (Haye et al 2014a). In Italy, the first individual was detected in September 2012 in the Province of Modena (Maistrello et al. 2014). Currently, *H. halys* is widely spread worldwide (Fig.3)

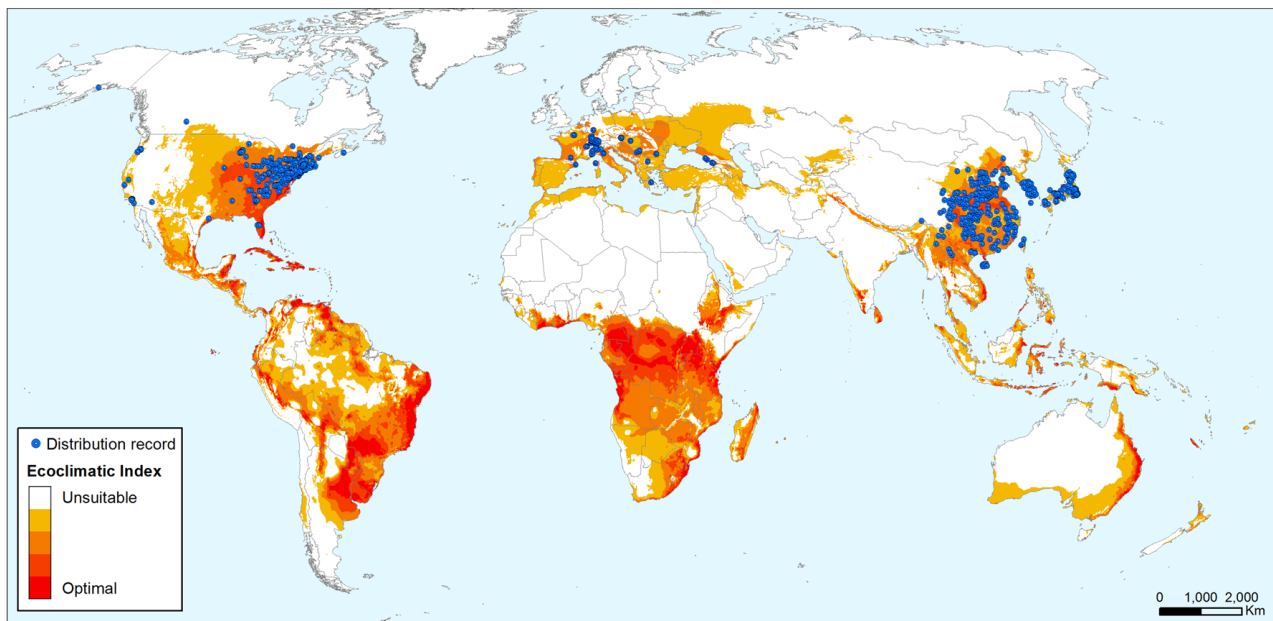
Figure 3. A. Adult of *Halyomorpha halys*, B. Current World distribution of *H.halys* (EPPO Global Database)



The ecological niche modeling (ENM) has been widely used to identify the potential distributions of species (Elith et al. 2006), with this aim, several studies with bioclimatic modeling based on distribution data in Asia (native range) and climate data hosted suggested that the most favorable conditions for the establishment of *H. halys* exist, in particular, in Mediterranean countries of Europe and the Middle East, South America with Argentina that results highly climatically suitable. In Australia, the highest risk for *H. halys* establishment would be along the east coast of the continent, and the north of Tasmania, where most horticulture is practiced.

In New Zealand, most of the North Island appears climatically suitable as well as the South (Fig. 4) (Kriticos et al. 2017). Model-based on the native-range source population successfully identified the earlier established *H. halys* populations in North America, but, for example, could fail to forecast later range expansion, for this reason, caution is therefore warranted when using the source population to predict invasion potential. (Zhu et al. 2016).

Figure 4. Modelled climate suitability (CLIMEX Ecoclimatic Index) for *Halyomorpha halys* globally, including reported distribution locations. Note the three outlying distribution records in the USA and Canada have been investigated and found to be transient populations associated with human transportation (Kriticos et al. 2017)



Biology, Ecology and Behavior

Halyomorpha halys presents many biological and ethological characteristics that make it a successful invasive species, these include primarily *r*-selected life history and association with human-modified ecosystems. Once established, its high dispersal capacity, polyphagy, and ability to compete with endemic species assist its worldwide spread (Sakai et al. 2001).

H. halys adults overwinter in hidden locations including natural refuges and human-made structures (Watanabe et al. 1994, Inkley, 2012, Lee et al. 2014). Oviposition starts when critical diapause-termination indices are encountered. Females overwinter reproductively immature and unmated, indeed, these are synovigenic and become reproductively after a developmental delay (Yanagi and Hagihara, 1980; Nielsen et al. 2008) continuing to oviposit and mating throughout their life span (Kawada and Kitamura 1983; Nielsen et al. 2017; Nielsen et al. 2018). Females oviposit the eggs in egg mass (about 28 eggs/egg mass), predominantly on the underside of leaves. Some laboratory studies conducted in the U.S. found an average of 9.3 egg masses in a lifetime (Nielsen et al. 2018) whereas European field studies estimated fecundity to range from 2 to 15 egg masses per female (Haye et al. 2014). During oviposition, females transfer symbiont to the egg chorion surface, this bacteria is essential for the development and survival of later instars (Taylor et al. 2014). The complete development of *H. halys* includes 5 nymphal stages.

In total the Optimum temperatures for *H. halys* development in the Europe climate conditions range from 12.97° to 36.50°C (Haye et al. 2014). The voltinism such as population size and structure will

be determined by the combination of the temperature requirements and critical diapause cues with photoperiods estimated in a range of 12.7-13.5 h (Nielsen et al. 2016; Nielsen et al. 2017).

H. halys is polyphagous with a broad host range that includes over 300 plants mainly of agricultural importance such as fruit and vegetables of many economically important crops but this aspect will be discussed in greater detail below.

Throughout the growing season, *H. halys* is present in various wild (Nielsen and Hamilton 2009) and cultivated host plants (Bergmann et al. 2016) and moves among species based on plant phenology (Martinson et al. 2015).

The dispersal capacity and behavior of adults and nymphs have been quantified to better understand the potential for invasion. In fact, in addition to being extremely polyphagous, BMSB also enables long-distance flight (at least 85% of all individuals flew up to 5 km within 24h but also up to 117 km have been recorded) (Wilman et al. 2014; Lee and Leskey, 2015).

Economic, environmental, and social impact.

Halyomorpha halys causes injury to plants by inserting their feeding stylets into plant fruiting bodies, which are often the marketable portion of commercial crops. Similar to other pentatomids, *H. halys* secretes sheath saliva composed of both insect and plant-derived proteins but it is the watery saliva injected by the feeding stylet into the plant tissue that breaks down the cell and enables the consumption of liquid contents. Feeding may result in scarring, faded sunken areas, deformed fruits, seeds or pods, white spongy areas on the surface of fruits and tissue damage internally visible as discolored flesh (Leskey et al. 2009; Leskey et al. 2014; Kuhar et al. 2012; Nielsen and Hamilton 2009; Rice et al. 2014). In addition to reducing the quality of marketable produce, feeding injury can reduce fruit set and subsequent yield by aborting flower buds and young fruiting bodies. Feeding on nuts (such as hazelnuts) increases the incidence of empty shells (Hedstrom et al. 2014).

The host range of *H. halys* includes many economically important crops such as larger tree fruits (e.g. *Malus domestica* (apple), *Pyrus communis* (pear), *P. persica* (peach), *Prunus armeniaca* (apricot), *P. avium* (cherry), *P. domestica* (plum), *Citrus spp.*, *Morus spp.*, small fruits (e.g. *Rubus idaeus* (raspberry), *Corylus spp.* (hazelnuts); *Vitis vinifera* (grapevine), vegetables, *Lycopersicon esculentum* (tomato), *Capsicum spp.* (peppers), *Helianthus annuus* (sunflower), field crops: *Glycine max* (soybean), *Phaseolus vulgaris* (common bean), *Zea mays* (maize), and a number of forest and ornamental trees and shrubs (e.g. *Acer spp.*, *Fraxinus excelsior*, *Paulownia tomentosa*, *Ailanthus altissima*, *Buddleia davidii*, *Cupressus spp.*, *Hibiscus spp.*, *Lonicera spp.*, *Rosa rugosa*, *Salix spp.*) (Leskey et al. 2012, 2014; Lee et al. 2013; Northeastern IPM Center 2014c; Rice et al. 2014; Haye et

al. 2014b). In addition to its status as an agricultural pest, brown marmorated stink bug has become well-known as a nuisance pest, as adults often invade human-made structures sometimes in very large numbers—to overwinter inside protected environments (Watanabe et al. 1994; Hoebeke and Carter 2003; Hamilton 2009; Inkley, 2012). In addition to the general aversion to having insects in one's home, stink bugs also produce a distinctive, unpleasant odor, which results from the bug's defense chemicals (Inkley, 2012).

To date, the situation in Europe has been less severe than in North America (Haye et al. 2015). Exposure likely occurs through the debris of dead insects in house dust or direct contact when removing live *H. halys* from their homes. Large aggregations of *H. halys* can block ventilation ducts and intakes, and particles may be aerosolized and distributed throughout the house. Adults release chemical defense compounds, a mix of tridecane, 5-ethyl-2(5H)-furanone, and (E)-2-decenal, from their scent glands when threatened (Solomon et al. 2013). Clinical tests confirmed that *H. halys* is a novel, clinically significant indoor allergen which could play an important role in allergic disease in the US in the near future (Anderson et al. 2012). A similar scenario seems to be possible in European cities, where *H. halys* is an increasing nuisance problem.

Natural enemies of *Halyomorpha halys*

The insects introduced into foreign locations (hosts as invaders) such as *Halyomorpha halys*, can spread rapidly, offering novel resources to native predators and parasitoids. Communities of indigenous parasitoids and predators attacking invasive species, in the major of cases are “generalists” and have a limited population-level impact on the invader (Cornell and Hawkins, 1993). Furthermore, indigenous natural enemies often fail to immediately include the invasive species in their host/prey inventories and may have unsuitable, leading to “evolutionary traps” (Schlaepfer et al. 2005). In this case, the invasive host causes indigenous predators to invest energy or reproductive resources that result in lower profitability than that normally associated with the investment. So, evolutionary traps may have consequences for the population dynamics of indigenous parasitoids, their indigenous hosts, and the invasive alien host species through their mediation of indirect effects (Abram et al. 2014). The likelihood that this will occur depends also on whether enough heritable genetic variability exists within and among populations of the parasitoid for the tendency to reject the invasive host species and the capacity to successfully develop in them (Henter, 1995). However, these situations may often be temporary; in some cases, indigenous species have been shown to develop behavioral, morphological, or physiological adaptations, such as body size or generation time, that allow them to use the invader as a resource and/or mitigate its negative effects (Phillips and Shine, 2004; Berthon, 2015). Moreover, the impact of indigenous natural

enemies is likely to be variable and condition-dependent, differing among invaded regions, habitat types, and over time as natural enemies adapt to the new pest resource (Strayer et al. 2006; Berthon, 2015).

Among predators, the species responsible for egg mortality are much less commonly identified, the most common are generalist chewing and sucking predators. Depending on the type of predator, *H. halys* eggs can show different signs of damage, which are related to the morphology of the predator's mouthparts (Morrison et al. 2016). Predators attacking *H. halys* adults and nymphs in Europe and North America have not been extensively studied. One unpublished study has gathered some information on the consumption of newly emerged (1st instar) *H. halys* nymphs, which are clustered on egg masses for several days and potentially vulnerable as they are relatively immobile and soft-bodied. Predators of first instar nymphs belonging mostly to the family of Carabidae (Coleoptera) but also Salticidae (Araneae) and Reduviidae (Hemiptera). Morrison et al. (2017) conducted the study of adult *H. halys* predation by web-building spiders in and outside human dwellings founding that several families of spiders (particularly Theridiidae, Pholcidae, and Agelenidae) were particularly adept at ensnaring and consuming *H. halys* adults. To date, it is difficult to spot among predators a valid candidate for *H. halys* biological control because none of the detected possible predators is able to attack all life stages of this pest.

In the native range of *H. halys*, control is primarily provided by a large number of Hymenoptera egg parasitoids (Lee et al. 2013) belonging to the genera *Trissolcus*, *Telenomus* (Scelionidae) and *Anastatus* (Eupelmidae). In China, *Trissolcus japonicus* is an important biocontrol agent, causing parasitism rates as high as 80% (Li and Liu, 2004; Qiu, 2010; Yang et al. 2009; Zhang et al. 2017). Several studies explored the ability of parasitoids to attack *H. halys* eggs, both in its native distribution and in the newly invaded areas worldwide (Haye et al. 2015; Talamas et al. 2015; Roversi et al. 2016; Herlihy et al. 2016; Abram et al. 2017; Dieckhoff et al. 2017). Parasitism caused by indigenous egg parasitoids is generally low. In 87% of surveys conducted in North America and Europe, using both fresh and frozen sentinel egg masses exposed to different environmental habits, parasitism was lower than 5% (Abram et al. 2017; Costi et al. 2019). In a recent study conducted in Italy, Iacovone et al. (2022) show that despite the release of large quantities of *Aanastatus bifasciatus* specimens, the main indigenous parasitoid species, the rate of parasitism (16%) is relatively low.

To date, only Asian native species of egg parasitoids appear to be promising candidates as biological control agents, primarily *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae) (Zhang et al. 2017). However, the spread of *H. halys* has also provided an invasion opportunity for its parasitoids. In 2015 *T. japonicus*, commonly known as the samurai wasp, was detected in the eastern United States (Talamas et al. 2015), followed shortly by the discovery of a second, independently established population in the Pacific Northwest (Milnes et al. 2016). In 2017 and again in 2018, *T. japonicus* was

recorded in Switzerland, its first reported recovery in Europe (Stahl et al. 2018). In the same year, in fruit orchards in the Region of Friuli-Venezia-Giulia (northeastern Italy), during the survey of *H. halys* egg masses in the brown marmorated stink bug monitoring campaign, another species of egg parasitoid was detected, *Trissolcus mitsukurii* (Sabbatini-Peverieri et al. 2018). *Trissolcus mitsukurii* is widespread in Asia, and its distribution extends to the southern limit of eastern Australia (Johnson 1991).

This species has otherwise not been reported as an adventive parasitoid outside of its native range, but it is conceivable that its distribution will follow that of *H. halys*, as has occurred with *T. japonicus*. The phenomenon of parasitoids following in the footsteps of their invasive hosts has become a growing trend, particularly with species of *Trissolcus* Ashmead (Sabbatini-Peverieri et al. 2018).

During the numerous investigations conducted from 2017 and 2019 in Asia, USA, and Europe on native and exotic egg parasitoids of *H. halys* and other pentatomids, new associations were continuously discovered. After *Trissolcus japonicus* and *Trissolcus mitsukurii*, this trend continues with *Acroclisoides sinicus* (*Acroclisoides solus* **syn. nov.**), a pteromalid that was described in the 1980s from Asia. Recently this species was found both in northern Italy that in Switzerland, but also in southeastern USA (Sabbatini-Peverieri et al. 2019) but no information is provided about this species. *Acroclisoides* Girault and Dodd (Hymenoptera: Pteromalidae) was established in 1915 and currently comprises just over a dozen species in the Afrotropics, Australia, and South Asia.

A single species with a disjunct distribution, *Acroclisoides solus* Grissell & Smith, 2006 (Hymenoptera: Pteromalidae), was described from North America (Grissell and Smith; 2006; see Noyes, 2019). *Acroclisoides* includes species that are apparently facultative or obligate hyperparasitoids, in most cases of pentatomid eggs parasitized primarily by scelionids, but also by eupelmids (Clarke and Seymour 1992; Sureshan and Narendran, 2002; Grissell and Smith, 2006). In each of these cases, the found of these species in the new invaded area was made through the cooperative effort of scientists working in the disciplines of biological control, taxonomy, and biological diversity, highlighting the synergy and necessity of collaboration.

Aims

The Ph.D. research herein presented investigated the ecological and ethological aspects of the egg parasitoids associated with the *Halyomorpha halys*, focusing mainly on those exotic species recently found in the invaded habitat of the USA and Europe. The aims of the research are not only to provide an overview of the ecology and ethology of these species but also for providing fundamental information for the proper management of *H. halys* biocontrol programs.

In particular, in Chapter 1, the research focused on the assessing distribution of the egg parasitoid species in those invaded areas of Europe where *H. halys* is abundant and where it has created the greatest damage in agricultural contexts, Switzerland and Northern Italy. At the same time, to support management efforts a diagnostic tool based on detailed descriptions of the meconium and the characteristics of the emergence holes of the most frequent parasitoid species associated with *H. halys* was developed.

The low efficacy and high environmental impact of insecticide treatments, combined with the lack of effective indigenous natural enemies, led to consider classical biological control with native egg parasitoids a promising method for long-term management of *H. halys* in the invaded areas. The employment of an exotic organism as a candidate biological control agent, including redistribution of adventive populations, is strictly regulated by national and international rules (van Lenteren et al. 2006a; EPPO 2014; FAOIPPC 2017; European Directives 92/43/CEE and 2002/89/ CE; Italian DPR 102/2019). Therefore, the approval of a petition for the release of a biological control agent requires a thorough risk assessment, which provides detailed information on the biological and ecological traits of the agent, first of all, its host range (Hunt et al. 2008). For this reason, in Chapter 2, the research activity was focused on the assessment of the two promising candidates, *Trissolcus japonicus* and *Trissolcus mitsukurii*, for *H. halys* biological control in Europe. The studies of the physiological host range of each species were conducted under laboratory conditions with multidisciplinary approaches, including behavioral and chemical ecology assays.

Considering the importance of these species in the context of *H. halys* biological control and the co-occurrence of these species in the same invaded habitats, in Chapter 3, the behavioral responses and the way these species exploit common resources, under laboratory conditions, were studied in different interspecific competition scenarios to provide information that may help to understand how the population dynamics of these species could evolve and to improve useful information for *H. halys* biological control programs.

In conclusion, following the discovery in USA and in Europe of a third exotic parasitoid, *Acroclisoides sinicus*, frequently found associated with *H. halys* and its parasitoid guild, the reproductive and developmental biology of this species, until now completely unknown, were studied in detail, in Chapter 4, to understand its behavior and its role in the context of *H. halys* biological control and its possible impact on native species.

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Chapter 1: *Halyomorpha halys* egg parasitoids

1.1 Assessing the Distribution of Exotic Egg Parasitoids of *Halyomorpha halys* in Europe with a Large-Scale Monitoring Program

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Abstract: The brown marmorated stink bug *Halyomorpha halys* is an invasive agricultural pest with a worldwide distribution. Classical biological control has been identified as the most promising method to reduce the populations of *H. halys*. Adventive populations of two candidates for releases,

Trissolcus japonicus and *Trissolcus mitsukurii*, have recently been detected in Europe. To assess their distribution and abundance, a large-scale survey was performed. From May to September 2019, a wide area covering northern Italy and parts of Switzerland was surveyed, highlighting the expanding distribution of both *Tr. japonicus* and *Tr. mitsukurii*. Within four years after their first detection in Europe, both species have rapidly spread into all types of habitats where *H. halys* is present, showing a wide distribution and continuous expansion. Both exotic *Trissolcus* showed high levels of parasitism rate towards *H. halys*, while parasitization of non-target species was a rare event. The generalist *Anastatus bifasciatus* was the predominant native parasitoid of *H. halys*, while the emergence of native scelionids from *H. halys* eggs was rarely observed. The presence of the hyperparasitoid *Acroclisoides sinicus* was also recorded. This study provided fundamental data that supported the development of the first inoculative release program of *Tr. japonicus* in Europe.

Keywords: biological control; BMSB; exotic biological control agents; invasive species; natural enemies; *Trissolcus japonicus*; *Trissolcus mitsukurii*

Introduction

The brown marmorated stink bug *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) is an invasive species from Central Asia that nowadays is present in North and South America (Faúndez and Rider, 2017; Xu et al. 2014), Europe (Maistrello et al. 2018) and the Caucasus (Musolin et al. 2018), causing severe damage on many agricultural crops (Leskey and Nielsen, 2018). Due to the negative effects of frequent use of broad-spectrum insecticides (e.g., disruption of Integrated Pest Management, pesticide resistance), biological control has been identified as the most promising mid- and long-term solution (Leskey and Nielsen, 2018). Both the use of native natural enemies (inoculation and inundation biological control) and the more promising introduction of exotic *H. halys* egg parasitoids from Asia (classical biological control) are among the key management strategies for sustainable control of the invasive pest (Abram et al. 2018; Conti et al. 2020).

Support for biological control has regained momentum (van Lenteren et al. 2018), however the debate on its environmental safety continues. Strict regulations are in place to limit the potential impact on biodiversity, and several international organizations have contributed to the development of step-by-step procedures for risk analysis, import and release of exotic agents (EPPO. PM 6/2 (3), 2014; FAO and I.P.C.C. 201911). However, the increased focus on unpredicted negative effects has contributed to the observed decline of classical biological control application and not without consequences: inaction or delay in the control of an invasive pest are in fact associated with increased pesticide use, impacting society and ecosystem dynamics (Hajek et al. 2016). Non-target effects, a critical aspect in

classical biological control, can be predicted using host specificity testing, supporting decision making and reducing potential risks using cost/benefit analysis (Van Lenteren et al. 2006).

In North America and Europe, natural enemies that attack *Halyomorpha halys* include egg parasitoids that belong primarily to three Hymenopteran families: Scelionidae (*Telenomus* spp., *Trissolcus* spp., *Gryon* spp.), Eupelmidae (*Anastatus* spp.) and Encyrtidae (*Ooencyrtus* spp.), and generalist predators that feed on eggs and young nymphs (Abram et al. 2017). In Europe, the egg parasitoid *Anastatus bifasciatus* (Geoffroy) (Hymenoptera: Eupelmidae) has been identified as the only native egg parasitoid capable of successfully developing in *H. halys*, thus it has been considered as a candidate for augmentative biological control releases (Haye et al. 2019; Costi et al. 2019). However, experimental field trials involving sentinel egg masses showed a limited impact of the augmentative releases of *An. bifasciatus* (Stahl et al. 2019), slightly higher in field studies with eggs laid in exclusion cages (Maistrello et al. 2020).

Recently, adventive populations of the main candidate for classical biological control, the Asian egg parasitoid *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae), were detected both in North America (Talamas et al. 2015) and in Europe, including Italy (Sabbatini Peverieri et al. 2018; Moraglio et al. 2020) and Switzerland (Stahl et al. 2019). In addition, adventive populations of another Asian egg parasitoid, *Trissolcus mitsukurii* (Ashmead), were detected on *H. halys* egg masses in Italy (Sabbatini Peverieri et al. 2018; Moraglio et al. 2020; Scaccini et al. 2020; Zapponi et al. 2020). The scattered distribution of the two exotic *Trissolcus* species in Italy and Switzerland might have been caused by multiple introductions on the same pathways of entry of *H. halys*, as parasitized egg masses on plants or diapausing adults (Stahl et al. 2019). Furthermore, their post-introduction dispersal ability may have further contributed, as was proposed for *Tr. japonicus* in North America (Milnes et al. 2016). The presence of the two exotic egg parasitoids in Europe suggests that there is the hope for a future biological control solution for the management of *H. halys*. However, to date, the current distribution of both exotic *Trissolcus* species and their impact on *H. halys* populations in Italy and Switzerland are poorly understood. Considering the high expectations on their potential for controlling the pest, field investigations on the status of the adventive populations have been considered mandatory. In addition, knowledge of the current incidence and distribution will provide a baseline for future studies, investigating movement, spread, and impact of these parasitoids on *H. halys* and non-target species.

The objective of this study was to evaluate the distribution of the adventive populations of *Tr. japonicus* and *Tr. mitsukurii* in North Italy and Switzerland in a large-scale monitoring program. In addition, their parasitism rate, impact on non-target species, phenology and habitat were evaluated. Lastly, the collected data were used to support the petition for the final approval of *H. halys* biological control program and the release in the field of laboratory-reared *Tr. japonicus*.

Materials and Methods

Field Surveys

With the aim to cover a wide geographical area, a network of 21 partners from northern Italy and Switzerland was developed, including research institutes, universities and local phytosanitary services. The members of the consortium jointly developed the workplan and survey protocol, in order to standardize all the key steps (e.g., data recording, sample storing, and taxonomic identification).

Field surveys were performed from late May to mid-September 2019 and took place in agricultural, urban and semi-natural habitats. To increase the sampling coverage, several types of surveys were combined: (a) visual inspection in fixed locations, repeated at least three times during the season; (b) visual inspections in non-fixed locations where *H. halys* adults and nymphs were observed; (c) deployment of sentinel egg masses (see Table S1 for further details). For the first two survey methods, the underside of leaves of trees, shrubs and herbaceous plants were inspected, searching for egg masses of *H. halys* and other pentatomids, for at least 30 min. For sentinel egg masses, both fresh (24 h) and frozen (stored at -80°C for maximum a month) egg masses were used and deployed for 3 to 5 days; for further details, see Stahl et al. (2019) and Zapponi et al. (2020).

After collection, each egg mass was labeled, stored separately, and transported back to the laboratories for successive rearing of nymphs and/or parasitoids. For each sample, date, surveyor code, GPS coordinates and habitat (type of crop and host plant) were recorded.

Laboratory Handling of Collected Egg Masses

Egg masses were stored in 90 mm plastic Petri dishes or 50 mL plastic vials and kept at a temperature of $24\text{--}26^{\circ}\text{C}$ and L16:D8 photoperiod. Samples were checked daily, until nymph or parasitoid emergence was completed. For the identification of native pentatomid egg masses, field-collected adults were reared by several co-authors, in order to have fresh laid egg masses that were then compared with the field-collected ones, to ensure their correct identification (Moraglio et al. 2021). When a species could not be assigned, genus level was used.

Parasitoids were promptly transferred into ethanol and preserved for subsequent identification. For each egg mass, the total number of eggs, hatched nymphs and emerged parasitoids were recorded. All emerged parasitoids were counted and sexed.

To compare the parasitism rate of the most common parasitoid species, the number of parasitized eggs in an egg mass over the total number of intact eggs was calculated, excluding eggs that did not hatch and those that showed signs of predation (Bin and Vinson, 1991). The parasitism rate was calculated only on field-collected egg masses (data from sentinel egg masses were not included in

this analysis). Furthermore, only egg masses from which a single parasitoid species had emerged were considered, to avoid underestimation/overestimation of parasitism rates.

Taxonomic Identification

Parasitoids were first separated and identified at family and genus levels. Then, for *Anastatus* the keys proposed by Askew and Nieves-Aldrey (Askew and Nieves-Aldrey, 2004) were used, while for *Acroclisoides* the method of Sabbatini Peverieri et al. (2019) was followed. Among Scelionidae, *Trissolcus* species were identified using Talamas et al. (2017) and Tortorici et al. (2019). *Telenomus* species were identified, whenever possible, using Kozlov and Kononova (1983) and comparing the specimens with the images of the primary types (kindly provided by Elijah Talamas).

Data Analysis

The occurrence of stink bug and exotic parasitoids was analyzed using QGIS 3.14 (Development Team 2018, QGIS Geographic Information System), mapping the presence of *Tr. japonicus* and *Tr. mitsukurii* on a heatmap obtained from *H. halys* occurrence data. Descriptive analyses were performed with R Statistical Software (v.3.5.2) (R Core Team, 2018).

Results

Field Survey

In four months, a total of 4,348 and 285 egg masses of *H. halys* and of other pentatomids, respectively, were collected in an area of approximately 140,000 km², covering northern Italy and parts of Switzerland (survey methods a and b). Most egg masses were found in cultivated areas (51.58%) or urban environments (44.81%), while fewer egg masses (5.21%) were collected in semi-natural habitats (Figure S1, content). About 93.85% of the collected egg masses belonged to *H. halys*, while the rest belonged to several other Pentatomidae species, including *Palomena prasina* (L.), *Rhaphigaster nebulosa* (Poda), *Piezodorus lituratus* (F.), *Dolycoris baccarum* (L.) and *Nezara viridula* (L.) (see Table 1).

Parasitoid Species Composition

From the collected pentatomid egg masses, the eupelmid *An. bifasciatus* emerged as well as numerous Scelionidae belonging to the genera *Trissolcus* and *Telenomus*, specifically: *Tr. basalis* (Wollaston), *Tr. belenus* (Walker), *Tr. cultratus* (Mayr), *Tr. japonicus*, *Tr. mitsukurii*, *Tr. semistriatus* (Nees von Esenbeck), *Tr. viktorovi* Kozlov, *Te. turesis* Walker, and *Telenomus* sp. (see Table 1 for hosts and number of parasitized egg masses). The hyperparasitoid *Acroclisoides sinicus* (Huang and Liao)

(Hymenoptera: Pteromalidae) was also recorded, emerging from parasitized egg masses of *H. halys* and *N. viridula* (Table 1).

Table 1. Number of egg masses collected (N. EM) for each stink bug species and number of parasitized egg masses (N. PEM), in total and in brackets for each emerged parasitoid and hyperparasitoid species (survey methods a and b).

Species	N. EM	N. PEM	Species of Parasitoids	Species of Hyperparasitoids
<i>Carpocoris</i> spp.	8	4	<i>Telenomus</i> sp. (4)	
<i>Dolycoris baccarum</i>	22	7	<i>Anastatus bifasciatus</i> (1), <i>Telenomus</i> sp. (3), <i>Trissolcus</i> <i>belenus</i> (1), <i>Trissolcus</i> <i>mitsukurii</i> (2)	
<i>Eurydema</i> spp.	4	2	<i>Trissolcus viktorovi</i> (2)	
<i>Halyomorpha halys</i>	4348	974	<i>Anastatus bifasciatus</i> (624), <i>Telenomus</i> sp. (25), <i>Trissolcus</i> <i>basalis</i> (20), <i>Trissolcus</i> <i>belenus</i> (4), <i>Trissolcus</i> <i>cultratus</i> (14), <i>Trissolcus</i> <i>japonicus</i> (152), <i>Trissolcus</i> <i>mitsukurii</i> (325), <i>Trissolcus</i> <i>semistriatus</i> (4), Undet. (20)	<i>Acrodissoides sinicus</i> (69)
<i>Nezara viridula</i>	153	31	<i>Anastatus bifasciatus</i> (17), <i>Trissolcus basalis</i> (11), <i>Trissolcus mitsukurii</i> (3)	<i>Acrodissoides sinicus</i> (1)
<i>Palomena prasina</i>	74	38	<i>Anastatus bifasciatus</i> (11), <i>Telenomus</i> sp. (7), <i>Telenomus</i> <i>turensis</i> (2), <i>Trissolcus</i> <i>cultratus</i> (11), <i>Trissolcus</i> <i>japonicus</i> (5), <i>Trissolcus</i> <i>mitsukurii</i> (2)	
<i>Pentatoma rufipes</i>	6	2	<i>Trissolcus cultratus</i> (2)	
<i>Piezodorus lituratus</i>	7	1	<i>Trissolcus semistriatus</i> (1)	
<i>Rhaphigaster nebulosa</i>	11	2	<i>Anastatus bifasciatus</i> (1), <i>Telenomus</i> sp. (1)	

Considering only parasitized *H. halys* egg masses (974, representing 22.40% of total number of collected egg masses), the most common species that emerged were *An. bifasciatus*, *Tr. mitsukurii* and *Tr. japonicus*. Unhatched and predated eggs represented the 25.26% of the total number of eggs (21.51% for non-parasitized egg masses, 27.20% for parasitized egg masses). See paragraph 3.3 for results on the number of parasitized eggs per egg mass.

Concerning native pentatomid egg masses (88 parasitized egg masses in total), *Tr. japonicus* and *Tr. mitsukurii* emerged in low numbers. The only native species parasitized by *Tr. japonicus* was *Pa. prasina* (five egg masses, representing 13% of the parasitized *Pa. prasina* egg masses collected), while *Tr. mitsukurii* emerged from egg masses of *D. baccarum* (2; 28% of parasitized *D. baccarum* egg masses), *N. viridula* (3; 10% of parasitized *N. viridula* egg masses), *Pa. prasina* (2; 5% of

parasitized *Pa. prasina* egg masses), and from an egg mass ascribed to a pentatomid of the subfamily Asopinae

Parasitism Rate and Phenology
Comparing the number of collected *H. halys* egg masses parasitized by the three most common species (Figure 1), parasitism by *Tr. mitsukurii* was higher in June, with two slightly lower peaks in August. Parasitism by *Tr. japonicus* showed a similar pattern, but it reached its maximum in August. These patterns were consistent across the study area.

Across the surveyed regions, the percentage of parasitized egg masses was very variable for the three most common parasitoids: 0.45–53.85% for *Tr. japonicus*, 0.17–20.20% for *Tr. mitsukurii* and 0.85–19.55% for *An. bifasciatus* (Table 2).

Table 2. Number of egg masses (N. EM) and eggs (N. E) for *Halyomorpha halys* collected in the different survey areas (methods a and b), showing percentage of parasitized egg masses (PEM) and parasitized eggs (PE), and resulting parasitism rate (PR) for *Trissolcus japonicus* (A), *Trissolcus mitsukurii* (B) and *Anastatus bifasciatus* (C), calculated on egg masses from which only one parasitoid species had emerged.

A. <i>Trissolcus japonicus</i>						
Country	Region/Canton	N. EM	N. E	PEM (%)	PE (%)	PR (Mean ± SE)
Italy	Piedmont	1788	46,824	4.03	2.98	89.43 ± 2.97
	Veneto	669	16,966	0.45	0.29	100.00 ± 0.00
Switzerland	Basel	26	625	53.85	50.88	80.25 ± 6.95
	Zürich	25	673	32.00	25.41	71.43 ± 10.19
B. <i>Trissolcus mitsukurii</i>						
Country	Region/Canton	N. EM	N. E	PEM (%)	PE (%)	PR (Mean ± SE)
Italy	Emilia Romagna	629	16,707	3.66	2.75	97.40 ± 1.68
	Friuli-Venezia Giulia	589	13,874	20.20	14.49	96.21 ± 1.38
	Piedmont	1788	46,824	0.17	0.09	91.23 ± 8.77
	Trentino-Alto Adige	585	15,506	5.98	4.15	96.70 ± 2.92
	Veneto	669	16,966	6.88	4.66	88.59 ± 3.27
C. <i>Anastatus bifasciatus</i>						
Country	Region/Canton	N. EM	N. E	PEM (%)	PE (%)	PR (Mean ± SE)
Italy	Emilia Romagna	629	16,707	19.55	2.36	66.06 ± 2.78
	Friuli-Venezia Giulia	589	13,874	0.85	0.35	44.89 ± 19.54
	Piedmont	1788	46,824	15.72	7.79	73.97 ± 1.92
	Trentino-Alto Adige	585	15,506	11.97	5.62	76.09 ± 3.69
	Veneto	669	16,966	1.05	0.62	68.32 ± 15.15
Switzerland	Basel	26	625	3.85	3.36	75.00
	Zürich	25	673	4.00	0.15	3.57

The parasitism rate varied as well for *Tr. japonicus* (71.43–100%), *Tr. mitsukurii* (88.59–97.40%) and *An. bifasciatus* (3.57–76.09%). The emergence of more than one species from the same *H. halys* egg mass was observed multiple times (Figure 2), from 96 egg masses in total ($n = 2539$ total number of eggs), and in the 83.33% it involved combinations of *An. bifasciatus* and 1–2 species. *Anastatus*

bifasciatus emerged often together with *Tr. japonicus* ($n = 55$) and *Tr. mitsukurii* ($n = 28$), while *Ac. sinicus* was less frequent ($n = 12$ on *Tr. mitsukurii* and $n = 1$ on *Tr. japonicus*). Two other associations were observed once: *Tr. cultratus* and *Tr. japonicus*; *Tr. basalis* and *Tr. mitsukurii*. The emergence of two species generally coincided with the lack of emergence if *H. halys* nymphs (only 1.5% of eggs) and the presence of unhatched eggs (25% of eggs).

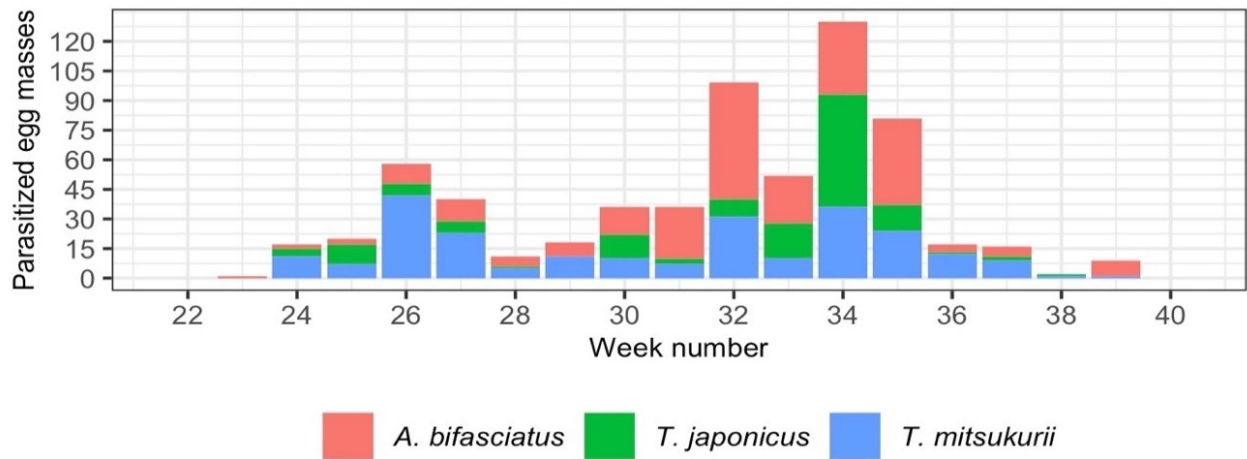


Figure 1. Number of parasitized egg masses collected throughout the sampling period (May–September 2019) for *Anastatus bifasciatus*, *Trissolcus japonicus* and *Trissolcus mitsukurii*

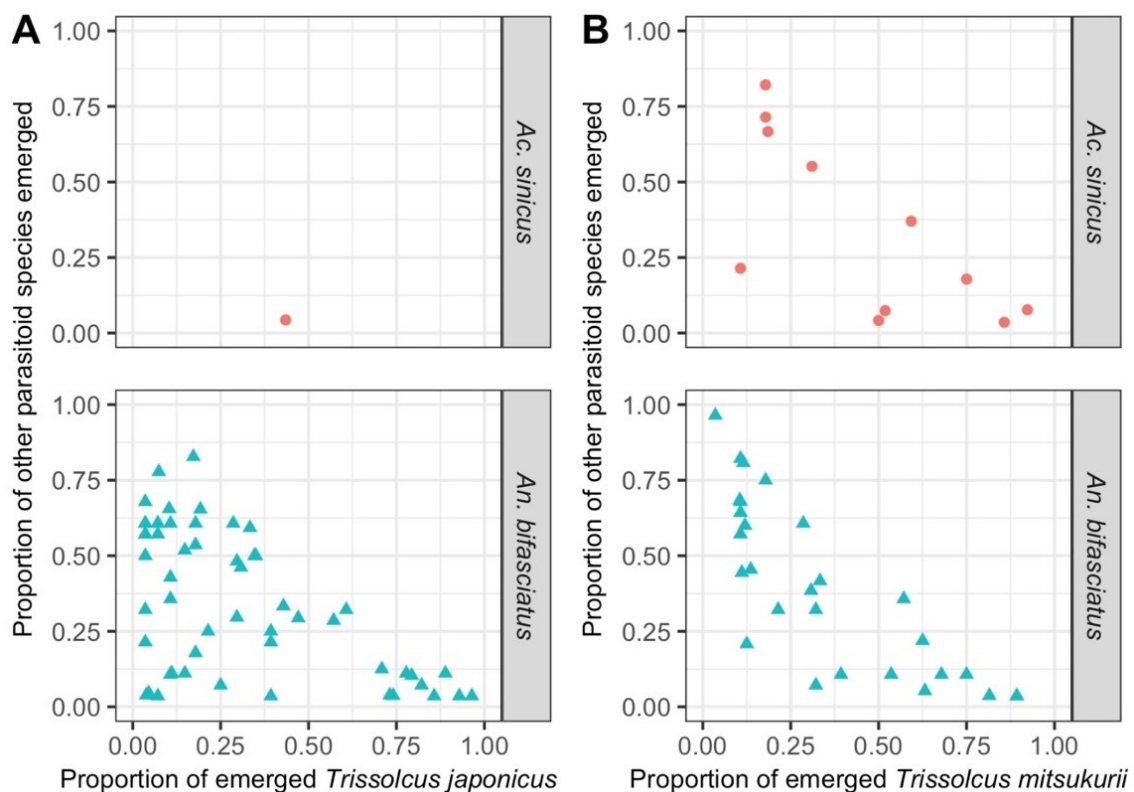


Figure 2. Multiparasitism of egg masses of *Halyomorpha halys* where the emergence of two species was observed (survey methods a and b): (A) proportion of emerged individuals for *Trissolcus japonicus* and other species (*Acroclisoides sinicus* and *Anastatus bifasciatus*) (N. of considered egg masses = 56); (B) proportion of emerged individuals for *Trissolcus mitsukurii* and other species (*Ac. sinicus* and *An. bifasciatus*) (N. of considered egg masses = 39).

Habitat, Host Species and Distribution

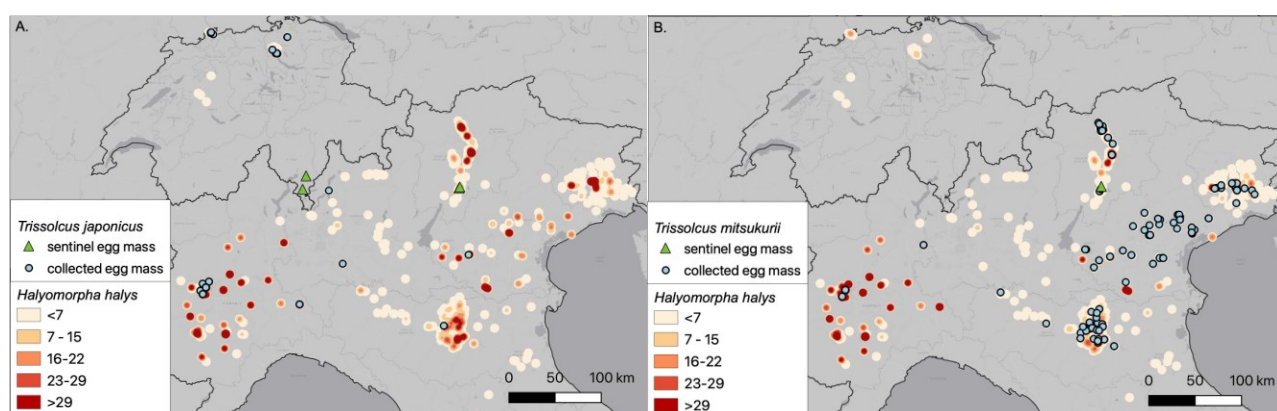
Both exotic parasitoids were found in agricultural and urban areas. In agricultural systems, parasitized egg masses were found in conventional, integrated pest management (IPM) and organic orchards and fields, both on the edge (e.g., perimetral shrubs, hedgerows) and in the inner part of the cultivated area. In urban areas, parasitized egg masses were located in gardens, parks, parking lots and street trees. Concerning host plants, *Tr. japonicus* was recorded on both cultivated plants (i.e., *Corylus avellana* L., *Prunus persica* (L.) Batsch, *Olea europaea* L.) and trees planted in hedgerows or along streets (i.e., *Acer* spp., *Acer campestre* L., *Catalpa bignonioides* Walter., *Fraxinus* spp., *Tilia platyphyllos* Scop.). See Table S2 for the complete list of *H. halys* plant host species.

Trissolcus mitsukurii was also recorded on cropland (on *Acer* spp., *Actinidia deliciosa* (A. Chev.) C.F. Liang and A.R. Ferguson, *Ailanthus altissima* (Miller) Swingle, *Corylus avellana* L., *Glycine max* (L.) Merr., *Malus domestica* (Suckow) Borkh., *Vitis vinifera* L., *Ziziphus jujuba* Mill.) and in urban areas (on *Acer* spp., *A. altissima*, *Fraxinus* spp., *Prunus spinosa* L., *Ziziphus jujuba* Mill.).

The surveys (methods a, b and c) proved that both species are widely distributed in northern Italy while in Switzerland only *Tr. japonicus* is currently present (Figure 3).

Anastatus bifasciatus and *Tr. mitsukurii* were recorded in all surveyed regions. The survey highlighted also that the distribution ranges of both exotic egg parasitoids are expanding southwards from the first Italian records (e.g., recording the first observation in Emilia-Romagna region).

Figure 3. Distribution map of the collected egg masses for *Halyomorpha halys*, showing the emergence of the two exotic parasitoids in northern Italy and Switzerland (survey methods a, b and c): (A) *Trissolcus japonicus* and (B) *Trissolcus mitsukurii*. The heatmap is based on the abundance of collected egg masses of *H. halys* where orange reflect a low number and red a high number of egg masses. Sentinel egg mass records are derived from Stahl et al. (2019) and Zapponi et al. (2020).



Discussion

Overall, the results of the large-scale monitoring demonstrated a wide distribution and continuous expansion of *Tr. japonicus* and *Tr. mitsukurii*, compared to previous studies (Sabbatini Peverieri et

al. 2018; Moraglio et al. 2020; Scaccini et al. 2020; Zapponi et al. 2020). Four years following their first detection in Europe, both species have rapidly spread into all types of habitats where *H. halys* has been surveyed. Host plants included a wide range of crop and non-crop species. The patterns of occurrence suggest that *Tr. mitsukurii* is currently more widespread than *Tr. japonicus* and more abundant in the eastern part of northern Italy, while in Switzerland *Tr. mitsukurii* appears to be still absent. This different expansion could be explained by the timing of arrival of the two species and first locality of accidental introduction, since the earliest official record of *Tr. mitsukurii* for northern Italy was traced back to 2016 (Scaccini et al. 2020), two years before the earliest detection of *Tr. japonicus* (Sabbatini Peverieri et al. 2020). Furthermore, the different abundance may be responsible also for the dissimilar observed habitat prevalence (agricultural/urban) observed for *Tr. japonicus* and *Tr. mitsukurii*. Whether other factors (e.g., host plant availability, sensitivity to chemicals) are responsible for the observed pattern merits further investigation.

As previously observed (Van Lenteren et al. 2006; Haye et al. 2015; Moraglio et al. 2020), our study confirmed that among native species, the generalist *An. bifasciatus* was the predominant parasitoid of *H. halys* in Italy, with a wide distribution and abundance. Conversely, the emergence of native scelionids was rarely observed. In the present study, *An. bifasciatus* emerged often from egg masses parasitized for other species as well. As previously pointed out (Viggiani, 2017; Konopka et al. 2017) the role of *An. bifasciatus* (as competitor or hyperparasitoid) should be further investigated.

Yet, the lack of detected emergence is no evidence that an attempt of parasitization has not taken place. Molecular methods proved that native scelionids may frequently parasitize *H. halys* eggs but fail to develop, resulting in an evolutionary trap (Konopka et al. 2019). Similarly, laboratory trials showed that native *Trissolcus* species are able to induce egg abortion (non-reproductive effects) in *H. halys* (Moraglio et al. 2021). Studies carried out on North American native parasitoids suggest that there is currently no evidence of intraspecific variation in traits that allow native parasitoids to successfully develop in *H. halys* eggs (Costi et al. 2020). However, the successful adaptation of native parasitoids to a new host may occur over larger timescales (i.e., decades) (Cornell and Hawkins, 1993) and should be further studied in Europe.

Field data from the native range of both *Trissolcus* species indicate that their host ranges are restricted to a few pentatomid species (Zhang et al. 2017; Arakawa and Namura, 2002). Both parasitoid species showed a favorable degree of specificity towards *H. halys* in the present study, even though they developed on other hosts (for example, *D. baccarum*, *N. viridula* and *Pa. prasina* for *Tr. mitsukurii*; *Pa. prasina* for *Tr. japonicus*), it was a relatively occasional event in this survey. Nevertheless, the data on native stink bugs were limited since the focus of the present study was on *H. halys*, and host plants of native pentatomids were only marginally sampled, resulting in a smaller number of collected egg masses. The use of sentinel egg masses produced in rearing facilities could overcome the potential

for a relatively low number of collected samples, but unfortunately such egg masses are less reliable since they tend to underestimate parasitoid impact (Jones et al. 2014).

The actual impact on native stink bugs should be evaluated with specifically designed field experiments, which should take into account hidden trophic interactions (Hepler et al. 2020) as well. Further investigations are needed to assess whether behavioral barriers (such as phenology, habitat preference and interspecific competition) exist and could prevent non-target parasitism in the field (Haye et al. 2020).

Both *Tr. japonicus* and *Tr. mitsukurii* showed notable levels of parasitism rate, which is helpful to build up populations. However, this first large-scale monitoring also showed a widespread presence of the hyperparasitoid *Ac. sinicus*, the expansion of which could cause possible problems in the future, limiting the impact of *H. halys* natural enemies. *Acroclisoides sinicus* was recently detected in many countries worldwide, but it is suspected to be an Asian species and its influence on the host–parasitoid trophic chain should be further investigated (Sabbatini Peverieri et al. 2019; Sabbatini Peverieri et al. 2020). The generalist parasitoid *An. bifasciatus* was frequently found throughout the survey region, but its impact on *H. halys* is considered low (Costi et al. 2019) and inundative releases of this species did not result in sufficient pest control (Stahl et al. 2019). Accordingly, future efforts should focus on the two coevolved biological control agents originating from the pest’s area of origin.

Studies assessing the long-term trend of host–parasitoid interactions between Pentatomidae and Scelionidae are scarce (Sabbatini Peverieri et al. 2018; Colazza, and Bin, 1995) and several important issues remain unexplored (such as the variation of parasitism rate with time, for adventive populations). Further investigations on population and community ecology are needed to provide a strong basis for biological control prediction and management (McEvoy, 2018). The present large-scale study (with shared survey protocols and analysis approaches) provides a baseline on parasitoid species composition and parasitism rate that may be used in the future to assess the impact of both adventive and released *Trissolcus* populations, and thus evaluate the output of classical biological control programs. In particular, data collected with the presented approach could also be used to understand whether laboratory predictions on non-target exploitation are consistent with what is observed in the field, in the area of introduction.

In Europe, the application of classical biological control is regulated by stringent risk assessments. Bureaucratic barriers, general criticisms due to poor public awareness, together with limited funding, may block the release of biological control agents (Barratt et al. 2018; Sheppard et al. 2006). As recently stated (Buffington et al. 2018; Peixoto et al. 2018), more coordinated efforts are needed, with taxonomists and researchers working together, to create the grounds for effective biological area-wide control programs. The cooperative character of the present study was fundamental to cope with the difficult task of performing a large-scale survey in a limited amount of time, and provide data for

Tr. japonicus risk assessment, essential for the ongoing release of the tested strain in Italy. The present approach could be applied to post-release studies as well, coping with logistically and economically challenging tasks. Biocontrol long-term development requires coordinated efforts, involving local and central governments, the engagement of the scientific community and public support (Wang et al. 2019). Thus, creating the means for large scale international collaborations represents a further step towards the implementation of sustainable agriculture.

Conclusions

Small founder populations of *Tr. japonicus* and *Tr. mitsukurii* initially recorded in Switzerland (Stahl et al. 2019) and in northern Italy (Sabbatini Peverieri et al. 2018; Moraglio et al. 2020; Scaccini et al. 2020; Zapponi et al. 2020) are currently expanding their range, following the path of their host *H. halys*. Bioclimatic models suggest that at least *Tr. japonicus* will be able to thrive and expand in all areas where *H. halys* already occurs, but also in nearly all areas where *H. halys* has been predicted to expand (Avila and Charles, 2018). Considering that *H. halys* control still largely relies on the use of broad-spectrum insecticides, the reduced risk of potential non-target effects on a limited number of native stink bug species should not hamper future releases of *Tr. japonicus* (Haye et al. 2020), but should however be carefully evaluated with the support of a cost/benefit analysis (Van Lenteren et al. 2006). The negative perceptions associated with classical biological control should not result in lost opportunities of increasing the sustainability of invasive pest management (Hajek et al. 2016).

Author Contributions:

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

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Abstract

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

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

Introduction

Halyomorpha halys (Stål) (Hemiptera, Pentatomidae) is an invasive stink bug native to Asia which causes significant damage in agriculture in different countries worldwide (e.g. USA, Italy,

Switzerland, Georgia) (Leskey and Nielsen 2018). Biological control with egg parasitoids is viewed as the most promising control method for long-term management, and several studies have focused on these natural enemies in the native range of the pest and in the newly invaded countries of USA and Europe (Haye et al. 2015; Herlihy et al. 2016; Roversi et al. 2016; Zhang et al. 2017). Hymenopteran species of *Anastatus* Motchulsky, *Trissolcus* Ashmead, *Telenomus* Haliday and *Ooencyrtus* Ashmead have been reported to attack eggs of *H. halys* in the native and invaded areas, although with varying degrees of successful development (Zhang et al. 2017; Abram et al. 2017). Unfortunately, in the newly invaded areas, the impact of the native egg parasitoids has not been sufficient to control *H. halys* (Haye et al. 2015; Abram et al. 2017; Dieckhoff et al. 2017; Costi et al. 2019). In Europe, the most common native egg parasitoid is *Anastatus bifasciatus* (Geoffroy) (Hymenoptera, Eupelmidae). Although parasitization is sometimes locally high, the overall parasitism by this species on *H. halys* egg masses laid naturally in the field is less than 6%–7%, too low to provide significant levels of biological control (Haye et al. 2015; Costi et al. 2019; Stahl et al. 2019a; Moraglio et al. 2020). Other native egg parasitoid species that are reported to attack *H. halys* in Europe include the scelionids *Trissolcus kozlovi* Ryakhovskii, *Trissolcus basalis* (Wollaston), *Trissolcus semistriatus* (Nees von Esenbeck) and *Telenomus turesis* Walker, all of which are reared infrequently from *H. halys* at low parasitism levels ranging from 0.1–0.7 % (Moraglio et al. 2020). In the native range of *H. halys*, its most important natural enemy is the egg parasitoid *Trissolcus japonicus* (Ashmead) (Hymenoptera, Scelionidae). This species causes high levels of parasitism (up to 80%) and is considered a classical biological control agent for potential release in the invaded ranges (Yang et al., 2009; Zhang et al. 2017).

Adventive populations of *T. japonicus* were recently found in Europe (Switzerland and Italy), and a second Asian egg parasitoid species, *Trissolcus mitsukurii* (Ashmead) (Hymenoptera, Scelionidae), was found also in Italy (Sabbatini Peverieri et al. 2018; Stahl et al. 2019b). The recent discoveries of these species have highlighted the possibility of effective control of *H. halys* in the near future in Europe, and studies aimed at defining the distribution, establishment and impact of the egg parasitoids are of significant interest. Moreover, recent field studies led to the discovery, both in North America and in Europe, of *Acroclisoides sinicus* (Huang & Liao, 1988) (Hymenoptera, Pteromalidae) (Sabbatini Peverieri et al. 2019). This pteromalid is native to Asia and is thought to be a hyperparasitoid due to its frequent association with *Anastatus* spp. (e.g. *An. bifasciatus* in Europe) and *Trissolcus* spp. (e.g. *T. japonicus* in Europe and Asia) within the same parasitized host egg masses (Clarke and Seymour 1992; Grissell and Smith 2006; Sabbatini Peverieri et al. 2019). All known egg parasitoids of *H. halys* are solitary idiobionts, with generally only one adult egg parasitoid emerging from each host egg. Monitoring the field occurrence of egg parasitoids by collecting and rearing host egg masses, followed by the identification or characterization of emerged parasitoids by taxonomists

and molecular biologists (e.g. Talamas et al. 2017) are crucial steps for successful biological control programs. When adult egg parasitoids have already emerged from host eggs prior to their collection, identification by molecular analysis of their DNA remaining in the host eggs is still possible (Garipey et al. 2014; Stahl et al. 2019c). However, a rapid visual method for species or genus identification is also desirable. For example, the characteristic exit holes of the different species of the *H. halys* egg parasitoid guild in Europe (i.e. *T. japonicus*, *T. mitsukurii*, *Ac. sinicus* and *An. bifasciatus*) were recently described (Sabbatini Peverieri et al. 2019).

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

Materials and methods

Collection and rearing of the insects

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

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

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

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

Microscope observations

Examination of the egg masses from which parasitoids emerged and meconium structure was conducted using a stereomicroscope (SMZ25) equipped with a digital camera (DSRi2) and the image acquisition software NIS-Elements (all from Nikon Corporation, Tokyo, Japan). Image postprocessing utilized Gimp (v. 2.10.04, GNU Image Manipulation Program). The samples of meconium were also analyzed by scanning electron microscopy (SEM) to obtain different details; images were taken with a JEOL NEO-SCOPE JCM-5000 equipped with an imaging system. Samples of meconium were prepared following the technique of Raafat et al. (2014). The mounted samples were coated with a thin layer of gold with a JEOL JFC-1300 Sputter Coater. Images were then taken under high vacuum at 10 kv with an enlargement ranging from 60X to 130X.

Results and discussion

A total of 12 egg masses of *H. halys* were collected in the field (298 eggs) which were partially or completely parasitized by at least one of the species of the egg parasitoid guild. Of these, 7.72% emerged in the field prior to collection, 8.05% of the eggs hatched to produce first instar *H. halys* nymphs, and 23.18% eggs died without any emergence. Overall, the parasitized egg masses produced 47 specimens of *T. japonicus*, 89 *T. mitsukurii*, 20 *An. bifasciatus* and 22 *Ac. sinicus*. Adults that emerged from the collected egg masses were used to establish lab-reared colonies. From these reared colonies, a total of 21 parasitized egg masses of *H. halys* were randomly selected for further analysis (four egg masses parasitized by *T. japonicus*, four by *T. mitsukurii*, five by *Ac. sinicus*/*T. japonicus*, three by *Ac. sinicus*/*T. mitsukurii* and five from *An. bifasciatus*). In total, 351 parasitized eggs were analyzed which included 104 adults of *T. japonicus*, 105 of *T. mitsukurii*, 97 *Ac. sinicus* (68 associated with *T. mitsukurii* and 29 associated with *T. japonicus*) and 45 *An. bifasciatus*.

Observation of parasitized egg masses revealed the presence of at least one type of meconium in each *H. halys* egg from which an egg parasitoid emerged, and in some cases the meconium (and pupae) could be observed through the intact egg chorion prior to emergence of the adult (Fig. 1).

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

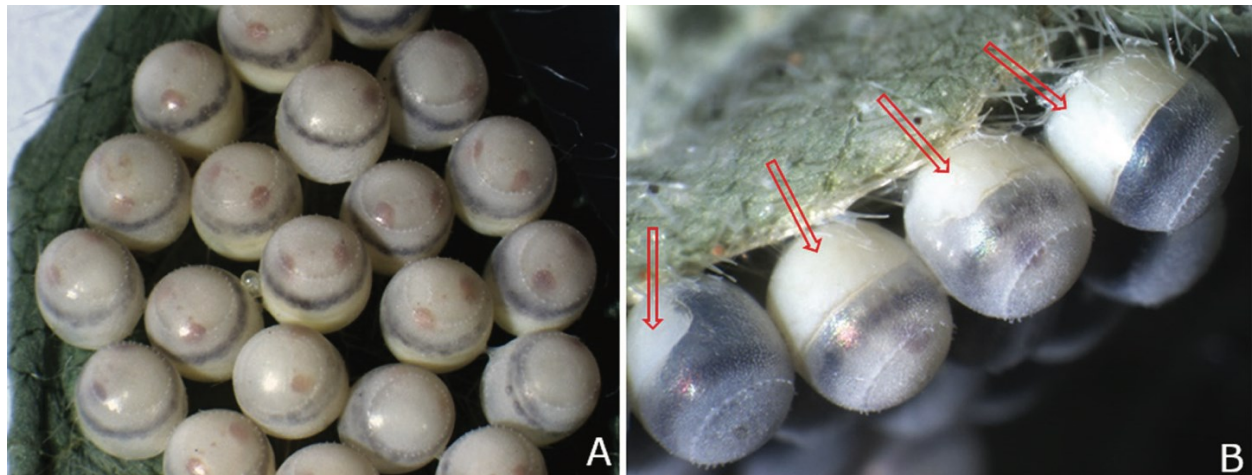
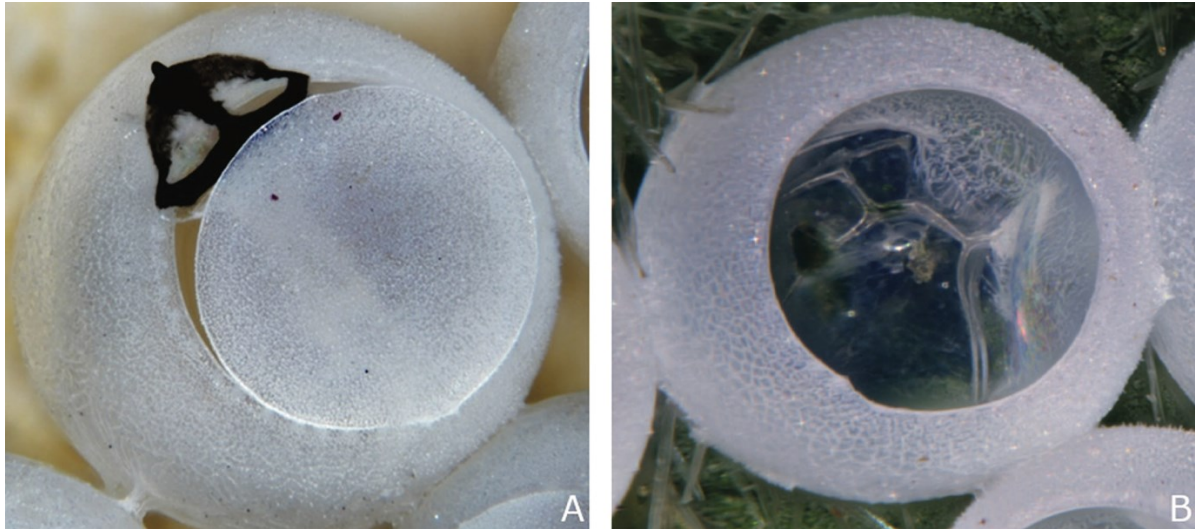


Figure 2. Exit holes of egg parasitoids of *Halyomorpha halys* in Europe and hyperparasitoids: *Acroclisoides sinicus* (A), *Trissolcus mitsukurii* (B), *Anastatus bifasciatus* (C), *Acroclisoides sinicus* partly emerged (D), *Trissolcus japonicus* (E), *Ooencyrtus telenomicida* (F, only from sentinel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



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

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



Meconium description

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

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

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

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

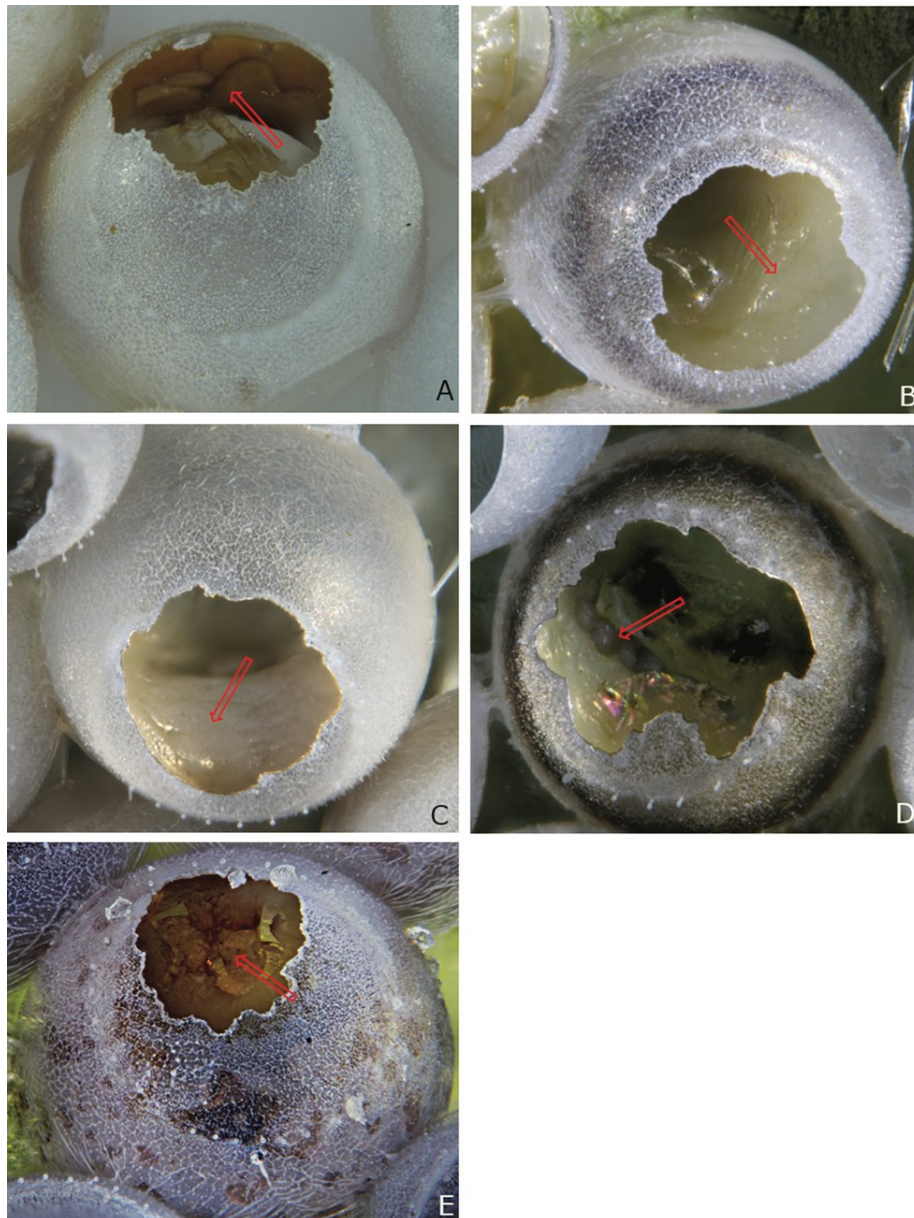


Figure 5. Meconium (arrows) of egg parasitoids of *Halyomorpha halys* visible through partially dissected host eggs: *Anastatus bifasciatus* (A); *Trissolcus mitsukurii* (B); *Trissolcus japonicus* (C); *Acroclisoides sinicus* on previous parasitized egg by *Trissolcus mitsukurii* (D); *Ooencyrtus telenomicida*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

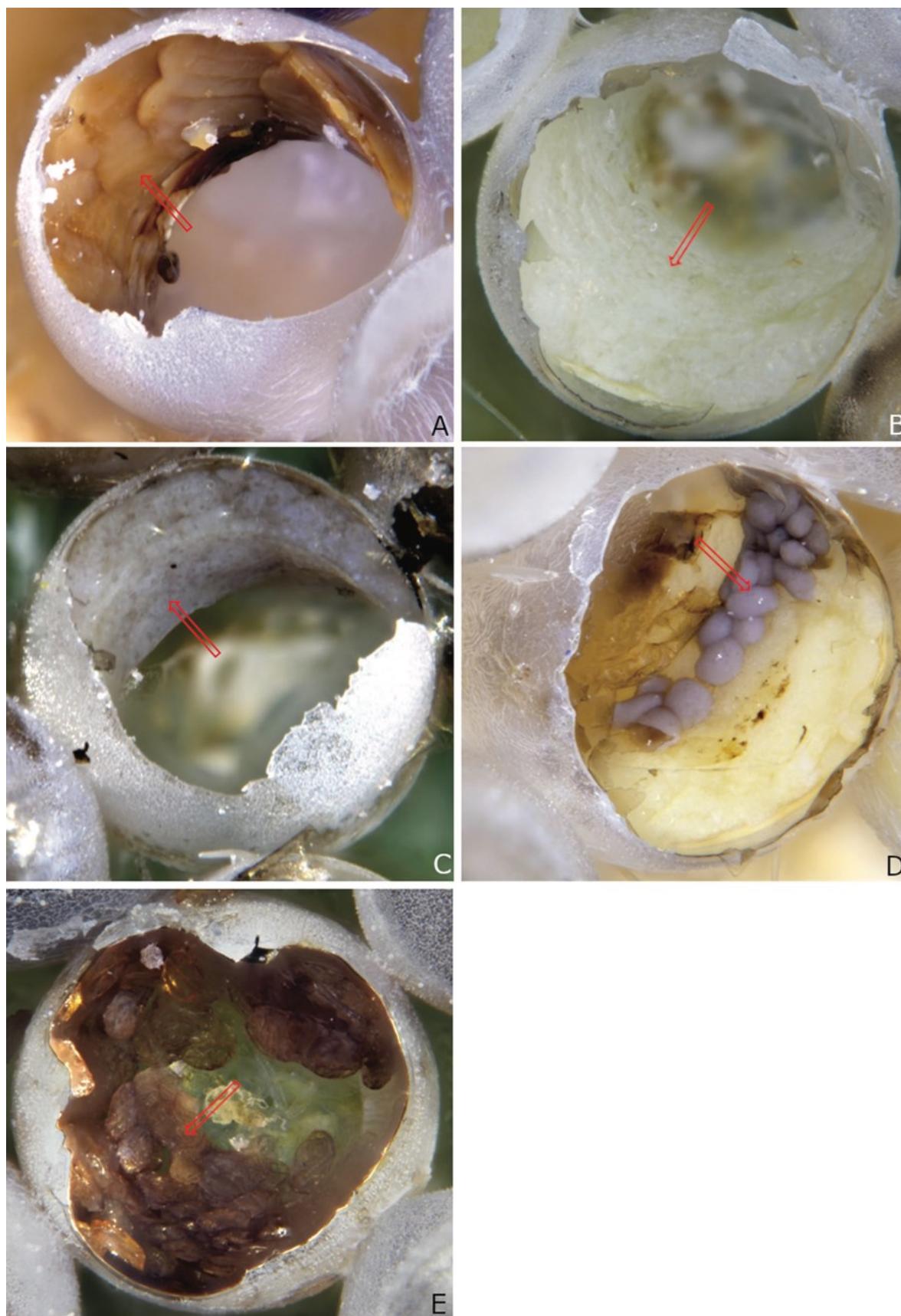
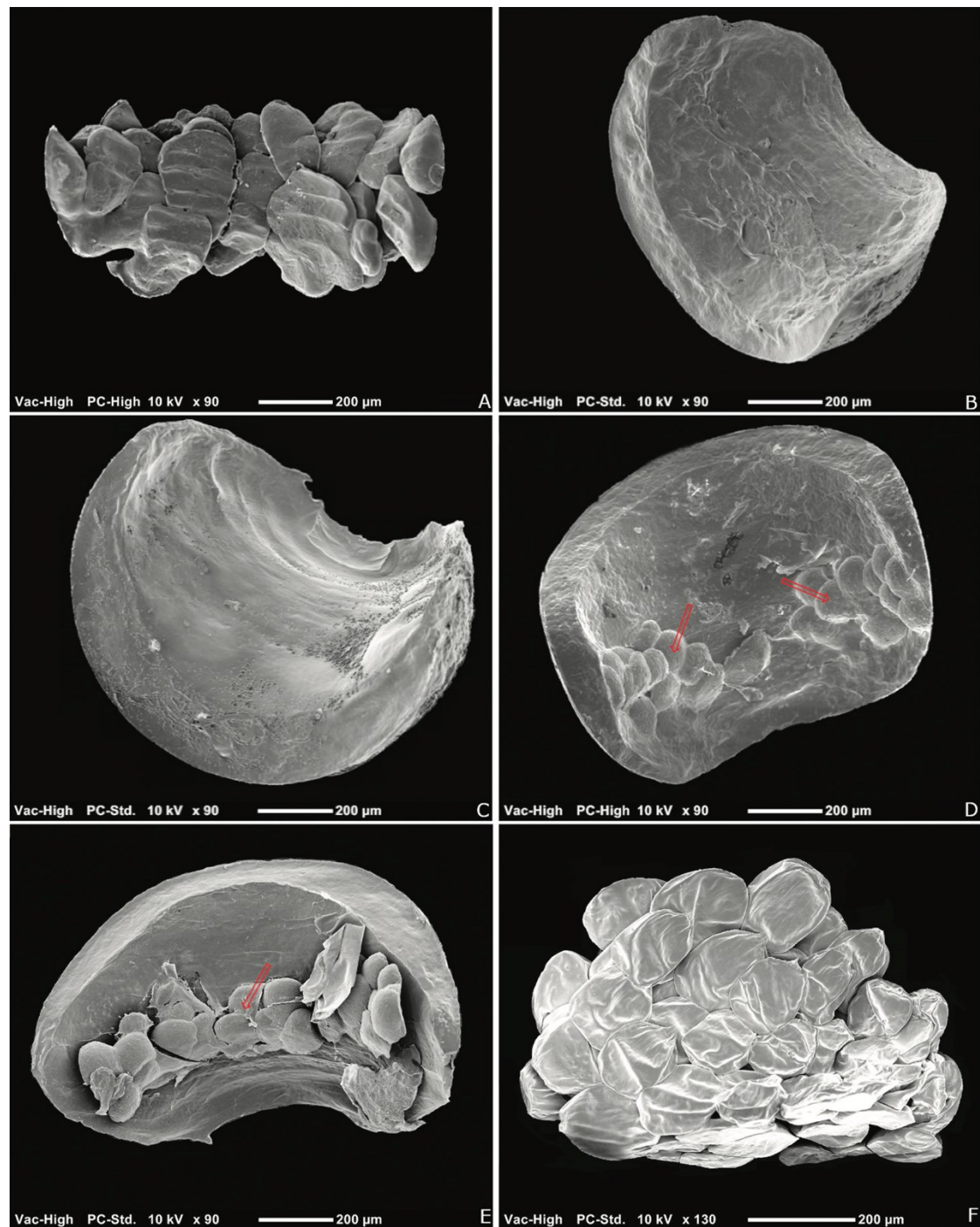


Figure 6. *Halyomorpha halys* egg parasitoid meconium extracted from the host egg: *Anastatus bifasciatus* (A); *Trissolcus japonicus* (B); *Trissolcus mitsukurii* (C); *Acroclisoides sinicus* (red arrows) on meconium of *Trissolcus japonicus* (D); *Acroclisoides sinicus* (red arrows) on meconium of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



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

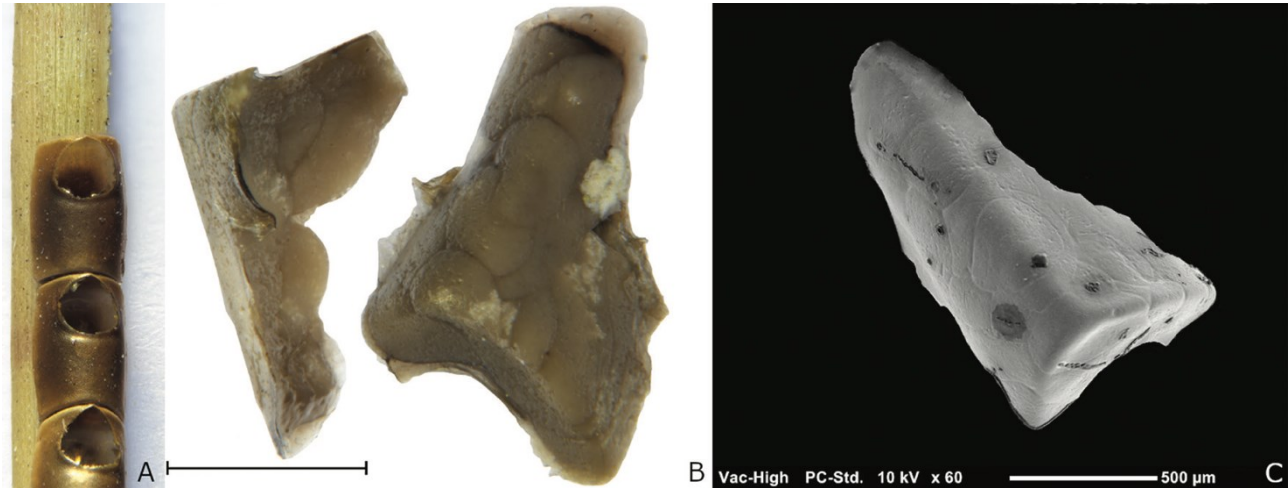


This suggests that hyperparasitism of *Trissolcus* by *Ac. Sinicus* occurs during a late stage of development of its host, when the larvae were mature, during the larvae-pupae transition, or at the pupal stage.

The meconium of *O. telenomicida* is different from all the other species of egg parasitoids previously considered, appearing as a mass of feces comprised of amber-brown discs that are randomly distributed inside the egg host (Figs 2, 4E, 5E, 6G, 7F). In contrast with the meconium of the egg

parasitoids described above, the individual pellets of *O. telenomicida* can easily be separated by breaking apart the mass with a brush (Fig. 6G).

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



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

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

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

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Author Contributions:

LG, GSP conceived and designed research. LG and GSP conducted experiments. LM, CB contributed for digital image acquisition. PFR involved in funding acquisition, resources and supervision. LG and GSP involved in data curation and writing original draft. LG, GSP, KAH, PFR involved in writing final version, in review and editing, LG, GSP, LM, GR, EC and PFR involved in review and editing. All authors read and approved the manuscript.

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Chapter 2: Non-target host risk assessment for *Trissolcus japonicus* and *Trissolcus mitsukurii* under different multidisciplinary approaches

2.1 Combining physiological host range, behavior and host characteristics for predictive risk analysis of *Trissolcus japonicus*

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Abstract

Halyomorpha halys is an Asian pentatomid that has recently invaded several countries worldwide, where it has become a severe pest. Classical biological control focused on the scelionid egg parasitoid *Trissolcus japonicus* appears to be the most promising long-term solution. However, non-target risks need to be included in cost/benefit analyses. Physiological host range tests were conducted by offering *T. japonicus* females a single hemipteran egg for short (2 h) and long (24 h) exposure periods, and recording the behavior and parasitization acceptance/success during the short exposures. Of the 16 different hemipteran species tested in no-choice experiments, 9 species were accepted and suitable for *T. japonicus* development. Among pentatomids, *Palomena prasina* and *Rhaphigaster nebulosa* were accepted at rates comparable to *H. halys*, while species accepted at lower rates included *Acrosternum heegeri*, *Carpocoris mediterraneus*, *C. purpeiripennis*, *Dolycoris baccarum*, *Piezodorus lituratus* and *Peribalus strictus*. The other 7 hemipterans tested, including two coreids and one reduviid, were never parasitized. The use of a short-term exposure of 2 h revealed no differences with the 24-h exposure that has been widely used in physiological host range testing. Thus, *T. japonicus* oligophagy observed in other studies was confirmed by using our extremely simplified laboratory protocol. Moreover, the host egg morphology was correlated with the acceptance rates, permitting us to propose a new predictive approach for non-target studies. However, further research with more complex experimental designs that account for environmental conditions is needed to confirm laboratory predictions of host specificity under actual field conditions.

Keywords: Biological control; Egg parasitoid; *Halyomorpha halys*; No-choice test; Pentatomidae; Non-target risk assessment

Introduction

Halyomorpha halys Stål (Hemiptera: Pentatomidae), a stink bug of Asian origin, was accidentally introduced during recent decades in many different regions worldwide (North and South America, Europe) and causes severe damage to many plants of agricultural, forest and ornamental interest (Leskey and Nielsen, 2018). Several pesticides showed relatively high insecticidal activities, but under field applications they led in many cases to unsatisfactory efficacy in the control of the pest (Leskey and Nielsen, 2018). Due to the limited control action provided by native natural enemies in the newly invaded countries, the management options for this pest over the long term have been focused with growing emphasis on classical biological control (Leskey et al. 2014; Abram et al. 2017; Buffington et al. 2018; Moraglio et al. 2020).

The egg parasitoid *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae) is considered the most important natural enemy of *H. halys* in its native area (China, Korea and Japan) (Zhang et al. 2017). Adventive populations of *T. japonicus* and *Trissolcus mitsukurii* (Ashmead), a second scelionid Asian egg parasitoid of *H. halys*, were recently discovered in Europe, and the future prospects for classical biological control of this pest are a matter of debate (Sabbatini Peverieri et al. 2018; Stahl et al. 2019).

The application of classical biological control is highly regulated by international and national policies. Proposals for the introduction of new exotic biocontrol agents are made by international research institutions and experts and submitted for review and approval by regulatory agencies (van Lenteren et al. 2006a; EPPO 2014; FAO-IPPC 2017; Barratt et al. 2018). Before introducing an exotic biological control agent (BCA), non-target risk analysis is one of the most important aspects to be considered. The possibility that the BCA might have negative effects on non-target hosts needs to be carefully evaluated in a cost/benefit analysis (Simberloff and Stiling, 1996; Bigler and Kölliker-Ott, 2006; EPPO, 2018). In some countries, the risks are considered more important than the benefits in evaluating possible impacts (Heimpel and Cock, 2018). Many studies are therefore designed to follow official guidelines, and they represent the most critical and difficult aspects in the risk assessment for the introductions of BCA (van Lenteren et al. 2006a). Since these studies can be difficult to conduct, potential non-target effects are evaluated based on studies with a limited number of species. Impediments to defining a suitable list of potential non-target hosts include collecting sufficient numbers of a species from the field and rearing them in the laboratory to produce viable and high-quality biological material for the laboratory tests (van Lenteren et al. 2006b). Furthermore, high quality biological material of the BCA and of the tested species for non-target risk assessment is

necessary to correctly interpret and compare the study results (e.g., physiological status comparable to field populations, high egg hatching rate) (Sands and van Driesche, 2003). Some species are uncommon and difficult to rear, and the ability to exploit the limited biological resources available (e.g., the stages expected to be attacked) is pivotal in such studies (Babendreier et al. 2006).

Host range studies on *T. japonicus* were initially performed worldwide (USA, Europe, New Zealand and China) by employing small testing arenas (Petri dishes or small vials) for choice or no-choice host egg masses exposure for 24 h, followed by larger-scale arenas in the laboratory or a semi-field setting. Several studies reported that *T. japonicus* is an oligophagous egg parasitoid (Zhang et al. 2017; Hedstrom et al. 2017; Botch and Delfosse 2018; Charles et al. 2019; Haye et al. 2020; Lara et al. 2019). To date, only one study has reported the impact on non-target species in the field in the USA (Milnes and Beers, 2019). Thus, more research is needed to predict the possible impact(s) caused by the introduction of *T. japonicus* in the field (Briese, 2005).

It is well known that laboratory tests are highly simplified trials that do not account for the complexity of factors that affect host selection in the field, such as the chemical footprints of hosts (Bertoldi et al. 2019; Boyle et al. 2019). Thus, laboratory tests most likely reflect the physiological (= fundamental) host range rather than the ecological (= realized) host range (Haye et al. 2020; Lara et al. 2019). To reduce uncertainty after conventional host-specificity testing trials, it is crucial to introduce additional experiments to predict the potential ecological host range of the candidate biocontrol agents (Haye et al. 2020). Conducting an exhaustive preintroduction risk analysis of parasitoids is a difficult challenge, inversely proportional to the specialization level of the useful organism. For this reason, non-target risk assessment in classical biological control is a complex field of study for which multidisciplinary approaches are needed, e.g., chemical ecology, electroantennography and olfactory studies.

Here we report a new approach for a simplified physiological host-range test of *T. japonicus* based on behavioral analyses using a single hemipteran egg as a test unit, as previously proposed by Babendreier et al. (2003) for tests with lepidopterans. Laboratory tests are severely constrained in terms of time and resources, so our first aim was to determine if such a simplified method—and especially a short egg exposure time (2 h), rather than 24 h—could be predictive for the physiological host range (see Babendreier et al. 2005). Moreover, the use of short duration exposures in choice tests would reduce multiple host parasitization in small arenas that can invalidate those replicates (see Haye et al. 2020). We also analyzed different behaviors of *T. japonicus* females exhibited during inspection and parasitization (acceptance or rejection) of the different hemipteran eggs offered. This approach allowed us to clearly define the behaviors of host egg inspection, oviposition and host marking and the overall level of interest displayed by females toward non-target eggs offered for oviposition.

Moreover, Babendreier et al. (2006) suggested introducing modelling approaches in risk assessment aimed at predicting potential risk. The pentatomid host species parasitized by *T. japonicus* display a wide range of egg features (e.g., volume, shape, chorion surface) (see Dieckhoff 2014a, b). Here, we present an assessment of *T. japonicus* host range based on the egg morphology of potential host species. This analysis offers a new tool to predict the potential suitability of different species and thus to foresee the potential impact of introduced parasitoids on the local fauna.

Materials and methods

Source of insects

The target species, *H. halys*, was collected at infested sites in northern Italy from May to August 2018–2019. Adult specimens of *H. halys* were reared in cages (BugDorm 4F4545, Insect MegaView Science Co. Ltd., Taichung, Taiwan) and colonies were maintained in the laboratory at + 26 °C and 16:8 L:D.

Non-target species were selected according to the information available in the literature and using the field host range survey method proposed by Kuhlmann and Mason (2003) and Kuhlmann et al. (2006). These species were collected during field surveys in the growing season in northern and central Italy, from May to August 2018–2019 (see Table 1 for a complete list of species and collection sites). Laboratory-reared colonies were established in cages and kept under the same standard conditions described above. Phytophagous pentatomids were fed with seeds (peanuts, *Arachis hypogaea*; sunflower, *Helianthus annuus*; pumpkin, *Cucurbita pepo*; fennel, *Foeniculum vulgare*), fresh vegetables (carrots, *Daucus carota*; green beans, *Phaseolus vulgaris*; corn, *Zea mays*), fresh fruits (apples, *Malus domestica*; kiwi, *Actinidia deliciosa*) and soybean plants (*Glycine max*) (all food were purchased from local markets, while potted plants were self-produced).

Entomophagous insects were reared on *Acanthoscelides obtectus* Say (Coleoptera: Chrysomelidae) adults and *Nezara viridula* L. (Hemiptera: Pentatomidae) nymphs. Water was provided with wet cotton. Food and water were replenished three times per week. As an oviposition substrate, paper towels were hung inside the rearing cage. Hemipteran eggs were collected daily (< 24 h) for use in all tests.

Laboratory-reared colonies of *T. japonicus* were established with specimens supplied by the USDA-ARS Beneficial Insects Introduction Research Unit, in Newark, Delaware, USA (population originally from Beijing, China) and imported into Italy under quarantine conditions and authorization number for laboratory studies DG/DISR/ DISR05/0013647-19/04/2018. Laboratory-reared colonies were established in a quarantine rearing facility, and specimens used in the tests were obtained from colonies reared over 10 generations. Pure honey droplets were used as an adult wasp food source and

were replaced twice a week; fresh egg masses (< 24 h) of *H. halys* were used for parasitization. Adults and parasitized host eggs were reared in climatic chambers at 26 ± 1 °C, $75 \pm 5\%$ RH and 16:8 L:D (HPP750, Memmert GmbH + Co. KG, Schwabach, Germany). All stages of the egg parasitoids were reared in glass tubes 15 cm long and 2 cm wide, closed on both ends by a plastic net of 250 µm mesh.

Table 1 List of species collected for the physiological host range tests with *Trissolcus japonicus* under laboratory conditions

Target and non-target species	Order / Family / subfamily / tribe	Collection site
<i>Halyomorpha halys</i> (Stål)	Hemiptera, Pentatomidae / Pentatominae / Cappaeiini	Codroipo (NE-Italy), Biella and Manta (NW-Italy)
<i>Carpocoris purpuripennis</i> (De Geer)	Hemiptera, Pentatomidae / Pentatominae / Carpocorini	Codroipo (NE-Italy),
<i>Carpocoris mediterraneus</i> Tamanini	Hemiptera, Pentatomidae / Pentatominae / Carpocorini	Trecate (NW-Italy)
<i>Dolycoris baccarum</i> (L.)	Hemiptera, Pentatomidae / Pentatominae / Carpocorini	Pozzolatico (Central Italy), Codroipo (NE-Italy)
<i>Palomena prasina</i> (L.)	Hemiptera, Pentatomidae / Pentatominae / Carpocorini	Pisa (Central Italy)
<i>Peribalus strictus</i> (Fabricius)	Hemiptera, Pentatomidae / Pentatominae / Carpocorini	Pozzolatico (Central Italy)
<i>Staria lunata</i> (Hahn)	Hemiptera, Pentatomidae / Pentatominae / Carpocorini	Pozzolatico (Central Italy)
<i>Rhaphigaster nebulosa</i> (Poda)	Hemiptera, Pentatomidae / Pentatominae / Pentatomini	Impruneta and Arezzo (Central Italy)
<i>Acrosternum heegeri</i> Fieber	Hemiptera, Pentatomidae / Pentatominae / Pentatomini	Impruneta (Central Italy)
<i>Nezara viridula</i> L.	Hemiptera, Pentatomidae / Pentatominae / Pentatomini	Impruneta and Pisa (Central Italy), Campasso (NW-Italy)
<i>Piezodorus lituratus</i> (Fabricius)	Hemiptera, Pentatomidae / Pentatominae / Piezodorini	Pozzolatico (Central Italy), Codroipo (NE-Italy)
<i>Sciocoris sidertis</i> Wollaston	Hemiptera, Pentatomidae / Pentatominae / Sciocorini	Pozzolatico (Central Italy)
<i>Eurydema ventralis</i> Kolenati	Hemiptera, Pentatomidae / Pentatominae / Strachiini	Arezzo and Florence (Central Italy)
<i>Coreus marginatus</i> (L.)	Hemiptera, Coreidae / Coreinae / Coreini	Pozzolatico (Central Italy)
<i>Gonocerus juniperi</i> Herrich-Schäffer	Hemiptera, Coreidae / Coreinae / Gonocerini	Cascine del riccio (Central Italy)
<i>Rhynocoris iracundus</i> (Poda)	Hemiptera, Reduviidae / Harpactorinae / Harpactorini	Pozzolatico (Central Italy)

Non-target tests and female behavior during parasitization

Non-target tests were performed in 0.5×7.0 cm glass vials. A single egg (< 24 h old) of the target or the non-target species (one egg per vial per female) was positioned on a thin paper strip using a wet brush. Single eggs used in the tests and exposed to the parasitizing female were gently separated from the egg mass and positioned on the paper strips. Eggs were carefully inspected to check the presence of any sign of manipulation damage and, in case, discarded. To verify that the handling of the eggs did not cause any detrimental effects, a batch of manipulated and unmanipulated eggs/ egg masses were reared without the parasitoid in a climatic chamber under the same standard conditions. All eggs were checked daily, and hatching success was recorded.

The test unit was set up as follows: we inserted the female of *T. japonicus* into the glass vial, followed by the paper strip bearing the egg; a drop of honey was added as a food source and the vial was closed with a small cotton ball. We randomly selected female wasps of five days age from the colony. The age of females is crucial in such studies to permit the optimal accumulation of eggs in the ovary (due to its synovigeny) and a homogeneous physiological parasitization tendency (Qiu et al. 2007; Haye et al. 2020).

The behaviors of the females in the vial during a first period of parasitization opportunity were video-recorded for two hours (Panasonic WV-BP330/GE, Suzhou Matsushita Co., Ltd., Suzhou, China) in the laboratory at room temperature of 26°C . After that, the vial containing the parasitoid female and the offered hemipteran egg was subsequently transferred to a climatic chamber and reared as described above for an additional time period of 22 h, for a total of 24 h exposure time. Following this, the eggs exposed for parasitization for 24 h were removed and reared in a second glass vial until egg hatching or parasitoid emergence, while the female was discarded; the status of the offered hemipteran egg was checked daily and hemipteran or parasitoid emergence was recorded. After 3 weeks post-exposure, unhatched eggs were classified as “dead eggs”. Dead eggs were dissected to determine whether developing wasps or nymphs failed to emerge; however, these data were not included in the statistics. By this method the failure of the parasitoid to complete development can be accurately assessed only at pupal or pharate adult stage, while juvenile mortality at egg or larval stage (especially at first or second instars) is difficult to determine and cannot be assessed by dissections, leading to a potentially important underestimation of the actual parasitization rates. To address this critical issue, Konopka et al. (2019) recently proposed molecular analysis of dead host eggs, but this procedure was not adopted in the present work. To verify the effective ability of the tested female wasps to parasitize, we offered them a single *H. halys* egg for 24 h under the same standard conditions the day after their nontarget tests. Replicates with females that did not show any ability to parasitize *H. halys* were not included in the successive analysis. The number of replicates varied depending on

the number of hemipteran eggs produced by the reared colonies in the laboratory. Replicates ranged from 8 to 43 (see results and Tables 2 and 3 for more details).

Table 2. Hemipteran host suitability and parasitization parameters of *Trissolcus japonicus*; asterisks within each column indicate significant differences among the non-target species and the control *H. halys* (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) (see supplementary material 1).

Species	Hatchability rate of hemipteran eggs (%)			Hemipteran egg fate offered for oviposition (24h test period)			
	unmanipulated eggs	manipulated eggs	N of total replicates	% of eggs with no parasitoid and no hemiptera emerged (dead eggs)	N of viable eggs (that produced a parasitoid or a nymph)	% of eggs hatching to nymphs (N)	% of successful parasitized eggs (N)
<i>H. halys</i> (control)	90.39	85.25	41	2.5	40	0 (0)	100 (40)
<i>Pi. lituratus</i>	87.98	87.21	27	14.81	23	95.65 (22)***	3.45 (1)***
<i>D. baccarum</i>	88.00	85.94	40	32.50**	27	88.89 (24)***	11.11 (3)***
<i>Ca. purpeiripennis</i>	74.86	75.00	32	25.00*	24	41.67 (10)***	58.33 (14)
<i>Ca. mediterraneus</i>	76.61	70.42	36	55.56** *	16	52.94 (9)***	45.75 (7)
<i>E. ventralis</i>	96.67	100	24	8.33	22	100 (22)***	0 (0)***
<i>Ra. nebulosa</i>	91.67	89.29	40	10.00	36	2.78 (1)	97.22 (35)
<i>St. lunata</i>	100	n.a.	13	0	13	100 (13)***	0 (0)***
<i>Pa. prasina</i>	97.56	87.27	26	3.85	25	4.00 (1)	96.00 (24)
<i>Pe. strictus</i>	n.a.	n.a.	18	0	18	94.44 (17)***	5.56 (1)***
<i>Sc. sidertis</i>	n.a.	n.a.	8	0	8	100 (8)**	0 (0)***
<i>A. heegeri</i>	94.03	96.55	21	9.52	19	47.37 (9)***	52.63 (10)
<i>N. viridula</i>	85.58	93.33*	43	18.61*	35	100 (35)***	0 (0)***
<i>Co. marginatus</i>	95.24	100	27	0	27	100 (27)***	0 (0)***
<i>G. juniperi</i>	100	98.11	24	0	24	100 (24)***	0 (0)***
<i>Rh. iracundus</i>	96.67	82.68	26	7.69	24	100 (24)***	0 (0)***

Table 3 Duration (in seconds) of behaviors of *Trissolcus japonicus* females during the first 2h of exposure to *Halyomorpha halys* and non-target species; asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) in pairwise comparison among non-target species and the control (*H. halys*); nc = not compared due to the low number of replicates (see supplementary material 1).

Species (among brackets, N of viable eggs)	time until first contact with egg ($\pm$ S.E.) (s)	inspection during first encounter with egg ($\pm$ S.E.) (s)	N of replicates with successful parasitization in the first 2h	inspection of egg before acceptance ($\pm$ S.E.) (s)	oviposition ($\pm$ S.E.) (s)	post-oviposition marking ($\pm$ S.E.) (s)	time span from test start to acceptance for oviposition ($\pm$ S.E.) (s)	A/C ratio
<i>H. halys</i> (control) (40)	170.20 ($\pm$ 33.36)	19.35 ($\pm$ 4.91)	40	29.18 ($\pm$ 5.54)	149.35 ($\pm$ 14.60)	26.03 ($\pm$ 1.12)	725.15 ($\pm$ 166.92)	0.27
<i>Pi. lituratus</i> (23)	188.70 ($\pm$ 59.63)	12.26 ($\pm$ 2.70)	1	-	125.00 ^{nc}	35.00 ^{nc}	327.00 ^{nc}	0.14 ^{nc}
<i>D. baccarum</i> (27)	158.67 ($\pm$ 36.35)	29.93 ($\pm$ 22.45)	3	37.6 ($\pm$ 12.21)	109.00 ($\pm$ 30.27) ^{nc}	23.00 ($\pm$ 2.89) ^{nc}	1189.00 ($\pm$ 315.11) ^{nc}	0.25 ^{nc}
<i>Ca. purpeiripennis</i> (24)	102.71 ($\pm$ 33.93)	5.29 ($\pm$ 1.57)**	14	36.14 ($\pm$ 8.03)	126.07 ($\pm$ 11.20)	26.79 ($\pm$ 1.15)	458.64 ($\pm$ 95.26)	0.11*
<i>Ca. mediterraneus</i> (16)	187.00 ($\pm$ 60.43)	15.75 ($\pm$ 4.37)	3	29.00 ($\pm$ 12.97)	195.00 ($\pm$ 90.01) ^{nc}	27.00 ($\pm$ 0.58) ^{nc}	369.00 ($\pm$ 113.60) ^{nc}	0.11 ^{nc}
<i>E. ventralis</i> (22)	1087.77 ($\pm$ 343.15) *	17.36 ($\pm$ 3.60)	0	-	-	-	-	-
<i>Ra. nebulosa</i> (36)	247.53 ($\pm$ 61.95)	29.25 ($\pm$ 5.73)	33	27.67 ($\pm$ 6.74)	234.70 ($\pm$ 21.66) ***	37.67 ($\pm$ 1.36)*	1248.45 ($\pm$ 285.24)*	0.15*
<i>St. lunata</i> (13)	247.53 ($\pm$ 61.95)	6.54 ($\pm$ 1.58)*	0	-	-	-	-	-
<i>Pa. prasina</i> (25)	1052.12 ($\pm$ 295.51) *	27.24 ($\pm$ 6.59)	21	36.27 ($\pm$ 10.36)	157.95 ($\pm$ 5.67)	31.57 ($\pm$ 1.75)*	1761.52 ($\pm$ 454.52)*	0.46
<i>Pe. strictus</i> (18)	296.22 ($\pm$ 98.41)	9.00 ($\pm$ 1.69)	1	30	40 ^{nc}	24 ^{nc}	1888 ^{nc}	0.06 ^{nc}
<i>Sc. sidertis</i> (8)	764.75 ($\pm$ 122.09) ***	2.00 ($\pm$ 0.50)	0	-	-	-	-	-
<i>A. heegeri</i> (19)	553.05 ($\pm$ 176.41) *	21.00 ($\pm$ 3.59)	6	22.71 ($\pm$ 9.77)	126.33 ($\pm$ 10.33)	42.33 ($\pm$ 8.97)*	493.83 ($\pm$ 150.99)*	0.24
<i>N. viridula</i> (35)	257.77 ($\pm$ 46.75)	7.65 ($\pm$ 0.85)	0	-	-	-	-	-
<i>Co. marginatus</i> (27)	601.07 ($\pm$ 146.42) *	6.48 ($\pm$ 1.442)*	0	-	-	-	-	-
<i>G. juniperi</i> (24)	755.92 ($\pm$ 229.88)	5.83 ($\pm$ 1.24)**	0	-	-	-	-	-
<i>Rh. iracundus</i> (23)	599.57 ($\pm$ 300.02)	3.52 ($\pm$ 0.51)***	0	-	-	-	-	-

Eggs characteristics and Trissolcus japonicus parasitization

To identify the possible association between egg morphological features and parasitization success/unsuccess of *T. japonicus*, and to produce a predictive model, we considered the following egg features of each tested species as described by Matesco et al. (2009, 2014): (1) disposition of the laid eggs (singly or in masses); (2) egg volume (mm^3); (3) egg shape (spherical, cylindrical, barrel-shaped, ovoidal); (4) visual patterning of the egg (uniform or striped); (5) egg chorion surface (smooth, spinose). For the egg volume, we used the parabolic barrel formula $V = \pi H(3r^2 + 4Rr + 8R^2)/15$, where H is the egg height, r is the radius of the operculum, and R is the radius at the center of the egg (Abram et al. 2015; Botch and Delfosse, 2018). Measurements were made using a stereomicroscope.

Polyphenism and female egg load of Trissolcus japonicus emerged from different host species

The dimensions (head width, thorax width, and length of the tibia of the third leg) of *T. japonicus* females emerged from target [*H. halys* (Hh)] and pentatomid non-target host [*Acrosternum heegeri* Fieber (Ax), *Carpocoris mediterraneus* Tamanini (Cw), *Carpocoris purpureipennis* (De Geer) (Cx), *Dolycoris baccarum* (L.) (Db), *Palomena prasina* (L.) (Pp), *Rhaphigaster nebulosa* (Poda) (Rn)] were analyzed to determine whether different hosts produce adults differing in body size.

To verify the effect on female fecundity associated with development in different host species, the egg load of ovaries of females emerged from *H. halys* and *D. baccarum* was assessed. For this, a colony of *T. japonicus* was established on eggs of *H. halys* and of *D. baccarum* using the same rearing method described above. Parasitized eggs of the two different hosts were checked daily, and at emergence, females were housed in glass tubes (separated per species and day of emergence) and fed with honey drops in a climatic chamber at standard conditions. From these colonies, on days 1, 3, 7, 14 and 21, females were randomly sampled ($n = 20$ per species and age) and ovaries were extracted after dissection. Eggs were stained with 1% toluidine bleu and counted under a stereomicroscope.

Data analysis and statistics

The hatching rates of manipulated and unmanipulated eggs of the different hemipterans tested were analyzed by pairwise Chi-squared tests. Frequency of successful parasitization (wasp emergence) of eggs that produced nymphs and of dead eggs (that produced neither parasitoids nor nymphs) was analyzed by pairwise Chi-squared tests to compare the non-target species with the target *H. halys*.

Video-recorded female behaviors during the 2-h exposures were analyzed by measuring the duration in time (seconds) of the following parameters (after Strand and Vinson, 1983; see Roversi et al. 2014; Haye et al. 2020):

- (1) Time from introduction of the egg into the vial and the first egg encounter by the female wasp;
- (2) Duration of the first egg inspection (antennal drumming and moving the antennae over the egg surface);
- (3) Oviposition duration (drilling and subsequent oviposition);
- (4) Duration of post-oviposition marking of parasitized eggs;
- (5) Time from the start of the test to the acceptance of the host parasitization (as evidenced by marking behavior).

Mean time of behaviors described in (1) and (2) were compared by pairwise Student's *t* tests between non-target species and the target *H. halys*. Data of parameters described in (3), (4) and (5) were analyzed only for the species showing at least five replicates of successful parasitization (although the entire data set was reported in Table 3). Data on parasitization behavior were analyzed by pairwise Student's *t* tests to compare means of non-target species with the target *H. halys*.

The latency time from the beginning of the test to the first egg encounters by the females (1) and duration of the first inspection (2) were compared between samples that were or were not successfully parasitized. Data were analyzed using a binomial mixed general linear model that included host species as the random factor with the “glmmPQL” function of the “MASS” R package (Ripley et al. 2013).

The differences in parasitization success displayed during the 2 h and 24 h were analyzed by pairwise chi-squared tests. The acceptance/contact ratio (a/c-ratio) proposed by Van Dijken et al. (1986) was estimated and compared among different host species tested.

To obtain an overview of the correlations among egg features and their possible association with parasitization success, we performed a principal component analysis (PCA) on the four categorical traits (disposition of the naturally laid eggs, egg shape, patterning of the egg, egg chorion surface) and the numerical trait of egg volume, using the “dudi.mix” function of “Ade4” R package (Dray and Dufour 2007). Components with eigenvalues > 1 were considered to explain a consistent fraction of variance. We then compared the parasitization success against egg traits by using nonparametric tests (Wilcoxon test for egg disposition, patterning and sculpture), Kruskal–Wallis for egg shape and Spearman correlation for an egg volume (“stats” R package). All analyses of egg features were performed using R 3.5.1.

For the polyphenism comparison, we carried out a preliminary PCA by using the “rda” function of the “vegan” R library on the three traits measuring body dimensions (head width, thorax width and length of tibia III) to obtain a single measurement of insect size. We then carried out a mixed generalized linear model by using *T. japonicus* female size as the response variable, with egg size of

host species (as assessed in the previous analysis) as a predictor and host species as a random factor. For polyphenism data analysis, we used the “lme” function of the “nlme” R package. We compared the egg load in the ovary of *T. japonicus* females that emerged from *H. halys* and *D. baccarum*, with Student’s t-test.

Results

Selected hemipteran species and their laboratory rearing

During the two years of field surveys in 2018–2019, we collected 16 hemipteran species, mostly in the subfamily Pentatominae (see Table 1). As taxonomically unrelated outgroup species, we used two species from the family Coreidae and one from the family Reduviidae. All the hemipterans collected were successfully reared in the laboratory. The viability of eggs of the different species was not substantially affected by their handling and manipulation (for statistics see Table 2 and Supplementary Material 1).

Non-target tests and female behavior during parasitization

Among the 16 tested species, nine pentatomids belonging to the tribes Pentatomini, Carpocorini and Piezodorini were suitable hosts for *T. japonicus*, although with differing degrees of suitability (Table 2). Successful parasitization was not recorded in Coreidae and Reduviidae, nor in four other species of pentatomids tested (two of them from the tribes Sciocorini and Strachiini, one from Pentatomini and one from Carpocorini), which therefore were confirmed as not physiological hosts for *T. japonicus*.

Halyomorpha halys exhibited a high rate of successful parasitization by *T. japonicus*, with a low rate of dead eggs (2.44%), and no nymphs emerged. Similar results were observed for *Pa. prasina* and *Ra. nebulosa*. In all other tested species that were successfully parasitized, the rates ranged between 3.45 and 58.33%. Differences among parasitization success were significant among the target host *H. halys* and all other tested species, except for *Pa. prasina*, *Ra. nebulosa*, *A. heegeri* and the two species of *Carpocoris* (see Supplementary Material 1 for statistics). Rates of dead eggs in non-target species varied between zero and 55.56%, and a significant difference compared to the target *H. halys* was detected only in *D. baccarum* and in the two *Carpocoris* species (Table 2). Dissection of dead eggs revealed the presence of dead pupae of *T. japonicus* only in four *D. baccarum* eggs and in one *E. ventralis* egg, while a clear cause could not be assigned in any of the other cases of dead eggs.

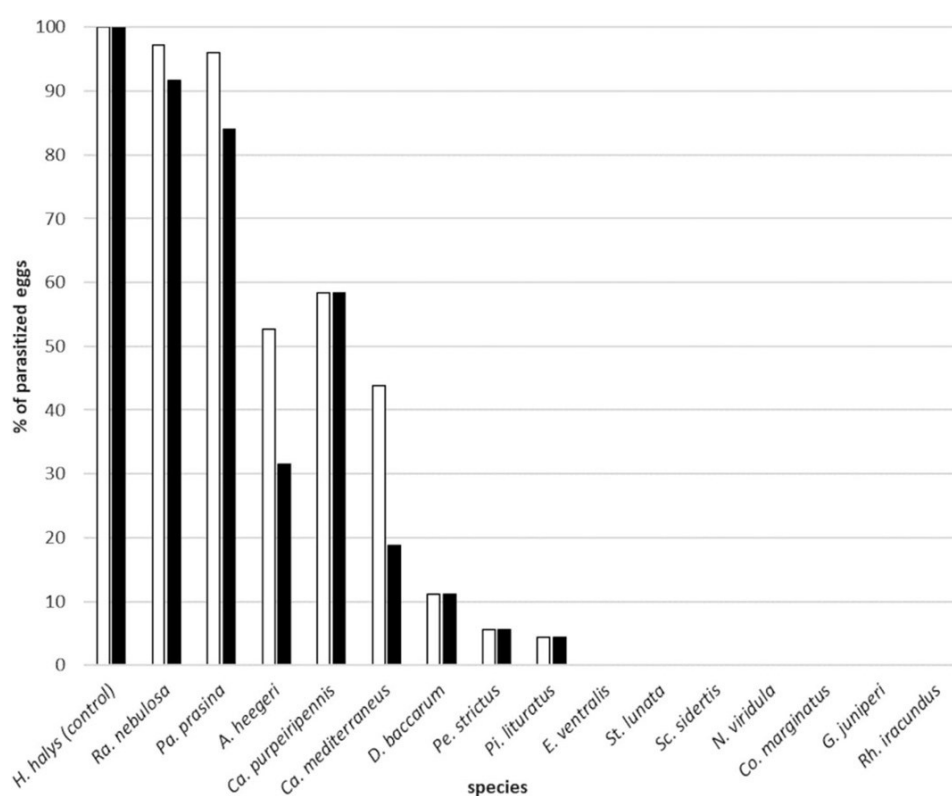
The time from the beginning of the test and the first contact with the egg did not differ between the eggs that were successfully parasitized ($316.58 \text{ s} \pm 42.90 \text{ SE}$) and the eggs that were not parasitized ($594.79 \text{ s} \pm 91.56 \text{ SE}$) (Estimate: 0.0003; SE: 0.0002; $df = 353$; $t = 1.668$; $P = 0.096$). Conversely, the duration of the first encounter inspection was longer for eggs that were successfully parasitized (19.91

$s \pm 3.06$ SE than for those that were not parasitized ($7.86 \text{ s} \pm 0.79$ SE) (Estimate: 0.060; SE: 0.013; $df = 353$; $t = 4.640$; $P < 0.001$).

All the females inspected the eggs at least once during the 2 h trial, and the first encounter egg inspection time ranged from 3.52 s (± 1.57 SE) for *Rhynocoris iracundus* (Poda) (Hemiptera: Reduviidae), to 29.93 s (± 22.45 SE) for *D. baccarum* (Table 3; see Supplementary Material 1 for statistics). Female oviposition time and marking time were not significantly different between non-target species and *H. halys*, except for *Ra. nebulosa* (Table 3). The time from the beginning of tests and acceptance for parasitization was significantly different from *H. halys* in *A. heegeri*, *Ra. nebulosa* and *Pa. prasina* (Table 3). Higher values of acceptance/contact ratio (a/c ratio) were observed for *H. halys*, *P. prasina* and *A. heegeri*, while lower values compared to the target species were observed for *Ca. purpeiripennis* and *Ra. nebulosa* (Table 3).

A comparison of the parasitization events that occurred within the short exposure time (2 h) with those occurring during the long exposure time (24 h) showed that all *H. halys* eggs were parasitized within the first two hours, as were most of other non-target species (Fig. 1). Only a few females of *T. japonicus* in the tests with *Ra. nebulosa*, *Pa. prasina*, *A. heegeri* and *Ca. mediterraneus* required more time to accept the egg. However, no significant differences in parasitism between 2 and 24 h of exposure were detected in any of the species tested (see Supplementary Material 1 for statistics).

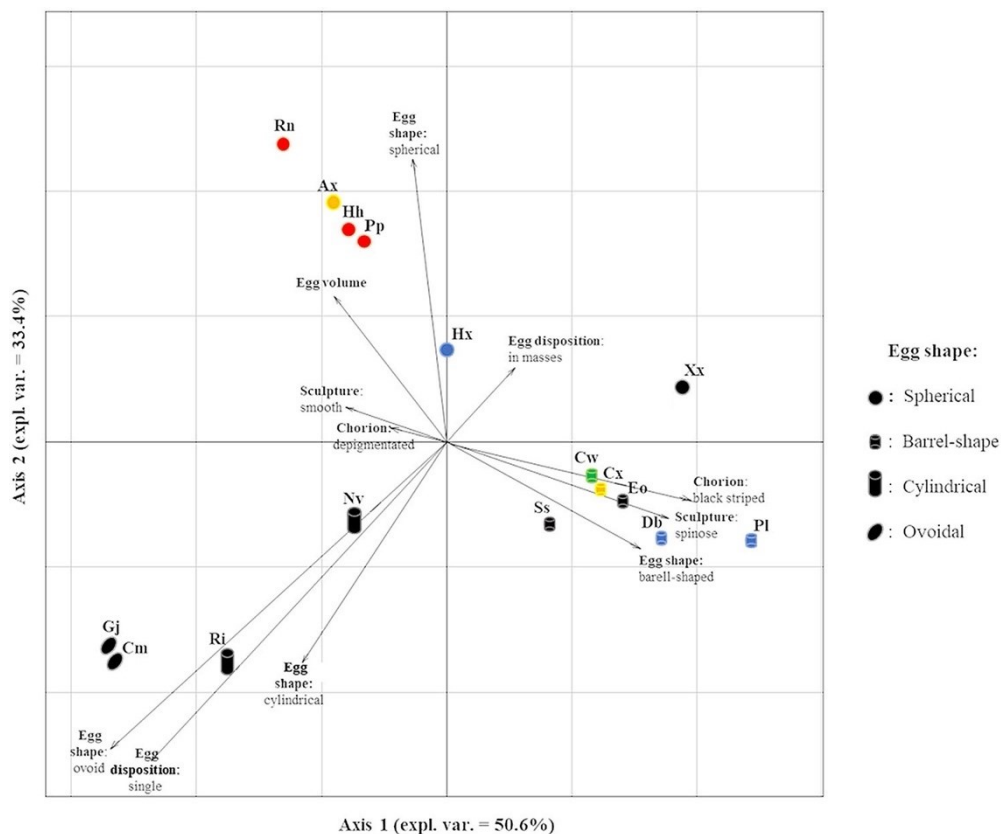
Fig. 1 Percentage of success of *Trissolcus japonicus* parasitization within 2 h (black histograms) and 24 h (white histograms) after egg exposure. Only data where eggs produced a *T. japonicus* adult or a hemipteran nymph are shown and included in the data analysis.



Egg characteristics and *Trissolcus japonicus* parasitization rate

The PCA analysis revealed four main groups of eggs: (1) large and spherical, (2) barrel-shaped, spinose and striped; (3) cylindrical and small; (4) ovoidal, small and laid singly (Fig. 2). The eggs showing the highest parasitization success are concentrated in the first group, the second group showed a lower parasitization rate, while the third and the fourth ones were not attacked.

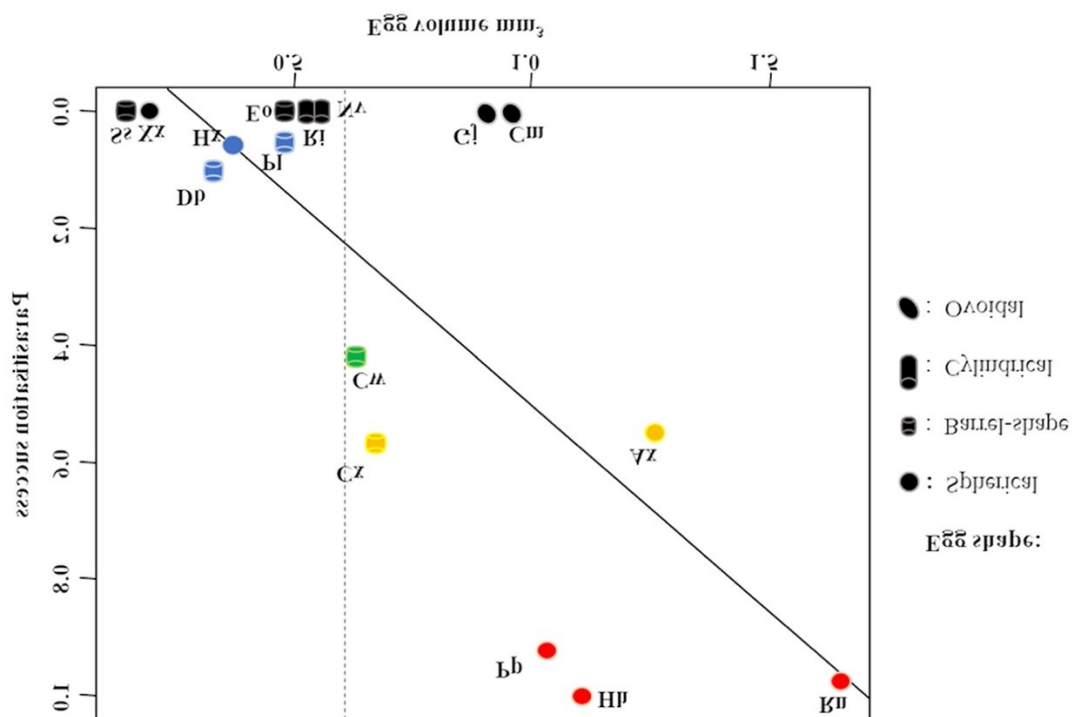
Fig. 2 A scatterplot for a dudi.mix PCA showing the correlation pattern among four egg traits (shape, disposition, sculpture, pattern). Marker shape indicates the shape of the eggs and the color represents parasitization success under laboratory conditions; Hh: *Halyomorpha halys*, Rn: *Rhaphigaster nebulosa*, Pp: *Palomena prasina*, Ax: *Acrosternum heegeri*, Cx: *Carpocoris purpureipennis*, Cw: *Carpocoris mediterraneus*, Db: *Dolycoris baccarum*, Pl: *Piezodorus lituratus*, Xx: *Staria lunata*, Eo: *Eurydema ventralis*, Hx: *Peribalus strictus*, Nv: *Nezara viridula*, Ss: *Scicocoris sideritis*, Cm: *Coreus marginatus*, Gj: *Gonocerus juniperii* e Ri: *Rhynocoris iracundus*. In the resulting scatterplot, we marked cases with different parasitization success using different colors to highlight the different parasitization percentages. Here, parasitization success (p.s.) was ranked in the following ranges: p.s. = 0% (black), 0% < p.s. ≤ 25% (blue), 25% < p.s. ≤ 50% (green), 50% < p.s. ≤ 75% (yellow), p.s. > 75% (red)



In comparisons of single variables, egg shape and disposition showed a trend in explaining the frequency of parasitization among species (Kruskal–Wallis test chisquared = 6.795, df = 3, P = 0.079; Wilcoxon test, W = 33, P = 0.068, respectively), while egg sculpture and patterning showed no significant effects (W = 19, P = 0.344, W = 15, P = 0.574, respectively). Larger eggs showed a

significantly higher level of success (Spearman test, $\rho = 0.600$, $S = 272.28$, $P = 0.014$). A scatterplot showing the correlations between egg volume and success in parasitization revealed that barrel shaped, cylindrical and spherical eggs smaller than 0.64 mm^3 had a very low probability (if any) of being parasitized (Fig. 3); the only exceptions were represented by the relatively large ovoidal eggs (0.92 mm^3) laid by the species of Coreidae, which were not parasitized at all (Fig. 3).

Fig. 3 A scatterplot showing the correlation between egg volume and parasitization success under laboratory conditions. Markers indicate the shape of the eggs, and the color represents parasitization success under test conditions; Hh: *Halyomorpha halys*, Rn: *Rhaphigaster nebulosa*, Pp: *Palomena prasina*, Ax: *Acrosternum heegeri*, Cx: *Carpocoris purpureipennis*, Cw: *Carpocoris mediterraneus*, Db: *Dolycoris baccarum*, Pl: *Piezodorus lituratus*, Xx: *Staria lunata*, Eo: *Eurydema ventralis*, Hx: *Peribalus strictus*, Nv: *Nezara viridula*, Ss: *Scicocoris sideritis*, Cm: *Coreus marginatus*, Gj: *Gonocerus juniperii* e Ri: *Rhynocoris iracundus*. In the resulting scatterplot, we marked cases with different parasitization success using different colors to highlight the different parasitization percentages. Here, parasitization success (p.s.) was ranked in the following ranges: p.s. = 0% (black), $0\% < \text{p.s.} \leq 25\%$ (blue), $25\% < \text{p.s.} \leq 50\%$ (green), $50\% < \text{p.s.} \leq 75\%$ (yellow), p.s. > 75% (red).

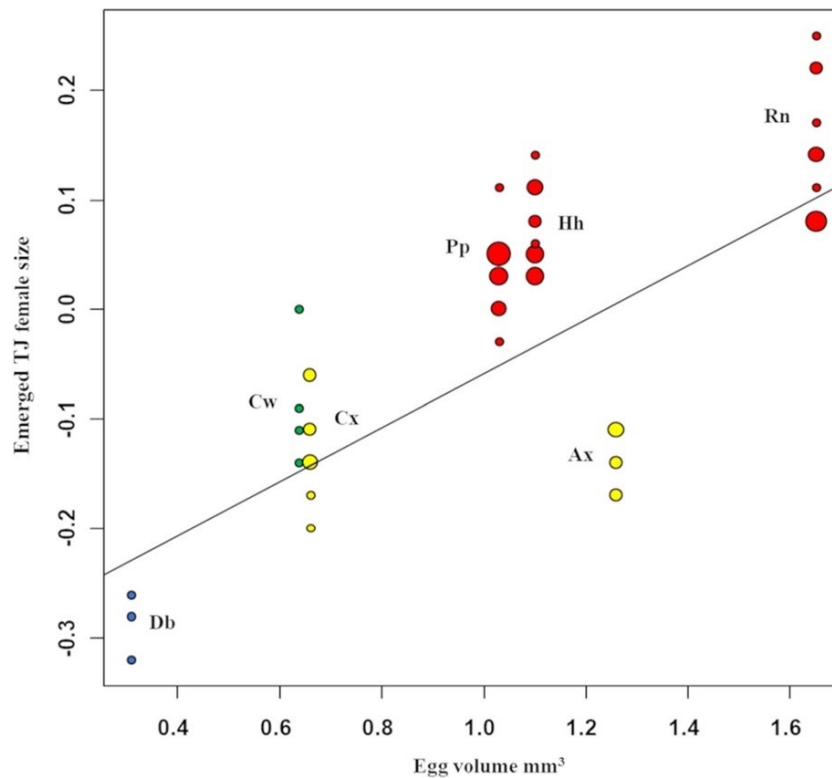


Polyphenism and female egg load of Trissolcus japonicus emerged from different host species

The hemipteran species which were most suitable hosts for *T. japonicus*, had an egg volume larger than 1 mm^3 : *Pa. prasina* (1.021 mm^3), *H. halys* (1.079 mm^3) and *Ra. nebulosa* (1.641 mm^3) (see supplementary material 2). The three body dimensions measured of *T. japonicus* females (head width, thorax width and length tibia III) revealed a high correlation with high body weight (> 0.5) in the PCA analysis, where a single factor showed an eigenvalue higher than 1. This allowed us to use this factor as an overall size indicator for female size. By including this factor in a mixed generalized

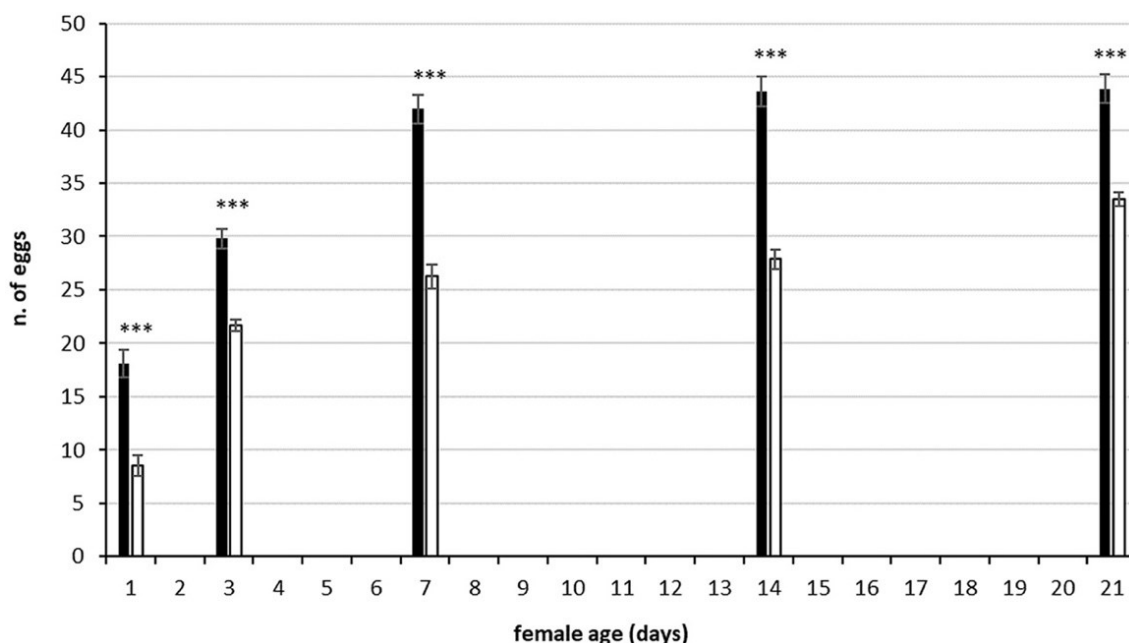
model, a significant relationship was detected between *T. japonicus* female size and host egg size ($F = 8.862$, $P = 0.031$, Fig. 4).

Fig. 4 A bubble scatterplot showing the correlation between egg volume and adult size of *Trissolcus japonicus* females emerging from eggs of different host species. *Acrosternum heegeri*: AX; *Carpocoris mediterraneus*: CW; *Carpocoris purpureipennis*: CX; *Dolycoris baccarum*: DB; *Halyomorpha halys*: HH; *Palomena prasina*: PP, *Rhaphigaster nebulosa*: RN. Circle size corresponds to the number of the females having the same values. Here, parasitization success (p.s.) was ranked in the following ranges: p.s. = 0% (black), $0\% < \text{p.s.} \leq 25\%$ (blue), $25\% < \text{p.s.} \leq 50\%$ (green), $50\% < \text{p.s.} \leq 75\%$ (yellow), $\text{p.s.} > 75\%$ (red)



The egg load in female wasp ovaries ranged from $18.15 (\pm 1.28 \text{ SE})$ in one-day-old females up to $43.85 (\pm 1.36 \text{ SE})$ in 14-day-old, host-deprived females when emerged from *H. halys* eggs, and ranged between $8.50 (\pm 0.97 \text{ SE})$ in oneday-old females and $33.5 (\pm 0.66 \text{ SE})$ in 14-day-old, hostdeprived females when emerged from *D. baccarum* (Fig. 5). Within each female age class, a higher egg load was detected in females emerged from *H. halys* respect to *D. baccarum* (see Supplementary Material 3 for statistics).

Fig. 5 Mean egg load of *Trissolcus japonicus* females of different ages reared from *Halyomorpha halys* (black histograms) and *Dolycoris baccarum* (white histograms) under host deprived conditions; bars indicate $\pm$ SE; asterisks display significant differences within the two host species at each scheduled day (***) ($P < 0.001$) (see Supplementary Material 3)



Discussion

In our study, *T. japonicus* was able to parasitize both the target and nine of the 16 non-target species tested but with differing degrees of success. Thus, we can confirm the oligophagous habit of this species as reported in other studies in the USA, New Zealand and Switzerland and in its area of origin (China) (Hedstrom et al. 2017; Zhang et al. 2017; Botch and Delfosse 2018; Charles et al. 2019; Haye et al. 2020; Lara et al. 2019). Our study included most of the western European species evaluated by Haye et al. (2020), and we obtained similar results by using a different experimental design.

Although the physiological host range appears to be relatively broad in *T. japonicus*, the success of egg parasitization was variable within the different non-target species we tested: the target host (*H. halys*) and a few other species were accepted and parasitized with a similar high rate of success (e.g., *Pa. prasina* and *Ra. nebulosa*), while other pentatomid species were less suitable (*A. heegeri*, *Ca. mediterraneus*, *Ca. purpeiripennis*, *D. baccarum*, *Eurydema ventralis* Kolenati, *Piezodorus lituratus* (Fabricius), *Peribalus strictus* (Fabricius)). Some species of pentatomids (*Sciocoris sidertis* Wollaston, *Staria lunata* (Hahn), *N. viridula*), the two coreids (*Coreus marginatus* (L.), *Gonocerus juniperi* Herrich-Schäffer) and the reduviid *Rh. iracundus* were never parasitized. However, in a few tested species, i.e., *Ca. mediterraneus*, *Ca. purpeiripennis*, *D. baccarum* and *N. viridula*, we reported a higher egg mortality (dead eggs) compared to the target species *H. halys*, thus suggesting potentially unsuccessful parasitization attempts by *T. japonicus*. However, we detected dead pupae only in *D. baccarum* and in one *E. ventralis* egg. This suggests that the failure in development in the other

species cited above, if parasitization takes place, occurs at the early developing stages of the egg parasitoid. The resulting host range of *T. japonicus* is apparently not closely correlated with the tribes of the subfamily Pentatominae. For example, one of the preferred species, *Pa. prasina*, belongs to the same tribe (Carpocorini) of less suitable species, such as *Ca. mediterraneus*, *Ca. purpeiripennis* and *D. baccarum*.

The acceptance/contact ratio (a/c ratio) was relatively high in *Pa. prasina* but was not statistically different from *H. halys* (the target species). The a/c ratios in *D. baccarum* and *A. heegeri* were similar to that of *H. halys*, and in both species of *Carpocoris* and *Ra. nebulosa*, this ratio was lower. A low a/c ratio indicates multiple rejections of an offered egg before its acceptance by *T. japonicus* females. Under a field scenario, after a first rejection of an egg mass of a non-target pentatomid a searching female might continue searching elsewhere for a more suitable host, thus reducing the impact on non-target species, as speculated by Botch and Delfosse (2018) and supported by Boyle et al. (2020). Therefore, this parameter could be helpful in a predictive introduction risk analysis (Van Dijken et al. 1986).

Trissolcus japonicus did not show a clear pattern in its parasitization behavior among the different species tested, as observed by Haye et al. (2020) and Botch and Delfosse (2018). The large variability in these behaviors may reflect a wide range in the individual physiological condition of the females during tests, although we tried to control this by using females of uniform age and lack of previous experience. Analysis of egg morphology and success in parasitization revealed that the shape and the volume of the host egg are features correlated with the host acceptance rate: *Pa. prasina* and *Ra. nebulosa* have eggs similar in shape and size to *H. halys* and showed similar parasitization rates. The only exception was *A. heegeri*, whose eggs are similar to *H. halys* but had a lower parasitization rate. For predictive risk analysis, once characteristic host egg features (in particular volume and shape) of an untested pentatomid species are known, the species can be included in the graphical charts of the Figs. 2 and 3, and compared with the results of our investigated host species. For example, *Arma custos* (Fabricius) (Hemiptera, Pentatomidae) lays smooth, spherical eggs with a volume of 0.98mm³ (L. Tavella and S.T. Moraglio, personal communication) and is similar to *H. halys* and *Pa. prasina*, leading to the prediction that this species may be at risk of being parasitized at a similar rate. Indeed, as observed by Haye et al. (2020), *A. custos* effectively showed a high parasitization rate in nochoice tests like that of *H. halys*. With the same approach, *Graphosoma lineatum* L. (Hemiptera, Pentatomidae) that has spherical eggs of about 0.56 mm³ volume and not having a smooth surface appears within the group of less-attacked species, as it was also observed by Haye et al. (2020) using a different experimental design.

In our experiments, the comparison between 2 and 24 h exposures showed no significant differences in parasitization rates. In our small arena, tested females accepted the offered host eggs after a mean

time of about 12 min for *H. halys* and within approx. 30 min for *Pa. prasina*. In only a few cases did females of *T. japonicus* accept the host egg after more than two hours (e.g., in *Ra. nebulosa*, *Pa. prasina*, *A. heegeri*, *Ca. mediterraneus*). Considering that the time of parasitization per egg (inspecting, ovipositing and marking) lasts about 4 min, egg masses of 28 eggs like that of *H. halys* may require less than 2 h to be completely parasitized. Thus, also considering the rapid acceptance of hosts following initial exposure to *T. japonicus*, testing periods in future studies can be reduced to a few hours, greatly reducing the time needed to conduct replicated tests.

Polyphenism in egg parasitoids is a common phenomenon that has previously been reported for *T. japonicus* by Medal and Smith (2015) and Botch and Delfosse (2018) and more recently by Boyle et al. (2020). We found a positive correlation between the egg volume of several different host species and the size of female adult wasp, except for *A. heegeri*; here, smaller adults were obtained from eggs that were larger than those of *H. halys*. In this case, we speculate that an unsuitable biochemical content of the host egg could result in lower suitability for a complete development. Moreover, in our study, smaller females obtained from *D. baccarum* had a lower egg load in the ovaries, corroborating the findings of Botch and Delfosse (2018), who observed that smaller females originating from non-target hosts (with smaller sized eggs) produced a lower number of offspring.

Overall, using a single host egg as a test unit, we demonstrated the physiological suitability of non-targets to be accepted and to sustain the development of the egg parasitoid *T. japonicus*. However, the complete egg parasitization rate within an entire egg mass cannot be assessed using this method, but it can be investigated with successive semi-field tests, permitting researchers to focus on a restricted list of species that display a high degree of parasitization success. It is also important to standardize the physiological status of *T. japonicus* females used in tests (e.g., ovary egg load). The varied results obtained in offspring rates when *H. halys* is used as host (from 46.9 up to 100%) in several studies (Hedstrom et al. 2017; Botch and Delfosse 2018; Charles et al. 2019; Haye et al. 2020; Lara et al. 2019) could be due to variation in the tested females physiological status, as argued by Lara et al. (2019). Temperatures during tests or in the pre-conditioning phase might also play a relevant role in parasitization. These and other potential complicating factors should be carefully evaluated and standardized to obtain robust and comparable results (Sands and Van Driesche 2003; Van Lenteren et al. 2006b). Our results demonstrate that using single host eggs with short exposure times is a valid alternative for the first step of a non-target analysis (the physiological host range), and the need for a reduced number of eggs for tests will be useful in cases of rare species or those that cannot be easily or successfully reared in the laboratory. For the European pentatomid species we tested, there is a clear acceptance threshold in egg size that, combined with egg shape, can predict the parasitization rate with high confidence. With increasing information on other non-target hosts, this predictive model can then be refined to improve its predictive power. Overall, our simplified test

(shorter observation time and use of single eggs per replicate) with the possibility to predict the parasitization success will facilitate the determination of the physiological suitability of potential hosts. In turn, this will allow researchers to more rapidly assess key potential hosts in more complex semi-field experiments, as discussed by Haye et al. (2020) and produce more reliable scenarios of non-target impacts. Although the results of our experiment showed which species were more susceptible to parasitization of *T. japonicus*, the model still requires validation with assessment of additional non-target species and their traits beyond those examined in this work. At this stage, it may be regarded as a tool to predict likely non-target impacts to be integrated within the framework of other experimental predictive studies, in order to mitigate as much as possible the risk of underestimating environmental impacts.

Author Contributions:

GSP conceived and designed research; GSP, LB, GM, FP, LG and LM conducted experiments; LD, GSP and LB performed statistical analysis; GSP, LB, GM, LD and KAH wrote the manuscript; GSP, LB, GM, FP, LM and PFR collected non-target species for establishing laboratory colonies; GM identified non-target species; KAH provided the founding colony of *Trissolcus japonicus*; GSP and LG maintained the laboratory-reared colonies for experiments; PFR provided funding and led the laboratory where experiments were conducted. All authors read and approved the manuscript.

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2.2 Physiological host range of *Trissolcus mitsukurii*, a candidate biological control agent of *Halyomorpha halys* in Europe

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Abstract

The invasive stink bug *Halyomorpha halys*, native to East Asia, is a severe agricultural pest of worldwide importance, and chemical insecticides are largely sprayed for its control. Negative impact and failures of chemical pest management led to consider classical biological control as one of the most promising methods in a long-term perspective. The Asian egg parasitoid *Trissolcus japonicus* is the main candidate biocontrol agent of *H. halys*, but more recently a second species, *Trissolcus mitsukurii*, which shares the area of origin with *H. halys*, has drawn special attention after adventive populations were found in Europe. Despite its recent detection, intentional release or redistribution are bound to approval of national petitions, which requires detailed risk analyses. With the aim of providing a contribution for the future development of a risk assessment for release purposes, here we present the first study on the physiological (fundamental) host range of *T. mitsukurii* in Europe. Tests conducted on different hemipterans common to Southern Europe, using three different experimental designs, revealed a oligophagous host range, limited to Pentatomoidea and comparable with the host range displayed by *T. japonicus* under similar laboratory conditions. In addition to its coevolved host *H. halys*, *T. mitsukurii* successfully parasitized the majority of tested pentatomid species and one scutellerid, although with highly variable emergence rates. Notably high parasitization rates were detected on *H. halys*, *Acrosternum heegeri* and *Dolycoris baccarum*. Host egg sizes positively affected parasitoid size and female egg load. Although *T. mitsukurii* might be a promising biological control agent of *H. halys* in Europe, further studies (e.g., chemical ecology, field parasitism) are needed for assessment of non-target risks.

Keywords: Classical biological control; Non-target risk assessment; Brown marmorated stink bug; Pentatomidae; Scelionidae; Egg parasitoid

Introduction

The Brown marmorated stink bug, *Halyomorpha halys* Stål (Hemiptera, Pentatomidae), is a polyphagous invasive pest native of East Asia, currently present in several countries of the Americas and Europe, where it causes severe damage to many agricultural crops (Leskey and Nielsen, 2018). The low efficacy and high environmental impact of insecticide treatments, combined with the lack of effective native natural enemies, led to consider classical biological control with egg parasitoids a promising method for long-term management of *H. halys* in the invaded areas (Abram et al. 2017). Although recent reviews indicated limitations in the success of classical biological control of stink bugs, research in this field needs to highlight any potential approach to find effective pest control solutions (Abram et al. 2020; Conti et al. 2021). These solutions could emerge from the integration of classical biological control with techniques targeting different instars of the pest (Abram et al. 2020), plant resistance (Rondoni et al. 2018), exploitation of plant volatiles to improve parasitoid efficacy (Rondoni et al. 2017), manipulation of stink bug behavior and parasitoid conservation (reviewed by Conti et al. 2021).

The employment of an exotic organism as candidate biological control agent, including redistribution of adventive populations, is strictly regulated by national and international rules (van Lenteren et al. 2006a; EPPO 2014; FAOIPPC 2017; European Directives 92/43/CEE and 2002/89/ CE; Italian DPR 102/2019). Therefore, the approval of a petition for release of a biological control agent requires a thorough risk assessment, which provides detailed information on the biological and ecological traits of the agent, first of all its host range (Hunt et al. 2008). Other aspects should also be considered for risk analysis, including dispersal ability, interspecific competition and hybridization. Laboratory investigations are the first step for non-target risk analysis of the exotic natural enemy and allow to explore its physiological host range. The physiological host range is the range of species a natural enemy can parasitize in the laboratory, and is the foundation on which non-target risk assessment is build up, according to stepwise testing with increasing levels of complexity, including behavioral and chemical ecology assays (Van Lenteren et al. 2006b). Such studies are necessary for moving from a physiological host range outcome to an ecological host range perspective, which allows to prevent or minimize the risk of negative impact caused by a voluntary human intervention in the ecosystem (van Lenteren et al. 2006b).

The Asian egg parasitoid *Trissolcus japonicus* (Ashmead) (Hymenoptera, Scelionidae) is considered, so far, the most valid candidate for biological control of *H. halys* (Zhang et al. 2017; Conti et al. 2021). Its host range, outside the area of origin, was investigated in the USA, Europe and New

Zealand. In these different areas, *T. japonicus*, although exhibiting a general oligophagous behavior, has shown a greater preference toward *H. halys* (Hedstrom et al. 2017; Botch and Delfosse, 2018; Charles et al. 2019; Lara et al. 2019; Haye et al. 2019; Sabbatini-Peverieri et al., 2021). Adventive populations of *T. japonicus* were discovered recently in Europe and the USA (Talamas et al. 2015; Sabbatini-Peverieri et al. 2018; Stahl et al. 2019). Surprisingly, a second Asian egg parasitoid of *H. halys*, *Trissolcus mitsukurii* (Ashmead) (Hymenoptera, Scelionidae), was also found in Italy, as the first record outside its native range (Sabbatini-Peverieri et al. 2018). However, while *T. japonicus* ability to attack *H. halys* is well-recognized, little is known so far about the preferences and life traits of *T. mitsukurii* (Zhang et al. 2017; Arakawa and Namura 2002; Arakawa et al. 2004). This parasitoid species is known mainly from Japan, where it has been recorded parasitizing in the field eggs of about ten species of Pentatomidae (Yasumatsu and Watanabe, 1964; Hokyo et al. 1966; Ryu and Hirashima 1984; Arakawa and Namura, 2002). Additionally, in laboratory test conducted in Japan, *T. mitsukurii* was able to parasitize a different pentatomid species in the genus *Plautia* (Arakawa and Namura 2002). Therefore, *T. mitsukurii* seems to be an oligophagous egg parasitoid. In Japan, it was previously reported as one of the most active natural enemies of *Nezara viridula* L. (Hemiptera, Pentatomidae) (Kiritani and Hokyo 1962; Hokyo and Kiritani 1963), although more recent data from the same country reported this parasitoid as commonly associated with *H. halys* (Arakawa and Namura 2002; Arakawa et al. 2004). Instead, the high parasitization rate reported in China for *T. mitsukurii* on *H. halys* (Chu et al. 1997), due to an original misidentification, should be assigned to *T. japonicus* as stated by Yang et al. (2009).

Recent field surveys on adventive *T. mitsukurii* populations in Northern Italy showed high rates of parasitism of *H. halys* eggs, suggesting that this egg parasitoid could be a valid candidate for biological control of the invasive stink bug in addition to *T. japonicus* (Benvenuto et al. 2020; Scaccini et al. 2020; Zapponi et al. 2020). Additionally, field data from Northern Italy suggest low ability of *T. mitsukurii* to attack non-target pentatomid species, i.e., *Dolycoris baccarum* (L.), *Palomena prasina* (L.) and *N. viridula* (Zapponi et al. 2021). However, this is a first and partial observation, conditioned by the status of *T. mitsukurii* populations in the field and the distribution of its potential hosts in the environment. For a clear perspective of the parasitoid host range based on field data, years of observations would be necessary after the natural spread of this species in the area and the establishment of interspecific host-parasitoid relationships.

Thus, because the host range of *T. mitsukurii* outside its original area remains unclear, this should be clarified to initiate the first step of a risk analysis workplan, which is necessary before considering the implementation of this egg parasitoid as a biological control agent (van Lenteren et al. 2006a, b). The present work is the first contribution to the knowledge of the physiological host range of *T.*

mitsukurii in the invaded area, conducted comparing the parasitoid performance on the target pest *H. halys* and on non-target Hemipteran species that are common in Europe.

Material and methods

Field sampling, insect collection and rearing

Halyomorpha halys adults were collected in Northern Italy in 2020, both in overwintering sites and in the field during the growing season. A colony was given rise from about 2,500 overwintering specimens and replenished monthly with field collected specimens. Instead, adults of non-target Hemiptera species were collected on various herbaceous plants, shrubs and trees of fallow fields, hedgerows, bushes and orchards during the 2020 growing season, in *H. halys* habitats and surrounding areas of North and Central Italy. Non-target Hemiptera species were chosen based on the potential host range of the parasitoid and the information mined from the literature, according to the centrifugal phylogenetic approach (Kuhlmann and Mason 2003; Kulmann et al. 2006). Beside *H. halys*, the following non-target herbivorous Hemipteran species were investigated: the pentatomids *Acrosternum heegeri* (L.), *Aelia acuminata* (L.), *Ancyrosoma leucogrammes* (Gmelin), *Carpocoris pudicus* (Poda), *D. baccarum*, *Eurydema ventralis* Kolenati, *Eurydema oleracea* (L.), *Graphosma italicum* (Muller), *N. viridula*, *P. prasina*, *Piezodorus lituratus* (F.), and *Sciocoris* sp., the scutellerid *Eurygaster maura* (L.), and the coreid *Gonocerus juniperi* Herrich-Schäffer. Two predator species, *Arma custos* (Fabricius) (Hemiptera, Pentatomidae) and *Rhynocoris iracundus* (Poda) (Hemiptera, Reduviidae), were also considered (see Table 1). Number of non-target collected specimens in the field where highly variable, from few specimens up to several dozens, depending on species abundance in the field, collection opportunity and collection method, i.e., sweep net or hand picking on herbaceous plants and trees, or occasionally on other substrates (Tab. 1).

Laboratory colonies were established and maintained using rearing cages (BugDorm 4F4545, Insect MegaView Science Co. Ltd., Taichung, Taiwan) in rearing rooms at 26 ± 1 °C and 16:8 L:D. For the polyphagous phytophagous species selected for the present study, a variety of plants was offered as a bouquet of food sources: seeds (*Arachis hypogaea*), fresh vegetables (*Daucus carota*, *Phaseolus vulgaris*), fresh fruits (*Malus domestica*, *Actinidia deliciosa*). Instead, more specific substrates were used to rear adults of the oligophagous species. *Eurydema* species were reared on *Brassica oleracea* (cauliflower cut in pieces and potted plants) and *Capparis spinosa* (twigs), *G. lineatum* was reared on *Foeniculum vulgare* (potted plants and seeds), while wheat ears were offered to *E. maura*. Food was purchased from local markets. Plants were self-produced and were used when at least 10 cm tall. The predator species were reared on *Acanthoscelides obtectus* Say (Coleoptera, Chrysomelidae) adults, *N. viridula* nymphs and *Tenebrio molitor* L. (Coleoptera, Tenebrionidae) pupae. For all species, food and water (provided with wet cotton) were replenished three times per week.

Table 1. List of the European native and exotic Hemiptera tested for *Trissolcus mitsukurii* physiological host suitability and relative egg hatching rates in the rearing facility (n.a. = not assessed)

Target and non-target species	Family, subfamily, tribe	Ecological role and origin	Egg mass hatching rate in the rearing facility
<i>Acrosternum heegeri</i> Fieber	Pentatomidae, Pentatominae, Pentatomini	Phytophagous/Native	n.a. (94.0% [*])
<i>Aelia acuminata</i> (L.)	Pentatomidae, Pentatominae, Aelini	Phytophagous/Native	n.a.
<i>Ancyrosoma leucogrammes</i> (Gmelin)	Pentatomidae, Podopinae, Graphosomatini	Phytophagous/Native	n.a.
<i>Arma custos</i> (F.)	Pentatomidae, Asopinae, Asopini	Predatory/Native	77.67%
<i>Carpocoris pudicus</i> (Poda)	Pentatomidae, Pentatominae, Carpocorini	Phytophagous/Native	95.7% (76.61% [*])
<i>Dolycoris baccarum</i> (L.)	Pentatomidae, Pentatominae, Carpocorini	Phytophagous/Native	88.6% (88.0% [*])
<i>Eurydema oleracea</i> (L.)	Pentatomidae, Pentatominae, Strachiini	Phytophagous/Native	n.a.
<i>Eurydema ventralis</i> Kolenati	Pentatomidae, Pentatominae, Strachiini	Phytophagous/Native	93.1% (96.67% [*])
<i>Graphosma italicum</i> (Muller)	Pentatomidae, Pentatominae, Graphosomatini	Phytophagous/Native	83.33%
<i>Halyomorpha halys</i> (Stål)	Pentatomidae, Pentatominae, Cappaeiini	Phytophagous/Exotic	93.35% (90.39% [*])
<i>Nezara viridula</i> L.	Pentatomidae, Pentatominae, Pentatomini	Phytophagous/Exotic (naturalized)	92.34% (85.58% [*])
<i>Palomena prasina</i> (L.)	Pentatomidae, Pentatominae, Carpocorini	Phytophagous/Native	n.a. (97.56% [*])
<i>Piezodorus lituratus</i> (F.)	Pentatomidae, Pentatominae, Piezodorini	Phytophagous/Native	n.a. (88.0% [*])
<i>Sciocoris</i> sp. Wollaston	Pentatomidae, Pentatominae, Sciocorini	Phytophagous/Native	n.a.
<i>Eurygaster maura</i> (L.)	Scutelleridae, Eurygastrinae, Eurygastrini	Phytophagous/Native	90.15%
<i>Gonocerus juniperi</i> Herrich-Schäffer	Coreidae, Coreinae, Gonocerini	Phytophagous/Native	n.a. (100% [*])
<i>Rhynocoris iracundus</i> (Poda)	Reduviidae, Harpactorinae, Harpactorini	Predatory/Native	n.a. (96.67% [*])

Arma custos and *R. iracundus* are predators, whereas all other species are herbivores

^{*}Data from Sabbatini Peverieri et al. (2021) using the same rearing condition are reported within parentheses

Paper towels were hung inside the rearing cages as oviposition substrates, and eggs were collected daily. As a measure of standard quality of laboratory hemipteran rearing, the viability of eggs was

occasionally recorded (Tab. 1). At this scope, batches of egg masses produced by *H. halys* and non-target species were reared in climatic chamber (HPP750, Memmert GmbH + Co. KG, Schwabach, Germany) at 26 ± 1 °C, $60 \pm 5\%$ RH and 16:8 L/D up to egg hatching.

Trissolcus mitsukurii was obtained from about 60 parasitized *H. halys* egg masses collected in 2018 and 2019 in *H. halys* infested sites of Northern Italy, the sites where the adventive populations of the egg parasitoid were initially recorded (see Sabatini-Peverieri et al. 2018). These egg masses were reared in the laboratory, and the emerged *T. mitsukurii* individuals (about 500 specimens) were used to start a laboratory colony. Adults of the egg parasitoid were fed with pure honey droplets. Periodically, fresh egg masses (< 24 h old) of *H. halys* were offered as host for parasitization and juvenile development. The permanent colony of the egg parasitoid was established and maintained in climatic chamber at 26 ± 1 °C, $60 \pm 5\%$ RH and 16:8 L/D; such conditions were considered as standards for the present work and were used for all experiments. Adults of *T. mitsukurii* and parasitized egg masses were housed in glass tubes (15 cm length, 2 cm diam), closed on both ends by a plastic net (250 µm mesh). Establishment and maintenance of the egg parasitoid colony and all experiments (see below) were conducted under quarantine conditions at CREA facilities. Quarantine laboratories are authorized for studies on exotic beneficial organisms in the framework of *H. halys* biological control program (MiPAAF, DG/DISR/ DISR05/0013647–19/04/2018 and SFR Regione Toscana 203304 – 12/04/2018).

No-choice black box tests

A total of three different experimental conditions were considered for the no-choice black box tests. In the first and second experiment, host egg masses were handled as little as possible to avoid the introduction of external contaminants, and any adjustment of size (egg number per mass) was avoided, as to simulate the natural number of host eggs, per egg batch, that female parasitoids would likely encounter in the field (Hedstrom et al. 2017; Botch and Delfosse 2018). For *G. juniperi*, which lays individual eggs, ten eggs were aligned and glued (using non-toxic clear school Elmer's® glue, Newell Office Brands) on a cardboard (15 mm × 80 mm), each egg distanced 5 mm from the other. For the tests, one egg mass (or a row of ten single eggs) of a given host species was placed in a glass tube (15 cm length, 2 cm diam.), and one parasitoid female, 7-d old, was introduced for two different exposure times. Specifically, in the first experiment, females were kept for 24 h and a small drop of honey was added on the glass internal surface as food source. In the second experiment, the exposure time was reduced to 2 h and no food was provided.

In the third type of black box no-choice experiment, only one egg was provided to the female for a total time of 2 h. In this experimental condition, a small glass vial (7 cm length, 0.5 cm diam.) was used and closed with a cotton ball to prevent the parasitoid from escaping. This assay is an extreme

simplified exposure condition that has been previously shown to provide reliable results for no-choice tests with egg parasitoids (Sabbatini-Peverieri et al. 2021). Tested eggs were glued (Elmer's® glue) on the cardboard to permit a sufficient stability.

In all no-choice experiments, only *T. mitsukurii* females originating from *H. halys* eggs were tested, and only fresh eggs (< 24 h old) of *H. halys* and non-target species were employed. Before use, eggs and egg masses were inspected under a stereomicroscope to assess their suitability for the tests. In case of, e.g., non-characteristic color of the eggs, unusual egg arrangement in the batch, unswollen eggs in the mass, or unusual low number of eggs per mass (according to known natural egg amount per egg mass of tested species), the egg or the egg mass was discarded. Parasitoids were removed at the end of the allotted time (24 h or 2 h). Eggs were maintained in the glass vials (single eggs) or tubes (egg masses) and were reared in climatic chamber at standard conditions until nymph hatching or parasitoid emergence. Eggs that did not hatch nor produced a parasitoid after three weeks from tests, were classified as “dead eggs.” These were dissected and inspected under a stereomicroscope, and the content was eventually classified as dead parasitoid (pupae or pharate adult), dead Hemiptera nymph, or undetermined content. Emerged adult parasitoids were counted and sexed.

Effect of host egg size on parasitoid size and egg load

The effects of host eggs on the parasitoid size and egg load was evaluated using *T. mitsukurii* females emerged from *H. halys* and non-target hosts of the no-choice black box tests. For this purpose, the volume (mm³) of eggs of host species was calculated by the parabolic barrel formula $V = \pi H(3r^2 + 4Rr + 8R^2)/15$, where “H” is the egg height, “r” is the radius of the operculum, and “R” is the radius at the center of the egg (Abram et al. 2016; Botch and Delfosse 2018). The head and thorax width and the length of the hind tibia were measured from females emerged from *H. halys* and non-target hosts. For this purpose, insects were dissected under a stereomicroscope and body parts were measured using Nikon SMZ25 and NIS-Elements software (Nikon Corporation, Tokyo, Japan). Additionally, parasitoid females from *H. halys* and non-target host eggs were reared individually for 7 days at standard conditions in climatic chamber. At the seventh day of rearing, the females were dissected and the egg load in their ovary was counted after staining with 1% toluidine bleu (Sabbatini-Peverieri et al. 2020).

Paired choice black-box tests using parasitoids reared on different hosts

In a first paired-choice experiment, seven-day-old *T. mitsukurii* females reared on *H. halys* eggs were singly used in dual-choice tests in Petri dishes (9 cm diam.). A female was allowed to exploit two fresh egg masses (< 24 h), one of *H. halys* and one of a non-target species (n = 12). *Arma custos* or *D. baccarum* were chosen as non-target species because of the high parasitization rates that were

recorded in the no-choice tests (over 50% of successful parasitization). *Arma custos* was also chosen as this is a key species for the safeguarding approach in risk analysis. The number of eggs (Table 2) of tested egg masses was not adjusted in order to maintain the similar egg numbers that *T. mitsukurii* would encounter in the field (Haye et al. 2019), with the only exception of the egg masses of *N. viridula*, too large compared to the maximal egg load of *T. mitsukurii* in the ovaries (see Sabbatini-Peverieri et al. 2020). The two egg masses were placed at the bottom of the Petri dish and at the opposite sides of the arena. The position of the egg species was inverted at each replicate. The parasitoid female was placed at the center of the arena and tested for a 2 h time span in climatic chamber at standard condition. Such reduced time of egg mass exposure was adopted because in the previous no-choice experiment, the parasitoid females displayed to parasitize host egg masses within this short time interval, and because this would limit the opportunity for multiple parasitization, which is likely to occur in a 24-h exposure test (Haye et al. 2019). At the end of the exposure, the females were removed from the arena and the egg masses were reared in the climatic chamber at standard condition until the eggs hatched or the parasitoids emerged. Eggs that did not hatch nor produced a parasitoid after three weeks from the experiment were dissected to determine the contents as described previously. A second paired-choice experiment was conducted to evaluate possible effects of the rearing host on host preferences by *T. mitsukurii*. For this purpose, different colonies of *T. mitsukurii* were established and maintained on egg masses of the non-target species, *D. baccarum* or *A. custos*, with the same method used when reared on *H. halys* as host. The same experimental design as described above was applied using *T. mitsukurii* parasitoid females originated from host eggs of either *D. baccarum* or *A. custos*. When females did not make a choice, i.e., they failed to oviposit at least in one of the two offered egg masses, the replicate was excluded from the analysis.

Statistical analysis

In no-choice tests with host egg masses exposure for 24 and 2 h, the percentages of hatched Hemiptera eggs, successfully parasitized eggs (emergence of the parasitoid) and dead eggs were analyzed by Kruskal–Wallis and Dunn’s multiple comparison post hoc test using *H. halys* as control species. Data were analyzed only for the species showing at least five replicates (although the entire dataset was reported). Pairwise comparison among species in single host egg exposure for 2 h was performed using Chi-square test with Yates’ correction. Pooled data on parasitoid female dimensions (head width, thorax width, hind tibia length) were analyzed with Pearson correlation coefficient. The functional relationship between the egg size of host species, emerged parasitoid female size and ovaries egg load was analyzed through linear regression model. In choice test, the percentages of hatched Hemiptera eggs, successfully parasitized eggs (emergence of the parasitoids) and dead eggs were compared using the Mann–Whitney U test. Ovarian egg load between *H. halys* and non-target

species were compared with Kruskal–Wallis and Dunn’s multiple comparison post hoc test. Statistics were performed using Graphpad Prism 8.

Results

No-choice black box tests

When Hemiptera egg masses were exposed to *T. mitsukurii* for 24 h, most eggs were suitable or partially suitable for parasitoid development, with significantly different emergence rates from the diverse tested species ($\chi^2 = 112.7$; $P < 0.0001$) (Table 2). *Trissolcus mitsukurii* was able to parasitize and develop in 12 pentatomid species out of the 14 that were tested (85.71% of tested species), and in the only tested scutellerid, *E. maura*, although with highly variable success. Conversely, it was not able to exploit the remaining pentatomids *A. leucogrammes* and *E. ventralis*, the coreid

G. juniperi and the reduviid *R. iracundus* (Table 2). The emergence rate of *T. mitsukurii* from *H. halys* egg masses was very high, close to 100%, and not significantly different compared to emergence from the pentatomids *A. heegeri*, *A. custos*, *C. pudicus*, *D. baccarum*, *G. italicus*, *P. prasina*, *P. lituratus*, *Sciocoris* sp. (eight out of 13 tested non-target pentatomid species) and the scutellerid *E. maura* (Table 2; see Table S1 for statistics). Instead, emergences were significantly lower, compared to *H. halys*, when eggs of *Aelia acuminata*, *E. oleracea* and *N. viridula* were tested (Table 2; see Table S1 for statistics). Sex ratios were strongly female biased in most of the species tested (Table 2).

The percentages of Hemiptera eggs that hatched after exposure to *T. mitsukurii* were significantly different ($\chi^2 = 80.04$; $P < 0.0001$), and hatching rates were partially complementary to parasitoid emergence rates. Almost none of the eggs of *H. halys*, *A. heegeri*, *D. baccarum* and *P. prasina* hatched, while hatching percentages were relevant for the other pentatomid species, although only *A. leucogrammes* and *E. ventralis* showed significantly higher rates compared to *H. halys*. The percentages of dead eggs (no hatching and no parasitoid emergence) were significantly different among the tested species ($\chi^2 = 73.50$; $P < 0.0001$). Dead eggs were present in all Hemiptera species except *H. halys* and *P. prasina* (but only one egg mass of the latter could be tested), and percentages were notably higher in *A. acuminata*, *E. oleracea*, *E. ventralis* and *N. viridula* than in *H. halys* (Table 2; see Table S1 for statistics). Dead specimens of *T. mitsukurii* (pupae and pharate adults) were detected in eggs of suitable and partially suitable pentatomid host species, with the only exceptions of *H. halys*, *D. baccarum* and *P. prasina*. No dead parasitoid specimens were recorded in eggs of the scutellerid *E. maura*, although several eggs died for unknown reason (undetermined content). While no *T. mitsukurii* emerged from *A. leucogrammes* and *E. ventralis*, dissection of dead eggs revealed the presence of high numbers of dead parasitoid specimens (adults and pupae) in both species.

Table 2 Physiological suitability of Hemiptera eggs for *Trissolcus mitsukurii* in 24h black box tests. Asterisks within each column indicate significant differences among the non-target species and *Halyomorpha halys* (Kruskal-Wallis followed by Dunn's Multiple Comparison test, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.a. = not assessed) (see Table S1 for statistics)

Species (replicates)	n. eggs/egg mass (mean $\pm$ SE)	% of eggs with parasitoid emergence/egg mass (mean $\pm$ SE)	% of females (mean $\pm$ SE)	% of hatched eggs / egg mass (mean $\pm$ SE)	% of dead eggs (mean $\pm$ SE)	n. of parasitized egg masses /with parasitoid emergence/total n. of eggs exposed	total n. of dead eggs		
							with undetermined content	with dead hemipteran nymphs	with dead parasitoid
<i>Halyomorpha halys</i> (12)	27.42 $\pm$ 0.29	99.40 $\pm$ 0.60	90.22 $\pm$ 3.29	0.60 $\pm$ 0.60	0 $\pm$ 0.00	12 / 12 / 329	0	0	0
<i>Acrosternum heegeri</i> (12)	13.25 $\pm$ 0.46	98.32 $\pm$ 1.24	89.78 $\pm$ 1.37	0 $\pm$ 0.00	0.49 $\pm$ 0.49	12 / 12 / 159	0	0	1 (adult)
<i>Aelia acuminata</i> (12)	11.83 $\pm$ 0.24	7.20 $\pm$ 3.57***	72.00 $\pm$ 12.64	25.00 $\pm$ 13.06	67.80 $\pm$ 12.26***	7 / 4 / 142	93	0	13 (all adults)
<i>Ancyrosoma leucogrammes</i> (10)	11.50 $\pm$ 0.45	0 $\pm$ 0.00***	-	90.00 $\pm$ 10.00***	10.00 $\pm$ 10.00	1 / 0 / 115	3	0	9 (all adults)
<i>Arma custos</i> (12)	14.33 $\pm$ 0.63	66.32 $\pm$ 12.51	90.65 $\pm$ 1.54	25.00 $\pm$ 13.06	8.67 $\pm$ 5.05	9 / 9 / 172	0	0	14 (all adults)
<i>Carpocoris pudicus</i> (12)	14.08 $\pm$ 0.87	75.00 $\pm$ 13.06	92.22 $\pm$ 0.60	9.22 $\pm$ 7.37	15.78 $\pm$ 9.95	12 / 9 / 169	0	0	29 (28 pupae, 1 adult)
<i>Dolycoris baccarum</i> (12)	13.92 $\pm$ 0.34	98.29 $\pm$ 0.89	92.08 $\pm$ $\pm$ 1.02	0.55 $\pm$ 0.55	0.59 $\pm$ 0.59	12 / 12 / 167	1	0	0
<i>Eurydema oleracea</i> (10)	10.10 $\pm$ 0.38	21.38 $\pm$ 11.09**	82.22 $\pm$ 5.35	25.00 $\pm$ 11.33	55.21 $\pm$ 11.50***	4 / 4 / 101	42	0	12 (all adults)
<i>Eurydema ventralis</i> (12)	11.83 $\pm$ 0.32	0 $\pm$ 0.00***	-	63.81 $\pm$ 11.49**	36.19 $\pm$ 11.49*	5 / 0 / 142	12	0	41 (6 pupae, 35 adults)
<i>Graphosoma italicum</i> (12)	14.58 $\pm$ 0.45	78.57 $\pm$ 8.05	84.52 $\pm$ $\pm$ 2.85	15.16 $\pm$ 8.07	6.28 $\pm$ 2.74	11 / 11 / 175	1	0	10 (all adults)
<i>Nezara viridula</i> (12)	28.25 $\pm$ 0.97	1.90 $\pm$ 1.57***	100	29.04 $\pm$ 10.45	68.36 $\pm$ 10.89***	4 / 2 / 339	207	0	22 (all adults)
<i>Palomena prasina</i> (1)	12	100 n.a.	75 n.a.	0 n.a.	0 n.a.	01/01/12	0	0	0
<i>Piezodorus lituratus</i> (5)	10.60 $\pm$ 1.29*	68.24 $\pm$ 18.39	68.37 $\pm$ 7.29	22.97 $\pm$ 19.33	8.79 $\pm$ 5.77	04/04/53	0	0	6 (all adults)
<i>Sciocoris</i> sp. (8)	10.38 $\pm$ 0.57	81.60 $\pm$ 12.25	83.07 $\pm$ 2.72	10.94 $\pm$ 9.28	6.07 $\pm$ 3.29	08/07/83	1	0	5 (all adults)
<i>Eurygaster maura</i> (12)	11.67 $\pm$ 0.53	87.36 $\pm$ 8.32	90.74 $\pm$ $\pm$ 1.34	9.03 $\pm$ 8.30	3.61 $\pm$ 2.51	11 / 11 / 140	6	0	0
<i>Gonocerus juniperi</i> (12)	10.00 $\pm$ 0.00	0 $\pm$ 0.00***	-	97.50 $\pm$ 1.31***	2.51 $\pm$ 1.30	0 / 0 / 100	3	0	0
<i>Rhynocoris iracundus</i> (3)	32.22 $\pm$ 0.88	0 $\pm$ 0.00 n.a.	-	74.33 $\pm$ 21.16 n.a.	25.67 $\pm$ 21.16 n.a.	0 / 0 / 97	26	0	0

Instead, dissections of dead eggs of the coreid *G. juniperi* and the reduviid *R. iracundus* never reported the presence of dead pupae or adults. When the Hemiptera egg masses were exposed to *T. mitsukurii* for 2 h, results were similar to the 24 h exposure experiment and the emergence rates were significantly different among the tested species ($\chi^2 = 77.38$; $P < 0.0001$) (Table 3; see Table S1 for statistics). *Trissolcus mitsukurii* was able to parasitize and develop in six out of the nine pentatomid species that were tested (66.67% of tested species) and in the scutellerid *E. maura* (Table 3).

Specifically, the emergence rates of the pentatomids *A. heegeri*, *A. custos*, *C. pudicus*, *D. baccarum*, and *G. italicum* (five out of eight nontarget pentatomid species) and the scutellerid *E. maura* were not significantly different from that of *H. halys* (Table 3; see Table S1 for statistics). Sex ratios were strongly female biased in most of the species tested (Table 3). Hatching rates of Hemiptera eggs were significantly different ($\chi^2 = 46.52$; $P < 0.0001$) and complementary with parasitoid emergence rates and dead eggs. Percentages of dead eggs were also significantly different ($\chi^2 = 74.06$; $P < 0.0001$) and consistent with previous data. Dead pupae and adults of the parasitoid were observed in most of the tested species, but not in *H. halys*, *A. heegeri* and *D. baccarum*. Specifically, eggs of *A. acuminata* showed lower parasitization success compared to *H. halys*, with significantly lower parasitoid emergence and higher dead eggs containing dead parasitoids.

Similarly, eggs of *N. viridula* showed no parasitoid emergence and significantly higher dead eggs containing dead parasitoids. Instead, a high rate of nymphs emerged from *E. ventralis* eggs and no parasitoid emerged, while some dead parasitoids were found in the eggs (Table 3; see Table S1 for statistics).

When a single Hemiptera egg was exposed to *T. mitsukurii* for 2 h, six out of seven pentatomid species (85.71% of tested species), and the scutellerid *E. maura* were suitable for *T. mitsukurii* development (Table 4). Percentages of parasitoid emergence from eggs of non-target species were significantly lower compared to those from *H. halys* (Table 4; see Table S2 for statistics), except for *P. prasina* where 100% of emergence success was recorded, like in *H. halys*. No parasitoids emerged from eggs of *N. viridula* and no parasitoid pupae or pharate adults were found in dead eggs of this pentatomid species.

Effect of host egg size on parasitoid size and egg load

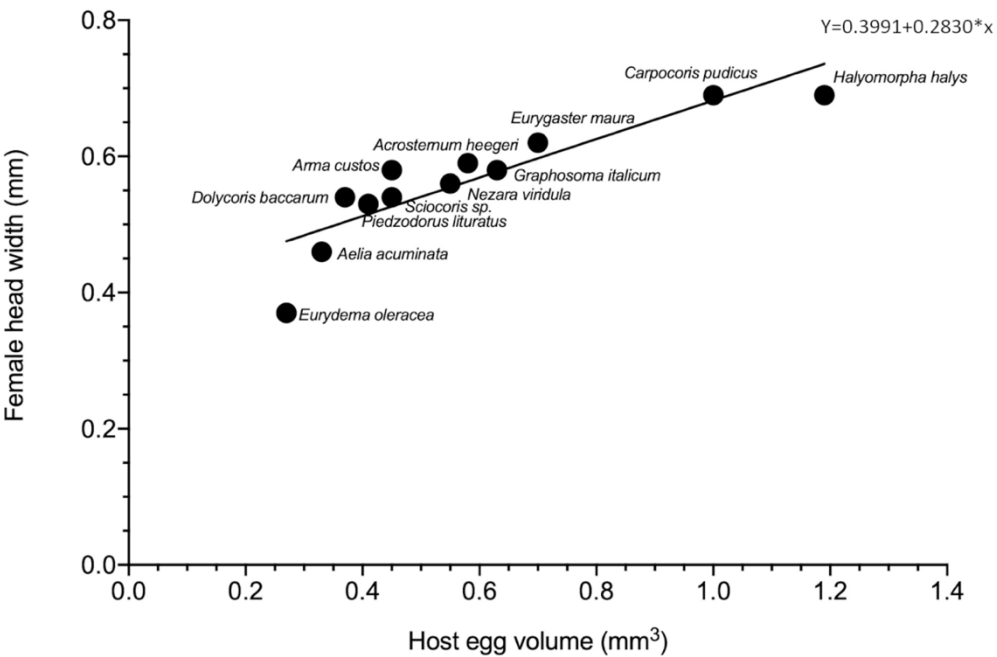
The three-body dimensions measured on *T. mitsukurii* females (head width, thorax width and hind tibia length, see Table S3) revealed to be highly correlated (head width vs. thorax width: $r = 0.96$, $P < 0.0001$; head width vs. hind tibia length: $r = 0.81$, $P < 0.0001$; thorax width vs. hind tibia length: $r = 0.81$, $P < 0.0001$). Within each Hemiptera host species, the female head width was used as main parameter to analyze in the linear regression model as a function of host egg volume. Regression

analysis displayed a positive trend between the volume of host eggs and the head width of the emerged parasitoid females ($r^2 = 0.7673$, $P = 0.0002$) (Fig. 1).

Table 3. Physiological suitability of Hemiptera eggs for *Trissolcus mitsukurii* in 2h black box tests. Asterisks within each column indicate significant differences among the non-target species and *Halyomorpha halys* (Kruskal-Wallis followed by Dunn's Multiple Comparison test, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) (see Table S1 for statistics)

Species (replicates)	n. eggs/egg mass (mean $\pm$ SE)	% of eggs with parasitoid emergence/egg mass (mean $\pm$ SE)	% of females (mean $\pm$ SE)	% of hatched eggs / egg mass (mean $\pm$ SE)	% of dead eggs (mean $\pm$ SE)	n. of parasitized egg masses /with parasitoid emergence/total n. of eggs exposed	total n. of dead eggs		
							with undetermined content	with dead hemipteran nymphs	with dead parasitoid
<i>Halyomorpha halys</i> (12)	27.58 $\pm$ 0.74	81.69 $\pm$ 3.41	94.76 $\pm$ 0.44	18.31 $\pm$ 3.41	0.00	12/12/331	0	0	0
<i>Acrosternum heegeri</i> (12)	14.50 $\pm$ 0.68	91.11 $\pm$ 8.30	89.68 $\pm$ 1.13	8.89 $\pm$ 8.30	0.00	11/11/174	0	0	0
<i>Aelia acuminata</i> (12)	10.92 $\pm$ 0.34	3.19 $\pm$ 2.53*	66.67 $\pm$ 13.61	23.41 $\pm$ 11.78	73.39 $\pm$ 11.46***	6/1/131	82	0	18 (all adults)
<i>Arma custos</i> (12)	15.33 $\pm$ 0.49	40.52 $\pm$ 14.47	92.84 $\pm$ 0.11	58.33 $\pm$ 14.87	1.15 $\pm$ 0.77	5/5/184	0	0	2 (all adults)
<i>Carpocoris pudicus</i> (12)	12.33 $\pm$ 0.33	88.46 $\pm$ 8.65	88.37 $\pm$ 1.09	8.97 $\pm$ 8.30	2.56 $\pm$ 2.56	11/11/160	0	0	4 (all adults)
<i>Dolycoris baccarum</i> (12)	14.00 $\pm$ 0.24	83.28 $\pm$ 4.41	85.85 $\pm$ 3.44	5.40 $\pm$ 3.53	11.40 $\pm$ 4.26	10/10/175	11	0	0
<i>Eurydema ventralis</i> (12)	11.67 $\pm$ 0.19	0 $\pm$ 0.00**	-	90.66 $\pm$ 3.78	9.28 $\pm$ 3.77	1/0/140	9	0	4 (all adults)
<i>Graphosoma italicum</i> (12)	14.75 $\pm$ 0.31	77.12 $\pm$ 5.84	86.85 $\pm$ 2.30	18.87 $\pm$ 6.36	4.00 $\pm$ 2.72	12/12/177	0	0	7 (all adults)
<i>Nezara viridula</i> (12)	34.25 $\pm$ 1.16	0 $\pm$ 0.00**	-	22.85 $\pm$ 10.62	77.15 $\pm$ 9.38***	5/0/411	260	0	27 (all adults)
<i>Eurygaster maura</i> (12)	13.58 $\pm$ 0.26	88.64 $\pm$ 8.13	92.48 $\pm$ 0.93	8.33 $\pm$ 8.33	3.02 $\pm$ 1.08	11/11/163	0	0	5 (all adults)

Figure 1. Regression analysis of *Trissolcus mitsukurii* females head width as a function of egg volume of Hemiptera host species



The egg loads (see Table S4) of *T. mitsukurii* females originated from the different Hemiptera host species were significantly different ($\chi^2 = 65.75$; $P < 0.0001$). Parasitoid females that developed in *H. halys* exhibited a higher number of eggs in their ovaries compared to other non-target pentatomid species (see Table S4 for statistics), except for *C. pudicus* and *A. heegeri* and the scutellerid *E. maura*. Regression analysis displayed a positive trend between host egg volume and the ovarian egg load ($r^2 = 0.8053$, $P < 0.0001$) (Fig. 2).

Figure 2 Regression analysis of *Trissolcus mitsukurii* female ovaries egg load (at 7 days in age) as a function of egg volume of Hemiptera host species

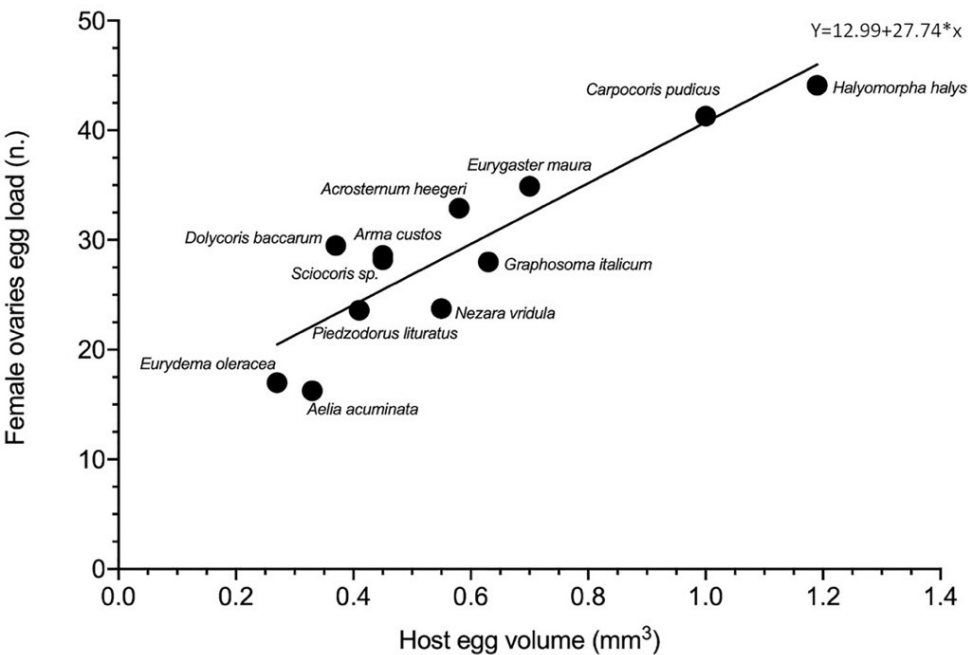


Table 4 Physiological suitability of Hemiptera eggs for *Trissolcus mitsukurii* in 2h exposure of a single host egg. Among brackets number of females tested; asterisks within each column indicate significant differences among the non-target species and *Halyomorpha halys* (Chi-square test with Yates' correction, *** $P < 0.001$) (see Table S2 for statistics).

species	% of emerged parasitoids	% of hatched eggs	% of dead eggs	
			with undetermined content or a dead hemipteran nymph	containing pupae or adults
<i>Halyomorpha halys</i> (31)	100	0	0	0
<i>Acrosternum heegeri</i> (30)	46.67***	53.33	0	0
<i>Arma custos</i> (43)	30.23***	55.81	9.31	4.65
<i>Dolycoris baccarum</i> (30)	20.00***	63.33	16.67	0
<i>Graphosma italicum</i> (30)	26.67***	16.67	0	56.67
<i>Nezara viridula</i> (30)	0***	56.67	43.33	0
<i>Palomena prasina</i> (26)	100	0	0	0
<i>Eurygaster maura</i> (34)	2.94***	26.47	61.77	8.82

Among brackets number of females tested; asterisks within each column indicate significant differences among the non-target species and *Halyomorpha halys* (Chi-square test with Yates' correction, *** $P < 0.001$) (see Table S2 for statistics)

3.4 Paired choice black-box tests using parasitoids reared on different hosts

In the *H. halys* vs. *D. baccarum* choice tests, all *T. mitsukurii* tested females, indifferently reared on *H. halys* or *D. baccarum*, successfully parasitized at least one host egg mass. While in the combination of *H. halys* vs. *A. custos* choice-tests, *T. mitsukurii* females failed to interact with host eggs in one case when the parasitoid females originated from *H. halys*, and in two cases when the females originated from *A. custos*.

When the parasitoid was reared on *H. halys*, emergence of the progeny of *T. mitsukurii* females showed no significant differences between *H. halys* and each of the two non-target host species (*H. halys* vs. *D. baccarum*, $U=48$, $P=0.1398$; *H. halys* vs. *A. custos*, $U=38.50$, $P=0.1339$). Percentages of dead eggs were not significantly different neither (*H. halys* vs. *D. baccarum*, $U=58$, $P=0.3811$; *H. halys* vs. *A. custos*, $U=45.50$, $P=0.3342$) (Fig. 3). A lower percentage of nymphs hatched from *H. halys* compared to *A. custos* eggs ($U=30$, $P=0.0434$), while similar percentages emerged from *H. halys* and *D. baccarum* eggs ($U=65$, $P=0.6924$).

When the parasitoid was reared on a non-target host (*D. baccarum* or *A. custos*), the emergence of the progeny of *T. mitsukurii* females showed no significant differences in the case of *H. halys* vs. *D.*

baccarum ($U=72$, $P>0.9999$) and in that of *H. halys* vs. *A. custos* ($U=30.50$, $P=0.1214$) (Fig. 4). Additionally, no differences were observed in the percentage of dead eggs (*H. halys* vs. *D. baccarum*, $U=57$, $P=0.8444$; *H. halys* vs. *A. custos*, $U=28$, $P=0.0927$) nor in the percentage of hatched nymphs (*H. halys* vs. *D. baccarum*, $U=65$, $P=0.6924$; *H. halys* vs. *A. custos*, $U=37.50$, $P=0.3541$).

Figure 3. Outputs of choice tests using *Trissolcus mitsukurii* females, originated from *Halyomorpha halys*, that were exposed for 2 h to egg masses of target and non-target species in pairwise comparisons (a = *H. halys* vs. *Dolycoris baccarum*; b = *H. halys* vs. *Arma custos*)

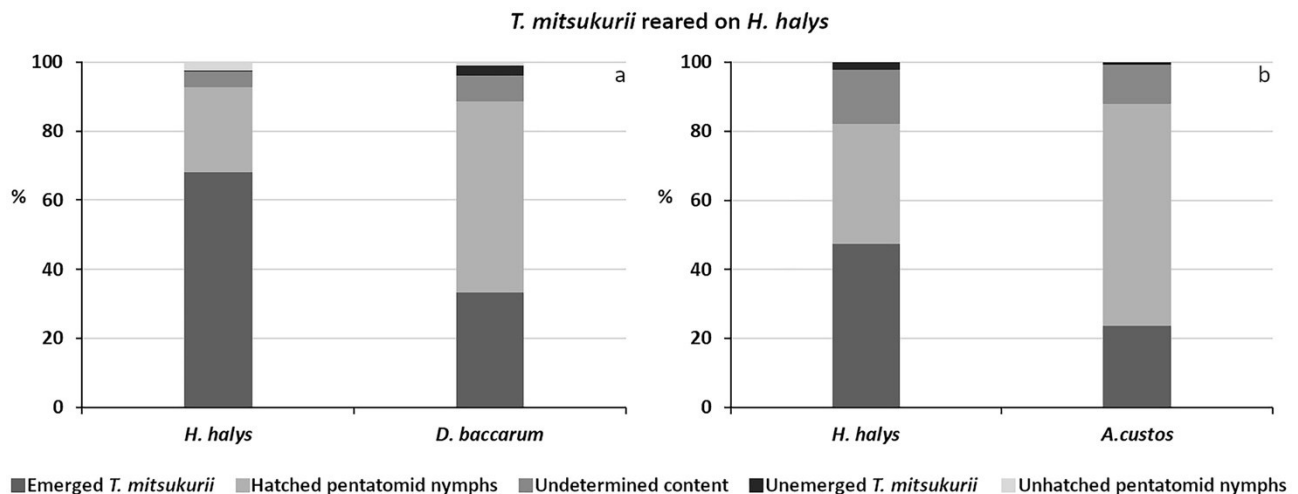
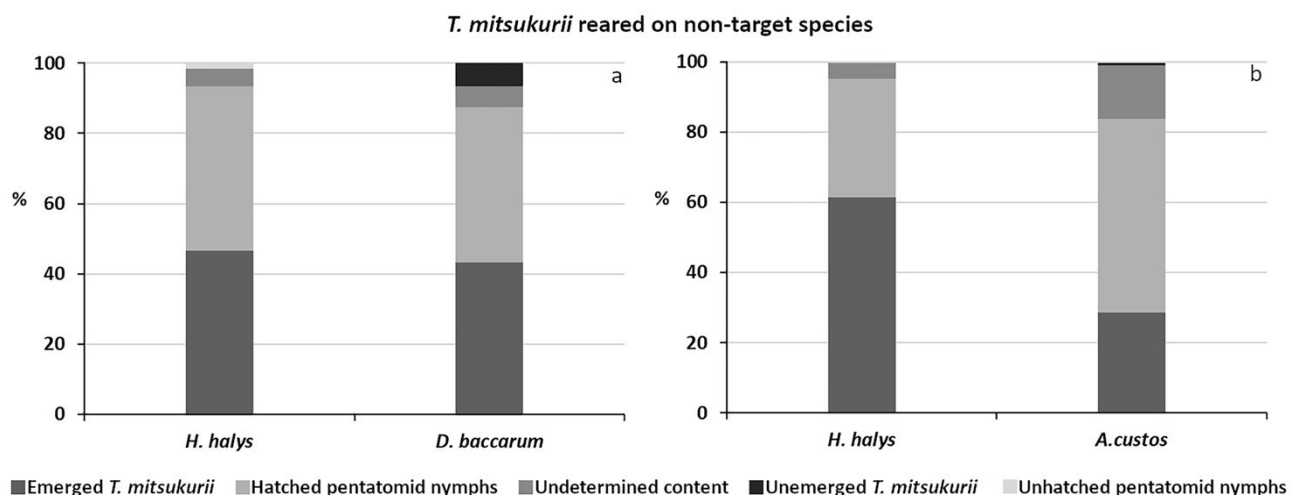


Figure 4. Outputs of choice tests using *Trissolcus mitsukurii* females, originated from the non-target species *Dolycoris baccarum* or *Arma custos*, that were exposed for 2 h to egg masses of *Halyomorpha halys* and non-target species in pairwise comparisons (a = vs. *D. baccarum*; b = vs. *A. custos*)



Discussion

The egg parasitoid *T. mitsukurii* has been poorly investigated till now as a candidate biological control agent of *H. halys*, especially when compared to other egg parasitoid species like *T. japonicus* and the non-coevolved *Anastatus bifasciatus* (Geoffroy) (Hymenoptera, Eupelmidae), on which a large

literature is available from China, Europe, USA and New Zealand. Field data from Japan indicate for *T. mitsukurii* an oligophagous behavior (Yasumatsu and Watanabe 1964; Hokyo et al., 1966; Ryu and Hirashima, 1984; Arakawa and Namura 2002), and data from the invaded areas in Europe (Northern Italy) evidenced the ability of this parasitoid to locate and successfully parasitize *H. halys* egg masses in the field (Sabbatini-Peverieri et al. 2018; Benvenuto et al. 2020; Scaccini et al. 2020; Zapponi et al. 2020). Furthermore, laboratory studies confirm that the reproductive biology of this parasitoid on *H. halys* is similar to that of *T. japonicus* on the same host (Sabbatini-Peverieri et al. 2020).

In our experiments under laboratory conditions and 24 h of egg mass exposure, *T. mitsukurii* displayed the ability to parasitize and develop successfully in eight (61.54%) out of 13 tested non-target pentatomid species, with no significant differences compared to the coevolved host *H. halys*, while additional three species were partially suitable although at a significantly lower level compared to *H. halys*. These results were largely confirmed when *H. halys* and non-target species were exposed to the parasitoid only for 2 h, as *T. mitsukurii* successfully parasitized five (62.50%) out of eight non-target pentatomid species with similar rates than when parasitizing *H. halys*. Additionally, the scutellerid *E. maura* was successfully parasitized in both the 24 h and 2 h tests, whereas the coreid *G. juniperi* and the reduviid *R. iracundus* were not. This can be at least partially explained if we consider that scutellerids belong to the same superfamily of pentatomids, i.e., Pentatomoidea. In previous laboratory investigations, *E. maura* was revealed to be physiologically suitable also for *T. japonicus* (Haye et al. 2019). However, because scutellerids were never found as hosts of neither *T. mitsukurii* nor *T. japonicus* in the field, it is possible to hypothesize that other ecological factors, e.g., habitat preferences, interspecific competition or oviposition periods, may limit the access to this host. Laboratory host range of *T. mitsukurii* can be considered similar to that of *T. japonicus*, which showed comparable results in previous host specificity tests on European non-target species, as 13 pentatomids were suitable for parasitoid development (Haye et al. 2019; Sabbatini-Peverieri et al. 2021).

The highly variable rates of *T. mitsukurii* emergence from non-target eggs, both in the 24 h and 2 h exposure experiments, could be discussed in terms of host egg recognition/acceptance and suitability, based on data on parasitoid emergence, egg eclosion, egg death and the presence of dead parasitoids in eggs. Thus, *H. halys*, *A. heegeri* and *D. baccarum* appear to be easily recognized by *T. mitsukurii* and highly suitable hosts for this parasitoid under laboratory conditions, as emergence rates were always very high, egg hatching low, and almost no dead eggs were recorded. Similar results were also observed in *P. prasina*, although this species was tested with only one egg mass due to the poor rearing success in the laboratory. Most of the other non-target species seemed to be less suitable, although statistically not different from *H. halys*, because of the high presence of dead eggs, often containing a dead parasitoid pupa or a pharate adult (*A. custos*, *C. pudicus*, *G. italicum*, *P. lituratus*,

Sciocoris sp.). Still, rates of successful parasitism of this species were rather high. High rates of parasitization successes under laboratory conditions were observed also for *T. japonicus* in China on *H. halys* and other pentatomid species (Zhang et al., 2017), while other studies in the USA and New Zealand reported lower rates both on the target, *H. halys*, and no targets, probably due to the different physiological status of tested females (Hedstrom et al. 2017; Botch and Delfosse 2018; Charles et al. 2019; Lara et al. 2019). In our study, a few non-target pentatomid hosts showed significantly lower *T. mitsukurii* emergence rates compared to emergence from *H. halys*. This was due either to low recognition and parasitization, as indicated by high egg hatching rates (*A. leucogrammes*), or to low suitability to parasitoid development, as indicated by the high rates of dead eggs (*A. acuminata*, *E. oleracea*, *N. viridula*), or to both reasons (*E. ventralis*). The occurrence of dead *T. mitsukurii* pupae or adults in several Hemiptera host eggs further reflects a low physiological suitability. Remarkably, dead parasitoids were found also in the eggs of pentatomid species that did not allow any parasitoid adult emergence, i.e., *E. ventralis* and *A. leucogrammes*. In the field, such cases might act as an evolutionary trap, as was stated for *T. japonicus* in similar cases, and unsuitable biochemical contentment of hemipteran eggs can here play a key role (Abram et al. 2014; Haye et al. 2019).

Surprisingly, as discussed above, the eggs of *N. viridula* showed a very low suitability rate for *T. mitsukurii*. This is a rather unexpected result because, although not coevolved due to the different geographical origin, *N. viridula* was addressed in the past as a main host for *T. mitsukurii* in its area of origin (Kiritani and Hokyo 1962; Hokyo and Kiritani 1963; Hokyo et al. 1966; Arakawa and Namura 2002; Arakawa et al. 2004). In our experiment, a very low parasitoid emergence rate and a high rate of dead eggs was detected, especially if compared with the high hatching rate observed in unexposed *N. viridula* egg masses. Moreover, in only about one tenth of the dead eggs, it was possible to find dead parasitoid pupae or adults, whereas the egg content could not be determined in the other cases. We can hypothesize that parasitization occurred also in these cases, but the parasitoid failed to survive already during the first instars, further suggesting that *N. viridula* eggs are not suitable for larval development of *T. mitsukurii*. A similar result was observed in *T. japonicus*, as the parasitizing females caused 100% mortality in *N. viridula* eggs, but no emergence of adults were observed, nor parasitoid presence could be clearly identified from egg dissection (Haye et al. 2019). The low suitability of *N. viridula* to *T. mitsukurii* deserves further investigations.

The extremely simplified experiment conducted using a single host egg, exposed to *T. mitsukurii* for 2 h, showed that among the seven pentatomid and one scutellerid species tested, most of them were detected as physiologically suitable, failing only in the case of *N. viridula*, which confirms its extremely low physiological suitability for *T. mitsukurii*. Remarkably, in such kind of test, only *H. halys* and *P. prasina* allowed 100% parasitism success by *T. mitsukurii*, while parasitoid emergence from all other non-target host species was significantly lower. Comparing the three methods of host

egg exposure to females of *T. mitsukurii* adopted in the present study (exposure of egg masses for 24 h and for 2 h and exposure of single eggs for 2 h) and considering only the Hemiptera species that were tested in all three experiments, the 2 h test with single egg exposure failed to detect host physiological suitability in one case, the less suitable host species *N. viridula*. Additionally, a high number of hatched eggs was observed in all species except *H. halys* and *P. prasina*. These results indicate that a single host egg was not easily recognized and probed by the parasitoid. Therefore, by exposing a single egg, the degree of host acceptance and physiological suitability might be more difficult to define since the effect of clustered eggs is not taken into account. However, the use of single host egg exposure was already adopted for host specificity tests with *T. japonicus* and the output of physiological host suitability range was comparable with the results from experiments conducted using egg mass exposed for 24 h using similar host species (Haye et al. 2019; Sabbatini-Peverieri et al. 2021). This simple method of exposure might permit to filter a first list of suitable non-target species, which can be later investigated using more complex procedures, thus optimizing resources and time. In future tests, the time of egg exposure to parasitoids can be reduced (depending on the size of arena and on complexity of tests) as a few hours are adequate rather than a 24-h experimental design. Here, considering outputs of the present work, three hours might act as optimal exposure time. The reduction in time might be a relevant factor in both choice and no-choice tests since this would increase parasitoid selectivity by reducing the number of encounters with egg masses, thus preventing host acceptance as a consequence of multiple contacts (Haye et al., 2019). Under field conditions, a first rejection of a potential host species might turn out in the parasitoid searching for more suitable hosts, while this is not permitted in the constrained environments under laboratory studies (Botch and Delfosse 2018; Boyle et al. 2020).

In our experiments, we also aimed at comparing host parasitism by *T. mitsukurii* in black-box choice tests under 2 h exposure and the effect of rearing host, using *H. halys* vs. either one of two suitable non-target species, *D. baccarum* and *A. custos*. No significant differences in host parasitism by *T. mitsukurii* females were observed, and the host species used to rear *T. mitsukurii* females did not play a significant role in host choice. However, behavioral investigations focusing on the parasitoid first choice might be useful to reveal possible preferences that cannot be evaluated in 2-h black box tests. Unlike *T. mitsukurii*, *T. japonicus* reared on *H. halys* was shown to prefer *H. halys* in two-choice tests conducted in small arenas, and partially also in more complex systems using large cages and plants as ovipositing substrates (Hedstrom et al. 2017; Botch and Delfosse 2018; Haye et al. 2019). On the other side, preferences seemed to vanish when *T. japonicus* was reared on non-target hosts. Although choice tests in simplified experimental designs are fundamental for a first understanding of the likelihood of parasitization, more complex systems are required for a higher certainty of results.

The last experiment was conducted to evaluate the effect of host egg size on the size and egg load of emerged *T. mitsukurii*. The results indicated that host egg volume significantly affected the size of emerging adult females and the number of the eggs in their ovaries, i.e., larger host eggs produced larger females with higher egg loads, confirming previous findings by Arakawa and Namura (2004). Since many non-target host species are smaller in size than *H. halys*, it can be assumed that a population of *T. mitsukurii* originating from non-target species results in females with lower reproductive ability. This was already speculated for *T. japonicus* by Botch and Delfosse (2018) and observed successively in laboratory tests (Sabbatini-Peverieri et al. 2021).

Summarizing results of the present study, *T. mitsukurii* displayed in laboratory tests no specific host specialization, showing capability to successfully parasitize most of tested Pentatomoidea species, including *A. custos*, an Asopinae predator, as was previously assessed also for *T. japonicus* (Haye et al. 2019). However, while in our laboratory experiments, *T. mitsukurii* showed similar host preference toward more than one pentatomid species, *T. japonicus* appeared to prefer its coevolved host *H. halys* both in laboratory and field conditions (Milnes and Beers 2019; Botch and Delfosse 2018). It is well-known that testing conditions in the simplified laboratory environment can be misleading in non-target risk analysis; therefore, the results must be carefully evaluated (Haye et al. 2019). Thus, more complex systems are needed to better simulate field environmental factors, and experiments with *T. mitsukurii* should adopt multidisciplinary and integrated approaches. These were considered in the case of *T. japonicus*, and the large literature on laboratory host range tests evidenced, on a global scale, that the physiological host range of this parasitoid embraces more than two dozens of species among tested pentatomids (Zhang et al. 2017; Hedstrom et al. 2017; Botch and Delfosse 2018; Charles et al. 2019; Haye et al. 2019; Lara et al. 2019). Meanwhile, by means of olfactometer bioassays to test volatiles from hosts and host-induced plant volatiles, a higher level of species selection might emerge, as chemical ecology experiments allow to predict what cues, from which host species, the parasitoids are able to exploit in the field during host location (Bertoldi et al. 2019).

Considering the topical interest of classical biological control of *H. halys*, new field data on host preference by *T. mitsukurii* in Europe are expected to appear soon in the literature. Combined with the information we provide here and with further studies involving olfactory cues from host and host plants, and this will help understand the actual host range of *T. mitsukurii* in its novel area of establishment, allowing evaluation of the possible risks associated with its use as a biocontrol agent of *H. halys*.

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Author Contributions:

LG, GSP and LM conceived and designed research. LG, GSP, LM, GR, EC, PFR collected material in the field. GL and GSP conducted experiments. PFR involved in funding acquisition, resources and supervision. LG, GSP and GR involved in data analysis. LG and GSP involved in data curation and writing original draft. LG, GSP, GR and EC involved in writing final version. LG, GSP, LM, GR, EC and PFR involved in review and editing. All authors read and approved the manuscript.

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2.3 Olfactory responses of *Trissolcus mitsukurii* to plants attacked by target and non-target stink bugs suggest low risk for biological control

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Abstract

In crop systems, successful management of invasive insect herbivores can be achieved through the introduction of exotic biocontrol agents, parasitoids or predators, having a coevolutionary history with the pest. To avert threats to local biodiversity, recent legislations require a risk assessment for the organism to be released. Evaluation of its ability to exploit, for host location, odours associated with target and non-target species is crucial for a better definition of its ecological host range. Using Y-tube olfactometer bioassays in a quarantine laboratory, we investigated the ability of the Asian egg parasitoid *Trissolcus mitsukurii* (Hymenoptera: Scelionidae) to exploit odours associated with the global invader *Halyomorpha halys* (Hemiptera: Pentatomidae) and with non-target stink bugs native to Southern Europe. We demonstrated that *T. mitsukurii* is attracted by plants exposed to feeding and egg deposition of the coevolved *H. halys* and the native *Nezara viridula*, while it is not attracted by physogastric (gravid) females or eggs alone. Remarkably, *T. mitsukurii* is repelled by plants bearing eggs of the beneficial *Arma custos*. Our results contribute to a more thorough and nuanced assessment of the potential non-target risks in the case of mass-release of parasitoids as part of a biological control programme for invasive stink bugs.

Introduction

Management of invasive arthropod species in agricultural settings encompasses multiple strategies, including the introduction and release of exotic natural enemies (Kenis et al. 2017). The release of a biocontrol agent that is coevolved with the target herbivore allows the restoration of ecological

processes, with positive implications for pest suppression (Hoddle, 2004). On the other hand, biocontrol agents can have negative effects on the community of native natural enemies and non-target herbivores, with consequences for local biodiversity that can be hard to foresee (van Lenteren and Loomans, 2006). Therefore, in the process of selecting a candidate biological control agent, a careful selection process with detailed bioassays is crucial for maximizing the benefits of pest control and, at the same time, minimizing ecological risks (Loomans, 2021). Recent national and international regulations require that applications for licensing candidate biocontrol agents are supported by thorough risk assessment documentation (Mason et al. 2017). Simple evaluation of the physiological host range provides a first understanding of whether a natural enemy will be suitable for use as a biocontrol agent of a given target pest (Sabbatini-Peverieri et al. 2021, Abram et al. 2021). Such protocols typically encompass no-choice and choice laboratory bioassays in small settings, i.e., Petri dish arenas, or larger insect cage tests (Haye et al. 2020). However, effective interactions of the introduced natural enemy in the field and the general impact on local insect communities depend on many ecological factors. Among these, the ability of the natural enemy to exploit odours from plants attacked by insects (synomones) and/or host instars (kairomones) at a long-range distance (Hilker and Meiners, 2008). In the hierarchical process of host/prey location, these odours can help the natural enemy to efficiently locate the host (Meiners and Peri, 2013; Conti and Colazza, 2012). But, when dealing with exotic species, a lack of coevolutionary history between members of the plant-host-parasitoid tritrophic system is expected, and may imply that host-associated odours are not reliable cues for parasitoids (Desurmont et al. 2014; Martorana et al. 2017). Therefore, incorporating evaluation of odour attractiveness in pre-release risk assessment can be crucial for determining the likelihood of species interactions in open field conditions (van Driesche and Murray, 2004; Conti et al. 2004). Y-tube olfactometer bioassays have been demonstrated to be very well suited for evaluating parasitoid response to odours (Conti et al. 2004; Ferracini et al. 2016; Avila et al. 2016; Wyckhuys and Heimpel, 2007; Gohole et al. 2003). Here, we explored this concept on an exotic egg parasitoid as a candidate biocontrol agent of an invasive stink bug and conducted olfactometer bioassays to investigate behavioural responses towards odours associated with target and non-target species.

The brown marmorated stink bug, *Halyomorpha halys* Stål (Hemiptera: Pentatomidae), is an economically important invasive pest of major concern worldwide (Leskey and Nielsen, 2018). Its aptitude to aggregate and hide in small spaces, e.g., shipping containers, facilitates its worldwide spread (Nixon et al. 2019). Native to East Asia, this species was accidentally introduced in the US and first detected in 1996 (Hoebeke and Carter, 2003). The feeding on many plant species, including a large number of crops and fruit plants, caused about US \$37 million losses to the apple crop in the US in 2010 (Leskey and Nielsen, 2018). In Europe, *H. halys* was first observed in 2004 (Switzerland) and since then discoveries have multiplied in European countries, including Italy (Haye et al. 2014;

Maistrello et al. 2018). After its establishment in Italy, *H. halys* has damaged field crops and orchards, causing serious economic damage with fruit losses that were estimated at nearly EUR 600 millions in 2019 (Bariselli et al. 2016; Rot et al. 2021). Current management strategies against *H. halys* include chemical, biotechnological and physical control, while classical and augmentative biological control are promising methods under investigation (Conti et al. 2021; Zapponi et al. 2021). The most effective natural enemy of *H. halys* appears to be the Asian egg parasitoid *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae), which exhibits high field parasitism rates in its area of origin (Zhang et al. 2017; Yang et al. 2009). Adventive populations of *T. japonicus* have been documented in North America and Europe, where this species co-occurs with *H. halys* in some of the areas invaded by the stink bug (Wyckhuys and Heimpel, 2007; Zapponi et al. 2021; Abram et al. 2019; Kaser et al. 2018; Moraglio et al. 2020). Additionally, adventive populations of another Asian egg parasitoid species, *Trissolcus mitsukurii* (Ashmead) (Hymenoptera: Scelionidae), have been recently detected in Northern Italy on *H. halys* egg masses (Moraglio et al. 2020; Sabbatini-Peverieri et al. 2018; Scaccini et al. 2020). Frequently described as a native parasitoid of *Nezara viridula* L. (Hemiptera: Pentatomidae) in Japan (Hokyo and Kiritani, 1963; Arakawa et al. 2004), *T. mitsukurii* also parasitizes *H. halys* and other stink bug species in China and Japan (Arakawa and Namura, 2002; Chen et al. 2020; Ryu et al. 1984). In Italy, adventive populations of *T. mitsukurii* have shown high parasitism efficacy on *H. halys*, comparable to that of *T. japonicus* (Scaccini et al. 2020). Considering this, *T. mitsukurii* represents a promising egg parasitoid of *H. halys* in Italy and neighbouring territories (Rot et al. 2021; Bout et al. 2021), and is currently under evaluation for the development of a pre-emptive biological control program of the stink bug in Australia and New Zealand (Caron et al. 2021). A detailed screening of *T. mitsukurii* host range using choice and no-choice experiments in Petri dishes was recently conducted and revealed oligophagy of this parasitoid limited to members of the Pentatomoidea superfamily (Giovannini et al. 2021). However, the capability of this parasitoid to exploit odours from the plant-host system for locating stink bug eggs in the field is unknown. Results from previous studies revealed that *Trissolcus* spp. are primarily attracted by oviposition-induced plant synomones (Bertoldi et al. 2011; Colazza et al. 2004; Tognon et al. 2016; Borges et al. 2017; Conti et al. 2010). Here we hypothesized that the oligophagous behaviour shown by *T. mitsukurii* during assessment of its physiological host range (Giovannini et al. 2021) will be shown to be narrower with the use of chemical ecology bioassays. We hypothesize that only odours associated with those host species that exhibit coevolutionary history with *T. mitsukurii* would elicit behavioural responses of the parasitoid.

Therefore, we conducted olfactometer bioassays and tested whether the parasitoid responds differently to odours from plants exposed to oviposition of *H. halys* or non-target native stink bugs, specifically *Arma custos* F., *Dolycoris baccarum* L., *Eurydema ventralis* Kolenati (Hemiptera:

Pentatomidae) and *N. viridula*. Odours from physogastric (gravid) females and egg masses alone were also tested. Of the different species, *H. halys* and *A. custos* naturally occur in *T. mitsukurii*'s native area, hence it is likely that a certain level of coevolution has occurred (De Clercq, 2000; Hamilton et al. 2018). The other stink bugs evaluated, *D. baccarum*, *E. ventralis* and *N. viridula*, are of West Palearctic or Mediterranean origins. They occur in part of Asia, where their presence should be considered outside their native range (Panizzi et al. 2000; Rider, 2006). Results of this investigation would help to understand the possible ecological impact of the exotic parasitoid *T. mitsukurii* in novel ecosystems. Additionally, they will provide foundational data for the preparation of a risk assessment document, in support of a petition for releasing the parasitoid in areas that are seriously infested by *H. halys*.

Material and methods

Origin of insects and rearing.

Stink bug colonies were established from adults collected in spring and summer 2020 in Northern and Central Italy from fruit orchards, herbaceous crops and uncultivated areas. Adult stink bugs were collected by sweep netting or visual handpicking on grasses, bushes and trees. Field collected adults were transferred to the laboratory and reared in insect cages (BugDorm 4F4545, Insect MegaView Science Co. Ltd., Taichung, Taiwan) under environmentally controlled conditions (25 ± 1 °C, $60 \pm 5\%$ RH and 16:8 h L:D). Phytophagous stink bugs were maintained with a mixed diet based on fruits, vegetables and seeds, whereas the predatory *A. custos* was fed with *Tenebrio molitor* L. pupae (Coleoptera: Tenebrionidae). Food was replaced three times per week. A daily wetted cotton wool placed inside an opened Petri dish (9 cm diam.) was used for water provision. About 10 paper towels (20 cm × 20 cm) were added inside each rearing cage to provide an oviposition substrate.

Trissolcus mitsukurii populations were initiated from *H. halys* parasitized egg masses originally collected in fruit orchards in north-eastern Italy. The parasitoid colony was replenished yearly with new field-collected specimens (Sabbatini-Peverieri et al. 2018). For breeding maintenance, 1-d-old *H. halys* egg masses were exposed to a parasitoid female for 24 h. The wasps were held in glass tubes (2 cm diam. × 15 cm length) sealed on both sides with a plastic mesh. A diet of honey droplets was dispensed on a rectangular cardboard (2 cm large × 4 cm length), offered to parasitoids and replenished two times per week. Males and females were kept together to permit mating and 7-day-old females were isolated in glass tubes before bioassays. Maintenance of the egg parasitoid colony (at 25 ± 1 °C, $60 \pm 5\%$ and 16:8 h L:D) and all olfactometer bioassays (see below) were conducted under officially authorized quarantine conditions at CREA facilities (DG/DISR/DISR05/0013647-19/04/2018).

Plant rearing and exposure for stink bug oviposition.

Seeds of soybean, *Glycine max* (L.) Merrill, and cauliflower, *Brassica oleracea* var. *botrytis* L., were sown in plastic pots (6.5 cm height × 5.5 cm diam. at mid-height) containing a horticultural substrate (commercial name Radicom, Vigorplant Italia S.r.l., Fombio, Italy). Plants were grown and maintained in a rearing room (24 ± 2 °C, $55 \pm 10\%$ RH, 16: 8 h L:D) and irrigated every 2 d. A mixture (1.4 g/L) of fertilizer (5-15-45, NPK, Plantfol, Valagro, Italia) was added one week after plant emergence. Two plants, 2- to 4-weeks old (approx. 15–20 cm tall) depending on plant species, were placed inside an insect cage containing mated females of each colony of the stink bug species tested. Soybean plants were used for *A. custos*, *D. baccarum*, *H. halys*, and *N. viridula*, while cauliflower plants were used for *E. ventralis*, as this species has preference for crucifers (Bonnemaison, 1965). Plants were checked twice per day for the presence of egg masses laid on the abaxial leaf surface. Whenever eggs were detected, plants were removed from the cages and tested for their attractiveness within 24 h. If no eggs were found after 3 d of exposure, plants were replaced with new clean plants.

Odour sources tested in the bioassays.

Using a Y-tube olfactometer we tested the behavioural responses of *T. mitsukurii* to plant-host odours associated with native and non-native stink bugs. For each of the five stink bug species, i.e., *A. custos*, *D. baccarum*, *E. ventralis*, *H. halys*, and *N. viridula*, the following treatments were evaluated:

- (a) *Plant + Eggs*: a plant exposed to stink bug feeding and bearing 1 naturally laid egg mass (< 24 h old)
- (b) *Females*: a batch of four females in the ovipositional phase, i.e., with physogastric abdomen (79)
- (c) *Eggs*: about 100 eggs < 24 h old. Only egg masses laid on unbleached paper towels were tested.

Controls consisted of clean air (AIR) for treatments with females and eggs, or of an insect-unexposed soybean or cauliflower plant (CLEAN PLANT) for treatments with plants.

Olfactometer bioassays.

The female behavioural responses to the different odour stimuli were investigated in a Y-tube olfactometer (common stem: 90 mm length; arms: 80 mm length each at 100° angle between arms and equally distant from the common stem, internal section: 15 mm × 10 mm), carved in a plexiglass plate (200 mm × 190 mm × 10 mm thick) and sandwiched between two glass sheaths (each plate: 200 mm × 150 mm × 5 mm thick), which provided the upper and lower closure of the olfactometer (Rondoni et al. 2017). Two identical Y-tube olfactometers were simultaneously used, allowing to

observe the behaviour of two parasitoids at the same time. A stream of environmental air was inflated by a diaphragm pump (KNF Italia S.r.l., Milan, Italy) through an activated charcoal filter (260 mm length $\times$ 40 mm internal diam.). The airflow was humidified through a Dreschel bottle (250 mL volume) containing distilled water and split into two identical routes, each consisting of a flowmeter that regulated the airflow at 0.8 L/min and a glass chamber (30 cm height $\times$ 10 cm internal diam., $\sim$ 1.9 L volume) containing the odour source (treatment or control cue). The glass chamber was sealed at the base to a Teflon disk (7 mm height, 14 cm diam.) using Parafilm M® sealing film (Heathrow Scientific, Vernon Hills, IL, USA). The airflow was conveyed using tubes in Silicone (6 mm internal diam.). Tubes were connected to the chamber by means of plastic opened screw caps (Kartell Spa, Noviglio, Italy). The airflow coming out from the glass chamber was eventually split again into two and conveyed to one of the two arms of both olfactometers. A digital flowmeter (mod. GFM17, Aalborg, New York, USA) was used to measure the flow rate entering each olfactometer arm ($\sim$ 200 mL/min). The olfactometer device was surrounded by a black fabric curtain to minimize external cues from the room and was illuminated by two 36 W cool white fluorescent tubes located above the device.

About 30 min before the bioassays, parasitoids, stink bugs and plants were moved to the bioassay room, maintained at 25 °C, to acclimatize. Bioassays were conducted from 09:00 to 16:00. In detail, a parasitoid female was introduced into the central stem of the Y-tube and the behaviour was recorded. After 4 bioassays the position of the tubes entering the Y-tube arms was switched to avoid possible bias. Additionally, the glass plates were cleaned with a laboratory detergent (2% solution of Cleanilab LM1; Kartell Spa, Noviglio, Italy), rinsed with tap water and acetone, whereas the plexiglass part of the olfactometer was cleaned with detergent, rinsed with tap water, and finally rinsed with distilled water. Each *T. mitsukurii* female was observed for 10 min. Parasitoid females were evaluated once. The time spent in each olfactometer arm and in the common stem was visually recorded with JWatcher 1.086,87. For each treatment, depending on insect and egg mass availability, 3–4 replicates of the same odour stimulus (plant bearing an egg mass, stink bug females, or eggs) were tested. For the different stink-bug species, 56 to 77 parasitoids were eventually evaluated for each treatment with a plant bearing an egg mass, 44 to 70 parasitoids for stink bug female tests, and 32 to 69 insects for the stink bug eggs tests.

Statistical analyses.

The walking behaviour of the parasitoid in the olfactometer was described by the residence time, i.e., the time spent by the female in each olfactometer arm, and by the first choice, i.e., the olfactometer arm the parasitoid entered first. Females that did not make a choice or only entered either olfactometer arm for a limited time (< 30 s) were considered not responding and were discarded from the analysis

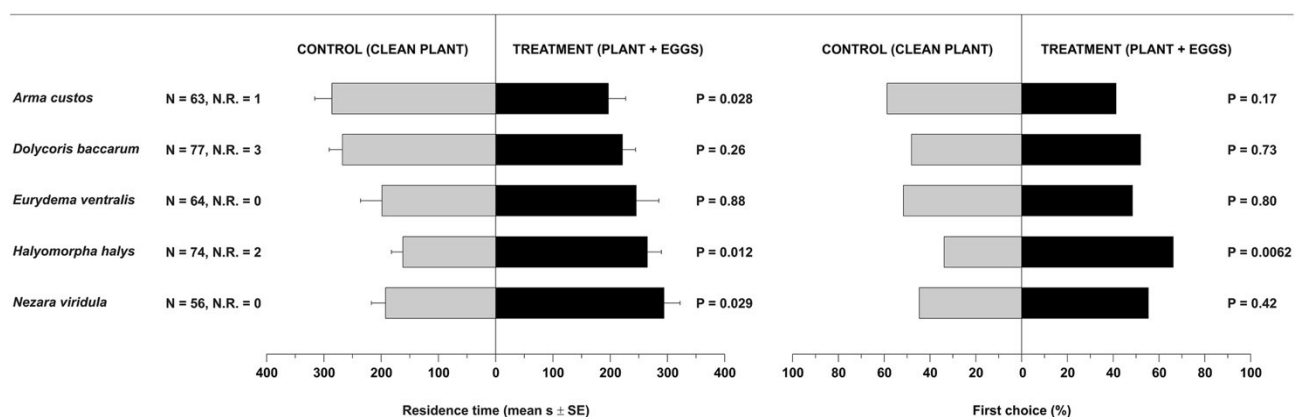
(similar to Peri et al 2011). For the analysis, the logarithmic transformation of the ratio between the residence time in the treatment arm versus the residence time in the control arm was calculated. This transformation (log-ratio) ensured that only one measure per insect was later analysed (Rondoni et al. 2017) . Generalized linear models (GLMs) with Gaussian error distribution (for residence time data) or with binomial error distribution (for first choice data) were fitted to test differences of treatment versus control within each odour source. Analyses were conducted in the R statistical environment, version 4.0.2 (R Core Team, 2020).

Results

Behavioural responses to odours from plants bearing an egg mass.

Trissolcus mitsukurii females responded positively to odours associated with the target host (soybean plant with an egg mass of *H. halys*) as their residence time in the treatment arm was higher compared to that in the control arm (contrast result for Gaussian GLM: $P = 0.012$) (Fig. 1, Table S1). Similarly, *T. mitsukurii* was attracted (higher residence time compared to control) to odours associated with *N. viridula* (soybean plant with an egg mass) ($P = 0.029$).

Figure 1. Residence time (means $\pm$ SE) and first choice (%) of *Trissolcus mitsukurii* females in Y-tube olfactometer exposed to odours from stink bug–plant systems. Treatments consisted of volatiles from soybean plants bearing an egg mass of *Arma custos*, *Dolycoris baccarum*, *Halyomorpha halys*, or *Nezara viridula*, or volatiles from cauliflower plants bearing an egg mass of *Eurydema ventralis*. Control consisted of a clean soybean or cauliflower plant. N number of responding insects. N.R. number of not-responding insects (discarded from the analysis). Planned comparisons were tested within GLM with Gaussian error distribution (residence time) or with binomial error distribution (first choice).



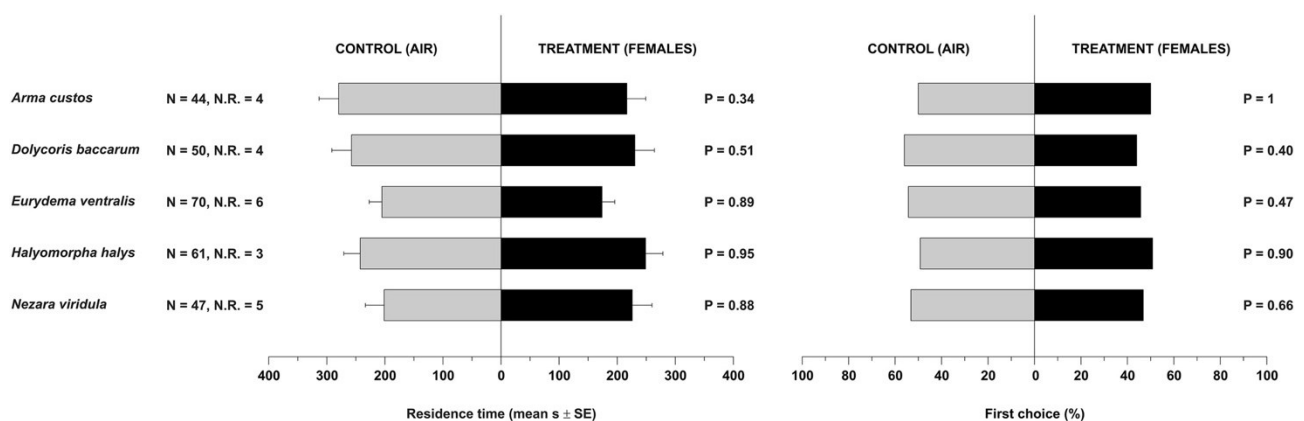
Notably, parasitoids appeared to avoid odours associated to *A. custos* (soybean with an egg mass), displaying a lower residence time in treatment vs. control arm ($P = 0.028$). In contrast, *T. mitsukurii* did not respond to odours associated with *D. baccarum* (soybean plant with an egg mass) or *E. ventralis* (cabbage plant with an egg mass), as residence time was similar between control and treatments ($P \geq 0.26$ for both comparisons). First choice data confirmed the preference of female egg parasitoids for plants carrying *H. halys* eggs (contrast results for binomial GLM: $P = 0.0062$) (Fig. 1,

Table S2). Conversely, first choices were similar between control and treatments for all the other tested species ($P \geq 0.17$).

Behavioural responses to odours from stink bug females.

Trissolcus mitsukurii females did not prefer *H. halys* ($P = 0.95$) nor those of the native species *A. custos*, *D. baccarum*, *E. ventralis* and *N. viridula* females as residence time was similar in the treatment and control arms ($P \geq 0.34$ for all the comparisons) (Fig. 2, Table S3). Correspondingly, first choice data did not differ between control and treatments ($P \geq 0.40$ for all comparisons) (Fig. 2, Table S4).

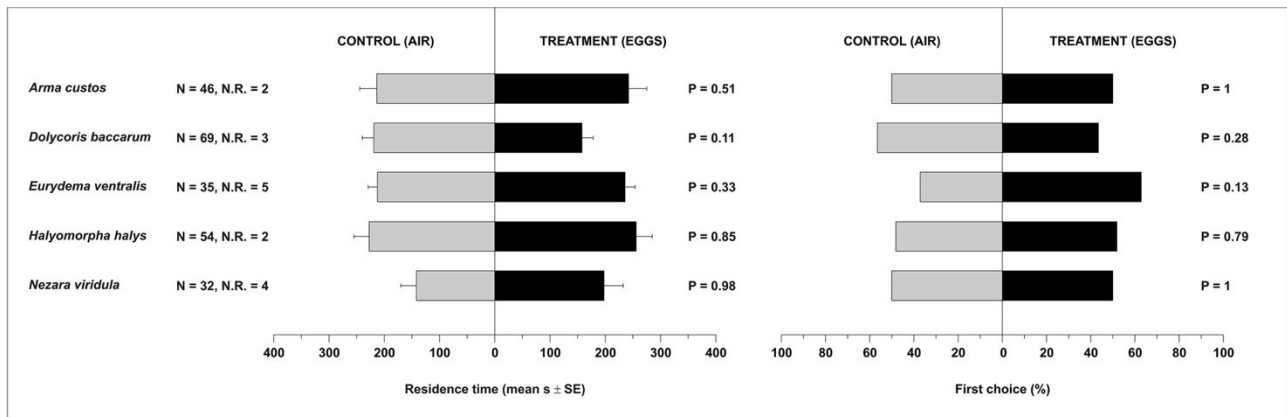
Figure 2. Residence time (means $\pm$ SE) and first choice (%) of *Trissolcus mitsukurii* females in Y-tube olfactometer exposed to odours from stink bug females. Treatments consisted of volatiles from females of *Arma custos*, *Dolycoris baccarum*, *Eurydema ventralis*, *Halyomorpha halys*, or *Nezara viridula*. Control consisted of clean air. N number of responding insects. N.R. number of not-responding insects (discarded from the analysis). Planned comparisons were tested within GLM with Gaussian error distribution (residence time) or with binomial error distribution (first choice).



Behavioural responses to odours from stink bug eggs.

Females of *T. mitsukurii* did not exhibit any attraction towards *H. halys* ($P = 0.85$), or any of the native stink bugs, as residence times in treatment and control did not differ ($P \geq 0.11$ for all the comparisons) (Fig. 3, Table S5). First choice analysis confirmed the absence of a significant attractivity of the eggs of all stink bug species tested ($P \geq 0.13$ for all comparisons) (Fig. 3, Table S6).

Figure 3. Residence time (means $\pm$ SE) and first choice (%) of *Trissolcus mitsukurii* females in Y-tube olfactometer exposed to odours from stink bug eggs. Treatments consisted of volatiles from eggs of *Arma custos*, *Dolycoris baccarum*, *Eurydema ventralis*, *Halyomorpha halys*, or *Nezara viridula*. Control consisted of clean air. N number of responding insects. N.R. number of not-responding insects (discarded from the analysis). Planned comparisons were tested within GLM with Gaussian error distribution (residence time) or with binomial error distribution (first choice).



Discussion

Among the different stink bug-associated odours tested in the olfactometer, only those from plants bearing an egg mass of *H. halys* or *N. viridula* elicited positive attraction in *T. mitsukurii* females. These results validated our hypothesis that a more in-depth evaluation of the parasitoid host specificity, through chemical ecology investigation, would further restrict the parasitoid host range that was depicted by physiological host range assays. Indeed, several non-coevolved hosts, although accepted under laboratory simplified conditions (i.e., no-choice and paired choice black-box tests), would be hardly located in the field due to a lack of suitable host-associated odours. This interpretation conceptually agrees with results from prior studies on *T. japonicus*, another *H. halys* exotic biocontrol agent that is currently being released in Italy (Zapponi et al. 2021). In fact, *T. japonicus* displayed a relatively wide physiological host range in laboratory choice and no-choice assays (Sabbatini-Peverieri et al. 2021; Haye et al. 2020; Zhang et al. 2017), but a more restricted oligophagy in olfactometer or in field conditions (van Driesche et al. 2004; Milnes and Beers, 2019). Our results also indicate that soybean plants exposed to *H. halys* feeding and egg deposition can emit volatile molecules that are detected by the coevolved parasitoid *T. mitsukurii*. This response is consistent among residence time and first choice data. Volatile emission induced by host oviposition represents an indirect defence for the plants, as already demonstrated in different systems involving *Trissolcus* egg parasitoids (Bertoldi et al. 2019; Colazza et al. 2004; Conti et al. 2010), and is an exceptionally reliable signal of the presence of target hosts in the canopy (Hilker and Meiners, 2008; Conti and Colazza, 2012). At the plant physiological level, induced defences have been demonstrated to involve JA- and/or SA-defensive pathways, depending on stink bug species, type and timing of attack (Peiffer and Felton, 2014; Rondoni et al. 2018; Giacometti et al. 2016; Timbó et al. 2014).

Induced plant volatiles act on long-distance range and are easily detected by the egg parasitoids, allowing them to rapidly locate the host that is suitable only for a short period (Colazza et al. 2004; Vet and Dicke, 1992). The ability of *T. mitsukurii* to exploit induced plant volatiles for locating *H. halys*, although it is investigated here for the first time, was recently hypothesized following results of open field surveys in northeast Italy and France (Bout et al. 2021; Zapponi et al. 2020). For instance, higher parasitization of *H. halys* by *T. mitsukurii* was detected in naturally-laid eggs compared to sentinel eggs (Zapponi et al. 2020). In two other studies, *T. mitsukurii* exhibited remarkable discovery efficiency of *H. halys* eggs laid on plant tissues, with observed parasitisation on egg masses that ranged from 27.3 to 46.5% (Scaccini et al. 2020; Bout et al. 2021). Noteworthy is also the positive attraction of *T. mitsukurii* towards plants bearing eggs of the non-coevolved *N. viridula*. Our result, together with the fact that *T. mitsukurii* positively responds to tracks of *N. viridula* females (Scala et al. 2021), would suggest high ability to locate eggs. Indeed, the parasitoid is considered a main enemy of *N. viridula* in Japanese areas where the stink bug has been established (Arakawa et al. 2004). Surprisingly, despite this remarked discovery ability, *T. mitsukurii* rarely emerged (successful development and emergence from host eggs) from laboratory-reared or field-collected eggs of *N. viridula* in the case of adventive Italian population(s) (Scaccini et al. 2020; Giovannini et al. 2021). Conversely, emergence of *T. mitsukurii* strains from *N. viridula* eggs in paddy fields or early-planting rice in Japan was variable (e.g., 12 to 51% of emerged offspring calculated on the total number of eggs) (Kiritani and Hôkyo, 1962; Hôkyo, 1966). The mismatch between the positive response of *T. mitsukurii* to cues associated with *N. viridula* and the inconstant suitability of this host for parasitoid development is partially consistent with lack of coevolution, as *N. viridula* is of Ethiopian-South Mediterranean origin (Esquivel et al. 2018). However, this does not explain why the parasitoid responds to cues associated with the novel host. A hypothetical explanation for this could be that *N. viridula* induces in soybean a defensive response that might be similar to that induced by other herbivores, like *Nezara antennata* Scott, native to Eastern Asia (Panizzi et al. 2000) and listed as host of *T. mitsukurii* (Ryu and Hirashima, 1984). It is known that *N. viridula* and *N. antennata* share common volatile compounds (Borges and Blassioli-Moraes, 2017), however, whether they also induce similar plant responses is unknown.

Previous choice and no-choice bioassays underlined high acceptance and suitability of *D. baccarum* for *T. mitsukurii* (Giovannini et al. 2021). Surprisingly, our data did not reveal any behavioural response of this parasitoid to odours from soybean plants bearing egg masses of *D. baccarum*. *Dolycoris baccarum* has a wide distribution throughout the Palearctic region (Panizzi et al. 2000), including Asia where it is a pest of several crops including soybean (Kobayashi, 1981; Nakamura and Numata, 2006). Although *D. baccarum* is listed in the host range of *T. mitsukurii* (Ryu and Hirashima, 1984), there are no quantitative data related to the prevalence rate of this parasitoid. On the other

hand, naturally-laid and sentinel egg masses of *D. baccarum* are highly parasitized in Chinese orchards by other parasitoids, i.e., *T. japonicus* (Zhang et al. 2017). Similarly, in Korea and Japan, *Trissolcus nigripedius* Nakagawa and *Telenomus gifuensis* Ashmead (both Hymenoptera: Scelionidae) are commonly found parasitizing *D. baccarum* eggs laid in crop fields, including soybean (Mahmoud and Lim, 2008), sometimes demonstrating high prevalence (Lim et al. 2007). Therefore, while some parasitoid species seem to effectively track host eggs of *D. baccarum* in the field, the same cannot be demonstrated for *T. mitsukurii*, suggesting that parasitization by this species might be occasional.

In no-choice black box experiments, *E. ventralis* eggs represented a poorly suitable host for *T. mitsukurii*, as parasitoids failed to develop inside its eggs (Giovannini et al. 2021). The lack of response towards odours from plants bearing an egg mass of *E. ventralis* in our experiments is consistent with the very low suitability of this species (Giovannini et al. 2021). The stink bug is of West Palearctic origin and is only marginally present in the native area of *T. mitsukurii* (Rider, 2006). Therefore, the risk that under field conditions *T. mitsukurii* would parasitize *E. ventralis* appears quite low.

One of the most desired aspects of risk assessment is that the candidate biocontrol agent has no or limited negative effect on beneficials. The fact that plants with *A. custos* eggs were less preferred by *T. mitsukurii* females compared to the control can be interpreted as a form of repellence towards the treatment odour (Gohole et al. 2003; Wäckers, 2004) and this may reduce the risk of non-target parasitisation. We may expect that in case of intentional release of (*T. mitsukurii*), the existence of such an ecological barrier would limit encounters with *A. custos* eggs in the field. The response of *T. mitsukurii* to *A. custos* appears at least partly similar to that of *T. japonicus*, which in no-choice tests successfully parasitized this stink bug predator, but in large cage tests, using plant bearing egg masses, preferred *H. halys* over *A. custos* (Hilker and Meiners, 2008; Giovannini et al. 2021). Similarly to herbivorous species, zoophytophagous Heteroptera, including *A. custos*, can feed on plant tissues to acquire water and nutrients (Gillespie and McGregor, 2000). It was demonstrated that some of these species can induce the activation of defensive signalling pathways in plants, with consequent release of volatile organic compounds, which can inform natural enemies of the ongoing attack (Bouagga et al. 2018; Martorana et al. 2019). Eventually, behavioural responses of natural enemies to such odour sources can vary in different systems. For instance, oviposition by the zoophytophagous *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae) induces the emission of plant volatiles which attract its coevolved parasitoid *Telenomus podisi* (Ashmead) (Hymenoptera: Scelionidae) (Martorana et al. 2019). In another trophic system, oviposition by the predatory *P. maculiventris* on tomato plants did not attract *T. japonicus*, although the parasitoid is able to successfully parasitize *P. maculiventris* eggs and develop inside the host (Bertoldi et al. 2019; Lara et al. 2019). The lack of a coevolutionary

history between the two species may explain the inconsistent behaviour of *T. japonicus* (Bertoldi et al. 2019). *Arma custos* (junior synonym *Arma chinensis* Fallou (Zhao et al. 2018)) is historically present in *T. mitsukurii*'s area of origin (Zou et al. 2012), hence we can speculate that the observed avoidance can be interpreted as a coevolutionary adaptation within the tritrophic system that might prevent the parasitoid to exploit the predator. Although *A. custos* eggs were highly suitable for *T. mitsukurii* in no-choice black box tests (Giovannini et al. 2021), development of the parasitoid larva is highly risky due to possible predatory (cannibalistic) behaviour of (*A. custos*) nymphs and adults, and considering that parasitized eggs tend to remain for a longer time compared to viable eggs (Zou et al. 2012; Zou et al. 2013; Wu et al. 2021). Hence, in the first step of the hierarchical process of habitat assessment, the parasitoid could use plant odours to avoid such a risky host. *Halyomorpha halys* viable eggs could be also exposed to cannibalism, but the stink-bug appeared to have developed a strategy to synchronize egg hatching in order to prevent sibling cannibalism (Endo and Numata, 2020).

The exploitation of adult-related chemical odours is quite common in egg parasitoids (Conti e Colazza, 2012; Afsheen et al. 2008; Rondoni et al. 2017), however in the present study we noticed a lack of *T. mitsukurii* response to females of *H. halys*. Although it is known that volatile and non-volatile cues from physogastric females may represent reliable information for some Scelionid wasps, kairomones from stink bug females elicit parasitoid responses mainly at short distance (Borges and Blassioli-Moraes, 2017; Bertoldi et al. 2021). For instance, a previous investigation demonstrated that *T. japonicus* responds to *H. halys* females only in a “short-distance” olfactometer and not in a “long-distance” olfactometer (as the one we have used here) (Bertoldi et al. 2019). In open arenas, *Trissolcus brochymenae* (Ashmead) (Hymenoptera: Scelionidae) responded to *Murgantia histrionica* (Hahn) (Hemiptera: Pentatomidae) physogastric females but not to males (Conti et al. 2008). Concerning *T. mitsukurii*, female extracts of *H. halys* seem to elicit a behavioural response of the parasitoid in Petri dish arenas, thus in a short-distance environment (Scala et al. 2021).

We did not detect any attraction of *T. mitsukurii* towards odours from eggs of the tested stink bugs. Kairomones from host eggs are typically present in small amounts, hence their role in host location is mainly expected at short distance (reviewed by Conti and Colazza, 2014; Fatouros, 2008; Vinson, 1998). In olfactometers, even though few species of egg parasitoids (e.g., *T. podisi*) were demonstrated to respond to odours directly emitted by eggs (Michereff et al. 2016), so far, investigated *Trissolcus* species did not respond (Bertoldi et al. 2019) except when a high number of eggs was placed very close to the air hole at the end of the olfactometer arm (Conti et al. 2003). Hence, we can hypothesize that such poorly detectable odours would only permit host location in close proximity, as shown for *T. brochymenae* using short-range bioassays in open arenas (Conti et al. 2003).

In conclusion, while previous host-acceptance investigations revealed a broad host range for *T. mitsukurii* in Europe (Giovannini et al. 2021), present results suggest that host location at long distance would likely favour parasitization of *H. halys* (or *N. viridula*), rather than the other stink bugs tested here, in particular the beneficial *A. custos*. Possibly, the presence of such an ecological filter would have positive implications for preventing undesired impacts on non-targets in case of intentional release of the parasitoid in biological control programs. On the other hand, there are no physiological impediments for *T. mitsukurii* to develop in some non-targets (e.g., *A. custos*), hence when such species co-occur in the field with *H. halys*, they would likely be more exposed to parasitization due to occasional encounters during foraging. Hence, the bioassays conducted here with *T. mitsukurii* following the host range studies (Giovannini et al. 2021), highlight the need of a multidisciplinary approach in pre-release risk assessment, where results from each step are part of the puzzle that will allow a reliable field scenario prediction. Dedicated field surveys of native and exotic stink bug eggs in those areas where *T. mitsukurii* have fortuitously established would likely help in estimating the relevance of such host-parasitoid interactions and provide better support for the definition of a risk assessment document, necessary for licensing parasitoid releases.

Author Contributions:

G.R., G.S.-P. and E.Co. conceived and designed research. G.R., E.Ch., L.G. and G.S.-P. conducted experiments. P.F.R. and E.Co. acquired funding, resources and supervised. G.R., L.G., and G.S.-P. analysed data. G.R., E.Ch. and E.Co. wrote original draft G.R., E.Ch., L.G., G.S.-P. and E.Co. wrote final version. All authors reviewed, edited the manuscript, read and approved the manuscript.

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Chapter 3: Behavioral responses of *Trissolcus japonicus* and *Trissolcus mitsukurii* under different competition scenarios

3.1 Interspecific competition between *Trissolcus japonicus* and *Trissolcus mitsukurii*, two promising candidates for biocontrol of *Halyomorpha halys*

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Abstract

The invasive stink bug *Halyomorpha halys*, native to east Asia, is a severe agricultural pest of worldwide importance and biocontrol is a promising method for its long-term management. The two Asian egg parasitoids *Trissolcus japonicus* and *Trissolcus mitsukurii* are considered to be effective candidates as biological control agents. These species can co-occur in the same habitats and interspecific competition for hosts could therefore have an important role in natural control. We assessed the foraging behavior and the progeny production of both species under laboratory conditions with three different interspecific competition scenarios. With indirect competition, *H. halys* egg masses previously parasitized by the female of one species were offered to the competitor females after 0, 3, 5, or 7 days. With direct extrinsic competition, females of each species were tested in arenas either when introduced simultaneously (synchronous release) or when half of the host egg mass had already been parasitized by the other species (asynchronous release). Both *Trissolcus* species were able to parasitize host eggs already parasitized by the other species, although the progeny production always favored the species that arrived first on the host egg mass. However, in the synchronous releases scenario, *T. mitsukurii* displayed more aggressive behavior and spent more time defending the host egg mass than *T. japonicus*, resulting in a higher progeny production. Our results showed that multiparasitism among *T. japonicus* and *T. mitsukurii*, at least under laboratory conditions, resulted in a general waste of searching time, eggs laid, and a lower reproductive potential by the second-arriving species on the host egg mass.

Keywords: brown marmorated stink bug, egg parasitoids, host discrimination, parasitization behavior, extrinsic competition, intrinsic competition

Introduction

The invasive Asian stink bug *Halyomorpha halys* Stål (Hemiptera, Pentatomidae) is a severe agricultural pest introduced accidentally in recent decades into several countries of the Americas, in Europe and in the Caucasus region (Leskey and Nielsen, 2018). Biological control is seen as one of the most effective long-term management approaches (Roversi et al., 2016; Abram et al., 2017). The solitary Asian egg parasitoid *Trissolcus japonicus* (Ashmead) (Hymenoptera, Scelionidae) is the main antagonist in the area of origin and is considered the most promising candidate for classical biological control in invaded regions (Zhang et al., 2017). This parasitoid has been extensively investigated as a biocontrol agent in the USA, Europe and New Zealand, and is the biocontrol agent targeted for release in the field after petition approvals by local authorities (Hedstrom et al., 2017; Botch and Delfosse, 2018, Haye et al., 2019, Sabbatini Peverieri et al., 2021). In Europe, Italy was the first country to initiate a large-scale release program of laboratory-reared *T. japonicus* (MiPAAF, 2020). Additionally, adventive populations of *T. japonicus* have also been recently detected in Europe and in the USA (Talamas et al., 2015; Sabbatini Peverieri et al., 2018; Stahl et al., 2019; Dieckhof et al., 2021). Moreover, adventive populations of *Trissolcus mitsukurii* (Ashmead) (Hymenoptera, Scelionidae), a less-studied egg parasitoid of eastern Asia origin, was recently found attacking *H. halys* in Italy and in neighboring countries of Slovenia and France, highlighting its role in the parasitoid guild of *H. halys* and leading researchers to consider it as a second candidate for biocontrol in the pest's invaded regions (Sabbatini Peverieri et al., 2018; Benvenuto et al., 2020; Rot et al., 2021; Yonow et al., 2021; Bout et al., 2021). While *T. japonicus* has been the subject of an extensive body of scientific literature, especially concerning risk analysis of its introduction into new areas, such studies about *T. mitsukurii* are currently in an embryonic stage (Giovannini et al., 2021a; Rondoni et al., 2022). Little is known about its lifecycle and its impact on the host species (Arakawa and Namura, 2002; Arakawa et al., 2004; Sabbatini Peverieri et al., 2020; Scaccini et al., 2020).

Trissolcus japonicus and *T. mitsukurii* co-occur in part of the Asian range of *H. halys*, but while *T. japonicus* is reported widely throughout this range, *T. mitsukurii* has been reported in the scientific literature mostly from Japan (Zhang et al., 2017; Yonow et al., 2021; Arakawa and Namura, 2002; Arakawa et al., 2004; Hokyo and Kiritani, 1963; Hokyo and Kiritani, 1963, 1966; Kiritani, 1964; Hokyo et al., 1966; Ryu and Hirashima, 1984; Yang et al., 2009; Nishimoto et al., 2015). Recent discoveries during an extensive monitoring program of both species in the same areas in Northern Italy, indicated that these species are sympatric there as well (Zapponi et al., 2021). Moreover, it is

expected that the adventive populations of *T. japonicus* and *T. mitsukurii* reported in this area will naturally spread into neighboring countries and/or new introductions will occur elsewhere (Rot et al., 2021; Bout et al., 2021; Dieckhoff et al., 2021).

Previous studies on interspecific competition between *T. japonicus* and native European and Asian egg parasitoids [*Anastatus bifasciatus* (Geoffroy), *Anastatus japonicus* Ashmead (Hymenoptera, Eupelmidae), and *Trissolcus cultratus* (Mayr) (Hymenoptera, Scelionidae)] found strong aggressive behavior by *T. japonicus* females, but also by *T. mitsukurii* against the latter, as recently reported by Costi et al. 2022. However, successful competition is also affected by the time of arrival to the host eggs (Konopka et al., 2017a; Haye et al. 2021; Mi et al., 2021). Similarly, studies on interspecific competition involving *T. mitsukurii* within the parasitoid guild of *Nezara viridula* L. (Hemiptera, Pentatomidae) [*Trissolcus basalis* (Wollaston) and *Telenomus nakagawai* Watanabe (Hymenoptera, Scelionidae)] documented its aggressive behavior in host patch defense (Hokyo and Kiritani, 1966; Hokyo et al., 1966; Field and Calbert, 1999; Nishimoto et al., 2015).

Interspecific competition in egg parasitoids can be “direct” or “indirect”. Direct (extrinsic) interspecific competition occurs when females of different species simultaneously attack the same host egg mass, or their juvenile stages are both present in the host egg (direct intrinsic competition) (Boivin and Brodeur, 2006; Cusumano et al., 2012). Indirect competition occurs when a female encounters a host egg mass already parasitized by a different species and reacts by adapting its foraging behavior (e.g., deciding to multiparasitize, or not). In direct competition, female interactions can be resolved in different ways: leaving the host patch, co-exploiting the patch, or engaging in aggressive physical interactions (e.g., fights using the mandibles). The resulting interactions may be negative for both competitors, by wasting searching time, loss of energy and risk of severe injury (Hokyo and Kiritani, 1966; Field et al., 1998).

Scelionid female behavior during host egg parasitization is typically stereotyped (Hokyo and Kiritani, 1966; Field, 1998) and can be summarized as follow: 1) host egg inspection by antennation; 2) egg acceptance with ovipositor drilling of the chorion; 3) oviposition; 4) marking of the parasitized host egg. Marking is adaptive by enabling recognition of already parasitized eggs to avoid self-superparasitism (Okuda and Yeorgan, 1988) and is a clear sign of successful oviposition in scelionid species (Hokyo and Kiritani, 1966; Field, 1998; Wilson, 1961; Van Baaren et al., 1994; Weber et al., 1996). Marked eggs are also recognizable by conspecifics (thus avoiding superparasitism), while in interspecific competition, in most of cases studied, the marked egg recognition appears uncommon (Boivin and Brodeur, 2006; Okuda and Yeorgan, 1988). During the post-ovipositional period, females may exhibit additional behaviors, such as continuing to inspect the egg mass, guarding, resting or grooming (Field, 1998).

The sympatry of *T. japonicus* and *T. mitsukurii* as reported by recent field data both in Italy (Zapponi et al., 2021) and Europe in general (see Bout et al. 2021; Dieckhoff et al. 2021; Rot et al. 2021), and the potential opportunity to use these species in biological control programs against *H. halys*, stimulated a number of studies on their interactions with each other or with different species of the parasitoid guild, in order to assess possible pest control outcomes, as firstly evidenced by Konopka et al. (2017a, 2017b), Haye et al. (2021), Mi et al. (2021) and successively by Costi et al. (2022). Indeed, interspecific competition can potentially disrupt biological control programs, or cause synergism, or it may have no influence at all (Boivin and Brodeur, 2006; Casula et al., 2006; Mills, 2006; Ode et al., 2022).

In laboratory experiments, we investigated the mechanisms and behaviors displayed during interspecific competition between *T. japonicus* and *T. mitsukurii* and the way these species exploit common resources. The outcomes focused on three different experimental scenarios which are potentially predictors for field occurrence: i) when a female of one species encountered a host egg mass previously parasitized by a female of the other species which had already departed (indirect competition); ii) when females of each species were released simultaneously in an arena containing a host egg mass (direct competition with synchronous release); iii) when a female of one species encountered a host egg mass already parasitized but still attended by a female of the other species (direct competition with asynchronous release). We then quantified and analyzed principal behavior patterns exhibited by *Trissolcus* females of each species under these competitive scenarios in order to estimate the success in host egg parasitization, the progeny production of each species, and the potential impact on host egg survival.

In view of the considerable interest in egg parasitoids of *H. halys*, similar studies on this subject were conducted concurrently by several research groups. Although the results obtained from such studies are similar to ours (e.g., see Costi et al., 2022), the different approaches used for the experimental designs (e.g., use of unmanipulated and naturally-sized egg masses, different timing in direct and indirect competition, synchronous and asynchronous release of females under direct competition, different ethological traits investigated, different origin/haplotypes of the parasitoid populations) provide additional and complementary information that may help to describe potential scenarios for biological control programs.

Material and Methods

Insect origins and rearing

Halyomorpha halys colony was established from individuals collected at infested sites in Northern Italy in 2020. Collected specimens were maintained in the lab in insect rearing cages (BugDorm ® 4F4545, MegaView Science, Taiwan, China) and fed with fresh fruits (apples, kiwi), vegetables

(carrots, green beans), potted plants (soybean), and seeds (peanuts and soybean); water was provided with wet cotton. *Trissolcus mitsukurii* females were obtained from laboratory colonies originally established with *H. halys* egg masses collected from infested sites in Northeastern Italy (*T. mitsukurii* strain H5). *Trissolcus japonicus* females were obtained from permanent lab-reared colonies of populations originally collected in Beijing (China) (*T. japonicus* strain H7). *Trissolcus mitsukurii* was selected due to the recent attention resulting from its occurrence in North Italy as a newly-reported member of the parasitoid guild of *H. halys*, while *T. japonicus* of Beijing origin was selected because this population is the most-investigated worldwide for classical biological control actions and has been mass-reared for field release. Colonies of both egg parasitoids were maintained on fresh *H. halys* egg masses (parasitized at age < 24 h); adult parasitoids were fed with pure honey droplets replenished twice a week. Adults and parasitized host eggs were maintained in glass tubes in climatic chambers under standard conditions of 26°C, 60%RH, and 16:8 L:D. A total of 156 *H. halys* egg masses were used in the tests (mean of 27.53 ± 0.19 S.E. eggs/ mass).

Indirect competition

To evaluate the parasitization behavior and the progeny production by *T. japonicus* and *T. mitsukurii* females when one species was provided with a *H. halys* egg mass already completely parasitized by the other species, a fresh *H. halys* egg mass was placed in a plastic Petri dish (30mm in diameter) and exposed to a single naïve female of *T. japonicus* or *T. mitsukurii* (7 d old post-eclosion for optimal accumulation of ovarian eggs, see Sabbatini Peverieri et al. 2020). Its foraging behavior in the arena was video-recorded (Panasonic WV-BP330/GE, Suzhou Matsushita Co. Ltd., Suzhou, China) for 2.5h starting from the first contact with the host egg mass in the arena. Preliminary analysis showed that 2.5h was a sufficient time-lapse to permit complete egg mass parasitization under non-competitive conditions for both egg parasitoids. Behavioral tests were conducted under laboratory conditions (25°C; 55% RH) and a constant cold light source.

For tests with *T. japonicus* as the first-arriving species, the egg mass was then removed from the arena and from the *T. japonicus* female and transferred to a climatic chamber for juvenile development as needed for the successive interspecific competition test. These egg masses were offered to *T. mitsukurii* females at four different intervals: immediately (T0), after 3 days (T3), 5 days (T5) and 7 days (T7). The development stage of parasitoid juvenile present at these intervals included: egg, developing larva (LI-LII), mature larva (including prepupa) and pupa stadium (Giovannini et al. 2021). At each scheduled time interval, a female *T. mitsukurii* (naïve and 7d in age) was introduced into the arena containing the *H. halys* egg mass previously parasitized by *T. japonicus*; its behavior was videorecorded for 2.5h. Each treatment was replicated 12 times.

The same experimental design was used when *T. mitsukurii* was given host eggs to parasitize first, then followed by *T. japonicus*. Following the experimental exposures to parasitoid females, the egg masses used were maintained in the climatic chamber until emergence of progeny. Additionally, twelve *H. halys* egg masses were offered to females of each species for parasitization as no-competition controls to compare progeny production and parasitization behavior. Offspring were identified to species and sexed; female development times were also recorded.

The following behaviors and parameters were recorded:

- a) time spent by the female inspecting the egg mass before beginning parasitization (acceptance time);
- b) percentage of host eggs parasitized successfully (oviposition and subsequent host egg marking);
- c) percentage of host eggs parasitized successfully before the first abandonment of the host egg mass;
- d) percentage of host eggs producing parasitoid progeny
- e) percentage of *H. halys* eggs hatched and dead.

Due to the complexity of the behavioral patterns shown by *Trissolcus* during the parasitization process, data on additional behavioral parameters are reported in Supplementary material S1. These data are relevant to the scope of our research and add ethological information for the studied species.

Direct competition with synchronous release of females

In this experiment, we simulated simultaneous arrival of two parasitoid females at the same *H. halys* egg mass. To evaluate parasitization behavior, species interactions and progeny production by the two parasitoids, a fresh *H. halys* egg mass was placed in a plastic Petri dish (30 mm in diameter) and one naïve female (7 days old) of each *Trissolcus* species was introduced simultaneously into the arena. To distinguish *T. mitsukurii* from *T. japonicus* females during behavioral analysis, the mesoscutum of each female was marked with a different color of acrylic tempera (Zenacolor®, BMS International, Grand Bay, Mauritius) using a fine brush (series 7 n. 000, Winsor and Newton®, London, UK) before being used in the tests. Preliminary observations showed that the marking on the mesoscutum did not affect mobility, parasitization behavior, longevity or fecundity. The behavior of both females in the arena was videorecorded for 2.5 hours and started when the females were introduced in the arena. At the end of the exposure the females were removed from the arena and the egg mass transferred to a climatic chamber and reared under standard conditions. The egg mass was checked daily until eggs hatched or parasitoids emerged. Offspring were identified to species and sexed, and the female development time was recorded. The experiment was replicated 12 times.

The elapsed time and numbers of the following behaviors and events were recorded for both species:

- a) time from release of each female in the arena to first contact with the host egg mass (discovery of egg mass);

- b) time spent by each female on the egg mass for parasitization (including egg inspection, antennation, drilling, egg laying and marking);
- c) time spent by each female standing motionless on the egg mass or moving across host eggs without further inspection (guarding);
- d) time spent by each female in aggressive interactions with each other (biting or wrestling);
- e) time spent by the female away from the egg mass after first contact;
- f) number of successful ovipositions in host eggs followed by marking;
- g) number of attempted ovipositions interrupted due to interaction with the competitor (not resulting in a complete oviposition with marking);
- h) number of times a female chased the other female away from the host egg mass (attacks);
- i) number of times a female won a physical contest;
- l) number of offspring produced by each of the two competing females;
- m) percentage of *H. halys* eggs hatched and dead.

Direct competition with asynchronous release of females

Direct (extrinsic) interspecific competition was also investigated with sequential release of the two species together in an arena containing an *H. halys* host egg mass. The same procedures used in experiment 2.3 were applied except that the second female was released into the arena after a short delay, permitting the first female introduced (hereafter the “resident” female) to attack a portion, but not all, of the host eggs in the mass before the second female was introduced. Previous analysis of parasitization behavior showed that *T. japonicus* takes less time to oviposit compared to *T. mitsukurii*, thus the females of *T. japonicus* and *T. mitsukurii* were allowed to parasitize the *H. halys* egg mass for 60 min. and 75 min., respectively. Preliminary observations showed that these intervals were sufficient to permit the parasitization of approximately half of the host egg mass. The second female was then introduced into the arena (hereafter the “intruder” female). The delayed arrival of the second female to the same egg mass produced an asymmetry in resource investment due to the partial parasitization of the mass by the first arriving species (Field and Calbert, 1999). Their direct competition was videorecorded for 90 minutes to analyze the behavior of both contestants. Twelve replicates of each succession combination (*japonicus* followed by *mitsukurii* and the reverse) were conducted.

The times and number of events of the following parameters were recorded for both species, as described for experiment 2.3:

- a) time spent for parasitization;
- b) time spent for guarding;
- c) time spent for fighting;

- d) time spent away from the host egg mass;
- e) number of attacks;
- f) number of times a female won a physical contest;
- g) total progeny produced;
- h) percentage of *H. halys* eggs hatched and dead.

Data analysis and statistics

The behavior of *Trissolcus* females during parasitization and direct extrinsic and indirect interactions was recorded with BORIS software (Friard and Gamba, 2016). Data obtained from videorecording and from rearing the parasitized *H. halys* host egg masses were used to build the data set in all three experiments.

Simple generalized linear models for binomial distribution with a logistic link function were adopted to evaluate the effect of the different exposure times on the behavioral responses recorded in indirect competition (including also the behaviors described in S1) and a Kruskal-Wallis test followed by Dunn's post hoc test ($p < 0.05$). The dead and hatched *H. halys* eggs, in absence of competition were analyzed by Mann-Whitney's test and pooled where no significant differences were detected. Release experiments, concerning both direct competition with synchronous and asynchronous release of females, were analyzed by a generalized linear model and a Kruskal-Wallis test followed by Dunn's post hoc test ($p < 0.05$). The $\ln(x + 0.5)$ transformation was used in this analysis to normalize the data in order to meet normality assumptions/criteria and stabilize the variance of the population. In the test of asynchronous release of females, Mann-Whitney's test was used to verify the homogeneity of the first arriving females parasitization. All statistics were performed using SPSS 20.0 software.

Results

Indirect competition

The total number of eggs parasitized was similar in the absence of competition and also in most of the competitive scenarios, regardless of the order of species succession and the time intervals between the two sequential parasitization exposures (Table 1); significant differences in parasitization behavior occurred only in treatment T7, where the second female attacked fewer *H. halys*, regardless of species order ($F_{17,198} = 5.810$, $p < 0.001$; $H = 45.140$, $p < 0.001$).

The mean range of parasitized eggs per egg mass before the first female departed the egg mass was variable between the 34% and 100% and no differences were detected among most of the combinations; also in this case, the only significant differences occurred in treatment T7 regardless of species successions ($F_{17,198} = 8.102$, $p < 0.001$; $H = 66.620$, $p < 0.001$). The mean acceptance time of

host eggs by females in the different combinations was variable and ranged from 42 and 109 seconds ($F_{17,198}=4.345$, $p<0.001$; $H=65.210$, $p<0.001$).

Table 1. Behavioral parameters and egg survival (mean±S.E.) of *Trissolcus japonicus* and *Trissolcus mitsukurii* at different time intervals, between successive parasitization exposures to *Halyomorpha halys* egg masses. Sequence day interval of the two successive parasitoid exposures: T0=immediate successive exposure, T3=3 days, T5=5 days; T7=7 days. Within each column, for each species behaviour, an asterisk indicates a significant difference between the treatment and the absence of competition; the dead and hatched eggs of *H. halys* recorded in each competition sequence treatment are compared with the absence of competition treatments (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) (data of dead and hatched eggs were pooled).

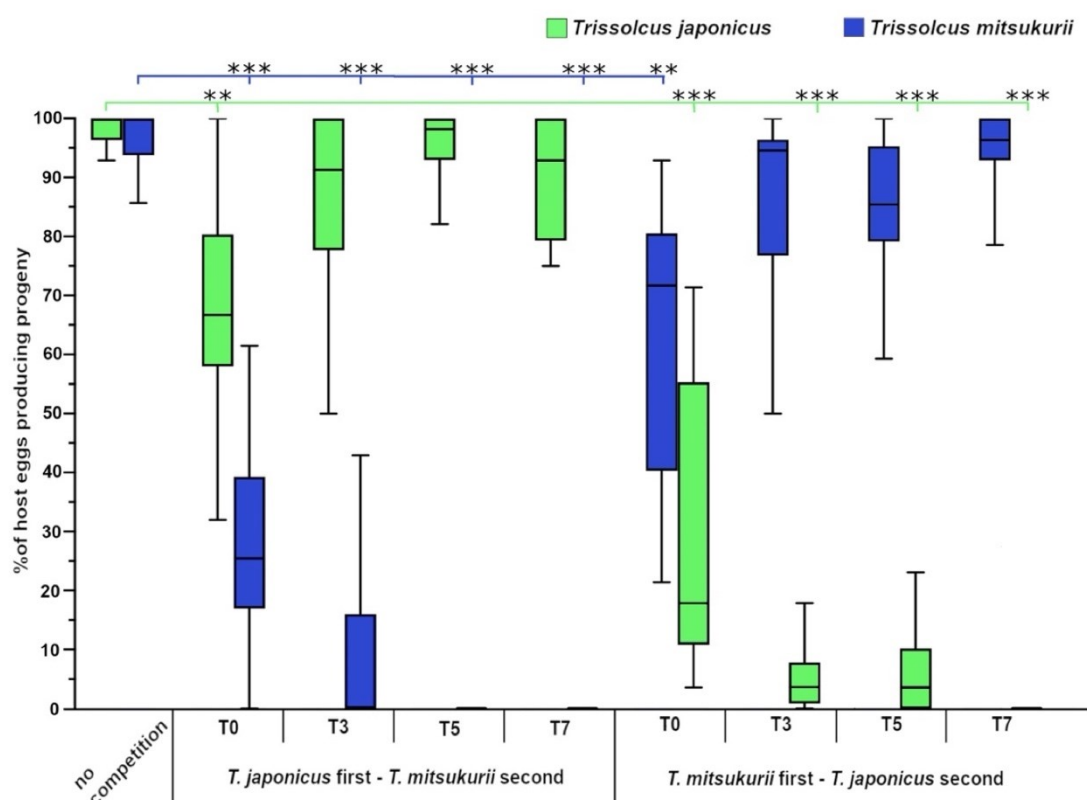
	Interval between initial parasitization and 2nd species introduction	% host eggs parasitized (host eggs marked)	% eggs parasitized during first encounter with egg mass	Host time acceptance (s)	% <i>H. halys</i> dead eggs	% <i>H. halys</i> hatched eggs		
no competition	Tj	100±0.00	100±0.00	43.83±10.05	0.00±0.00	0.00±0.00		
	Tm	98.51±0.69	83.63±7.57*	85.67±11.95**	1.49±0.67	0.00±0.00		
<i>T. japonicus</i> first – <i>T. mitsukurii</i> second	T0	Tj	96.07±2.99	96.07±2.99	41.75±5.93	4.24±1.58*	0.08±0.08	
		Tm	89.04±4.82	76.48±6.44	109.08±18.15			
	T3	Tj	100±0.00	97.88±1.44	42.42±10.88	5.28±2.23**	0.00±0.00	
		Tm	91.37±5.81	88.11±6.26	80.25±6.87			
	T5	Tj	100±0.00	100±0.00	45.50±6.23	4.53±1.29**	0.08±0.08	
		Tm	83.43±8.48	97.06±1.80	79.17±10.80			
	T7	Tj	98.40±1.60	98.08±1.92	64.17±16.01	9.41±3.94**	0.17±0.17	
		Tm	58.30±8.85***	34.74±10.19**	69.58±9.44			
	<i>T. mitsukurii</i> first – <i>T. japonicus</i> second	T0	Tm	95.78±2.12	78.31±5.75	85.75±11.57	3.05±0.89*	0.08±0.08
			Tj	100±0.00	97.22±3.13	54.17±6.76		
T3		Tm	98.85±0.49	86.70±6.79	83.92±8.52	9.70±2.99***	0.00±0.00	
		Tj	86.83±5.41	86.55±5.57	50.25±6.86			
T5		Tm	97.90±0.69	78.97±8.09	101.00±11.96	7.31±2.05***	0.17±0.11	
		Tj	96.68±2.32	90.12±7.96	107.50±46.45			
T7		Tm	99.39±1.11	87.13±7.81	92.92±13.70	4.77±1.78**	0.25±0.18	
		Tj	74.12±8.51**	37.22±11.66***	61.33±19.66			

Trissolcus mitsukurii females had a longer acceptance time than *T. japonicus* in absence of competition, but no differences were detected among competitive scenarios. The percentages of dead

eggs in absence of competition were extremely low both in *T. japonicus* and *T. mitsukurii* (zero and 1.49%, respectively) and not different ($U=48,000$, $p=0.093$); such data were pooled and compared with the treatments. Under the competitive interactions, higher numbers of dead eggs were recorded in all other combinations than in absence of competition ($F_{17,198}=12.370$, $p<0.001$; $H=68.350$, $p<0.001$). *Halyomorpha halys* eggs that hatched were observed at an extremely low rate ($< 0.1\%$ of all the exposed host eggs). No hatched eggs were recorded in absence of competition in both species, similarly to results observed among the competitive scenarios (Table 1; $F_{9,110}=0.818$, $p=0.601$; $H=1.39$, $p=0.592$).

Both *T. japonicus* and *T. mitsukurii* had similar high levels of progeny production in non-competitive control replicates, reaching a mean of 98.20% and 97.31% respectively ($F_{17,198}=103.700$, $p<0.001$; $H=183.400$, $p<0.001$). The second arriving females of both species produced a lower number of progeny than females in absence of a competition scenario. No significant difference in the number of progeny was recorded for the first arriving females except in the T0 treatment, where the progeny production was significantly lower than in the absence of competition (Fig. 1).

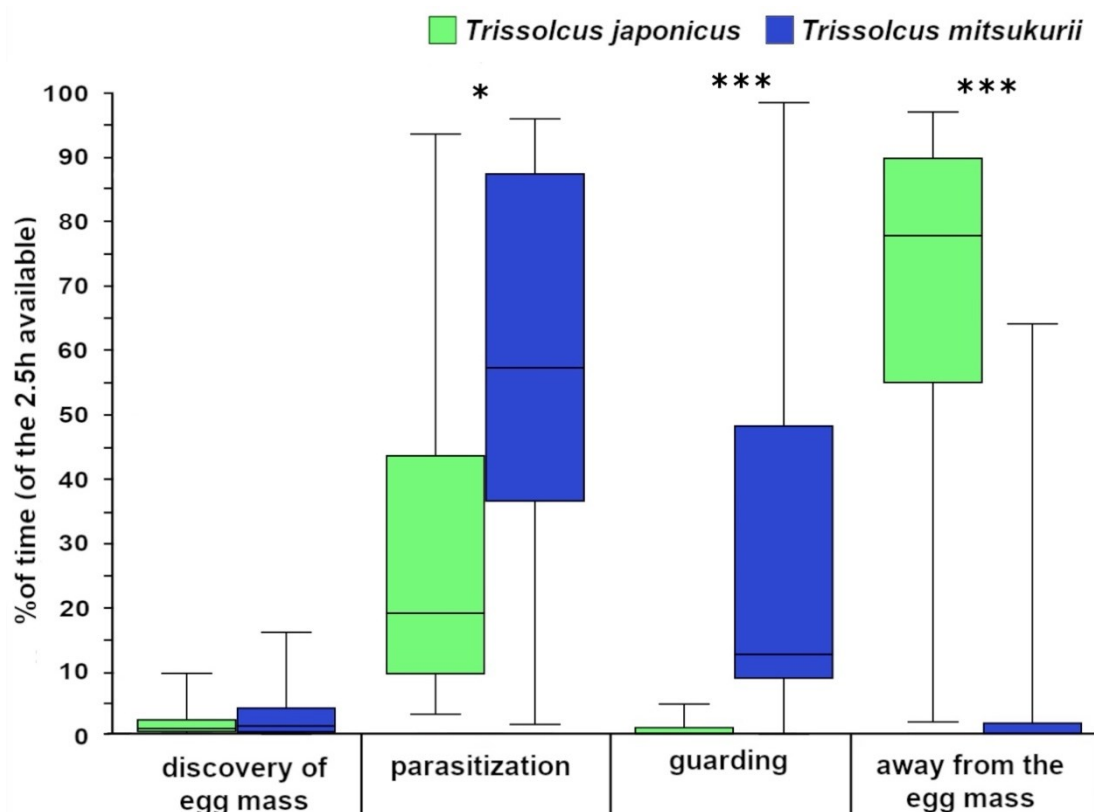
Figure 1. Progeny production following indirect interspecific competition between *Trissolcus japonicus* and *Trissolcus mitsukurii*: egg masses of *Halyomorpha halys* were first parasitized by one species and, then, offered to the other species, at different time intervals. Interval (days) between successive exposures: T0=immediate; T3=3 days; T5=5 days; T7=7 days. Asterisks indicate significant differences (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) for each treatment with competition absence. In the boxplots the boxes indicate the first and third quartile, the thick line in between shows the median, whiskers indicate $1.5\times$ the interquartile range.



Direct competition with synchronous release of females

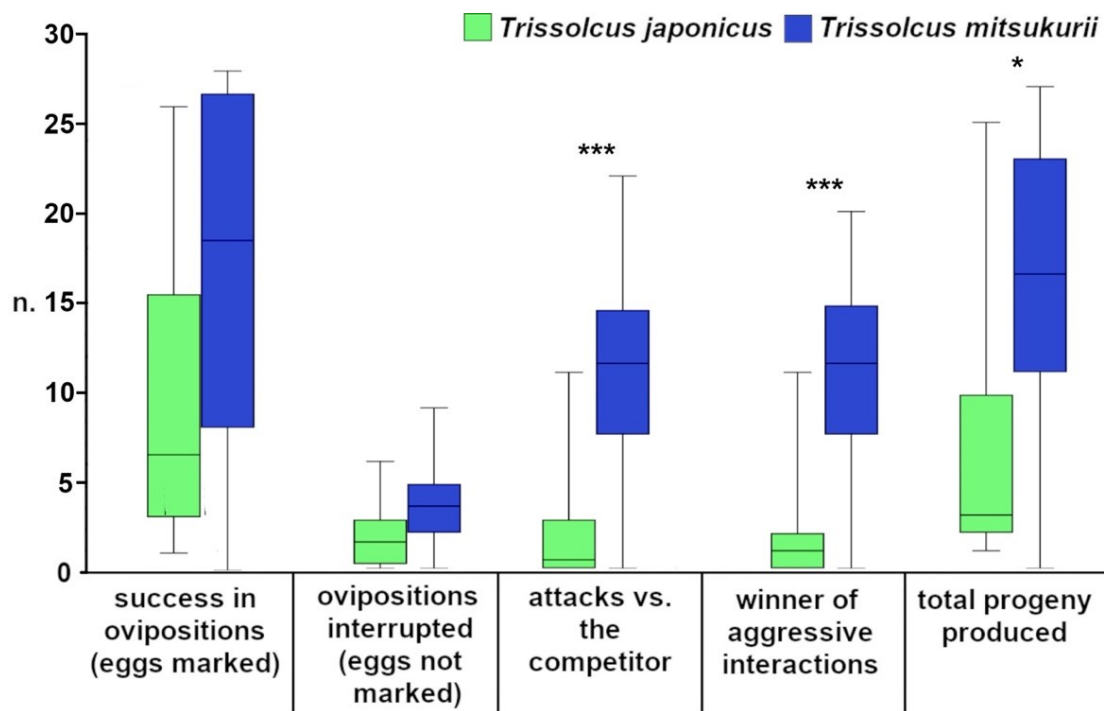
Trissolcus japonicus was the first to discover the egg mass in seven of the 12 replicates. *Trissolcus japonicus* and *T. mitsukurii* females both encountered the host egg mass within a few minutes of starting the test, on average 3.16 min and 4.27 min ($F_{1,22}=0.009$, $p=0.923$; $H=0.12$, $p=0.729$), respectively. *Trissolcus japonicus* spent significantly less time (31.79% of the total time observed) inspecting and parasitizing the egg mass than *T. mitsukurii* females (59.10%) ($F_{1,22}=3.102$, $p=0.092$; $H=4.201$, $p=0.040$) (Fig. 2). Duration of guarding behavior also differed between the two species ($F_{1,22}=20.420$, $p<0.001$; $H=12.610$, $p<0.001$). *Trissolcus mitsukurii* spent more time guarding the egg mass (28.03%) than *T. japonicus* (0.76%). The two species also differed in time spent away from the egg mass ($F_{1,22}=26.330$, $p<0.001$; $H=14.080$, $p<0.001$). *Trissolcus japonicus* was more often away from the host patch compared to *T. mitsukurii* (64.70% vs. 9.30% of the total available time). The total time spent in aggressive interactions (i.e., fighting), occupied less than a minute (0.59%) of the total time.

Figure 2. Behaviors of *Trissolcus japonicus* and *Trissolcus mitsukurii* females released simultaneously in the arena containing an egg mass of *Halyomorpha halys*: time spent in each behavior is reported as a percentage of the total time available (2.5h per test). Asterisks indicate significant differences (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) within each behaviour trait. In the boxplots the boxes indicate the first and third quartile, the thick line in between shows the median, whiskers indicate $1.5\times$ the interquartile range.



Trissolcus mitsukurii females were significantly more likely to exhibit aggressive behavior, initiating a greater number of attacks than *T. japonicus* ($F_{1,22}=20.070$, $p<0.001$; $H=11.020$, $p<0.001$), and were more likely to win the physical contests ($F_{1,22}=20.000$, $p<0.001$; $H=10.830$, $p<0.001$) (Fig. 3). The presence of a competitor on the egg mass sometimes induced females to interrupt oviposition, although such interruptions were infrequent and did not differ significantly between the two species ($F_{1,22}=3.944$, $p=0.059$; $H=3.968$, $p=0.043$). *Trissolcus mitsukurii* successfully completed more ovipositions (including marking of the host egg) (mean of 17.83 ± 2.67 S.E.) than *T. japonicus* (9.67 ± 2.56 S.E.) although this difference was not significant ($F_{1,22}=1.749$, $p=0.200$; $H=3.741$, $p=0.053$). Nevertheless, *T. mitsukurii* produced significantly more progeny (16.08 ± 2.42 S.E.) than *T. japonicus* (6.67 ± 2.18 S.E.) ($F_{1,22}=4.861$, $p=0.038$; $H=5.070$, $p=0.024$). No dead or hatched *H. halys* eggs were observed in any of the replicates.

Figure 3. Behaviors of *Trissolcus japonicus* and *Trissolcus mitsukurii* females simultaneously released in the arena containing an egg mass of *Halyomorpha halys* and numbers of progeny produced. Each category is expressed as the mean number of events recorded during test (2.5h). Asterisks indicate significant differences (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) within each behaviour trait. In the boxplots the boxes indicate the first and third quartile, the thick line in between shows the median, whiskers indicate $1.5\times$ the interquartile range.

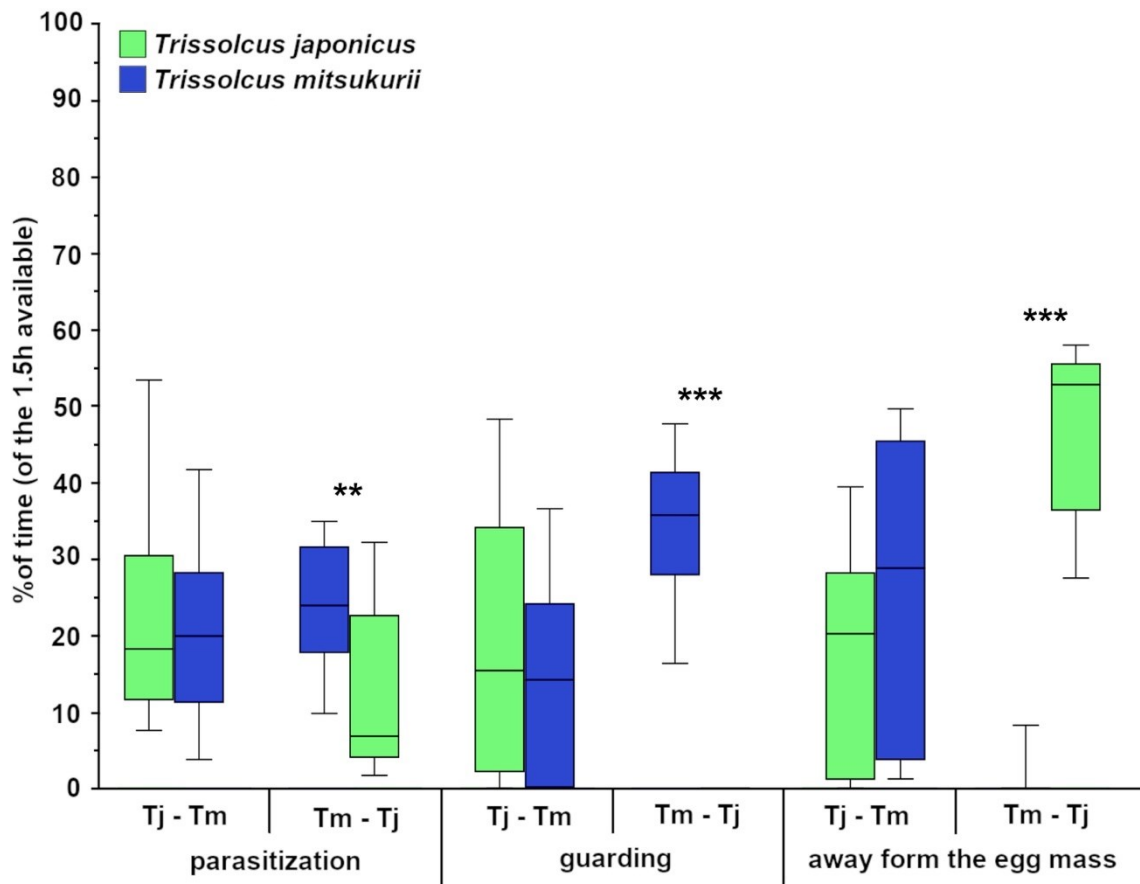


Direct competition with asynchronous release of females

A mean of 17.00 ± 1.22 (S.E.) and 17.58 ± 0.58 (S.E.) eggs were parasitized by *T. japonicus* and *T. mitsukurii*, respectively, before introducing the female of the other species ($U=56,500$, $p=0.384$). Parasitization behavior (including host inspection and oviposition) was not significantly different for the two species when *T. japonicus* arrived first (resident species) and *T. mitsukurii* was introduced as

second species (intruder) ($F_{1,22}=0.162$, $p=0.691$; $H=0.120$, $p=0.729$) (Fig. 4). In the reverse case, when *T. mitsukurii* was the first species introduced and *T. japonicus* was second, differences in parasitization behavior were significant ($F_{1,22}=11.200$, $p=0.003$; $H=6.750$, $p=0.009$). Similarly, guarding behavior was not significantly different when *T. japonicus* was the first species introduced in the arena ($F_{1,22}=1.200$, $p=0.285$; $H=0.563$, $p=0.451$), but when *T. mitsukurii* was the first species introduced, the females spent more time in egg mass guarding ($F_{1,22}=9790.000$, $p<0.001$; $H=17.280$, $p<0.001$). When *T. japonicus* was the resident species, no significant differences were observed in time spent away from the egg mass ($F_{1,22}=2.092$, $p=0.162$; $H=1.688$, $p=0.193$). In contrast, when *T. mitsukurii* was the resident species, *T. japonicus* spent significantly more time away from the egg mass ($F_{1,22}=115.700$, $p<0.001$; $H=17.280$, $p<0.001$).

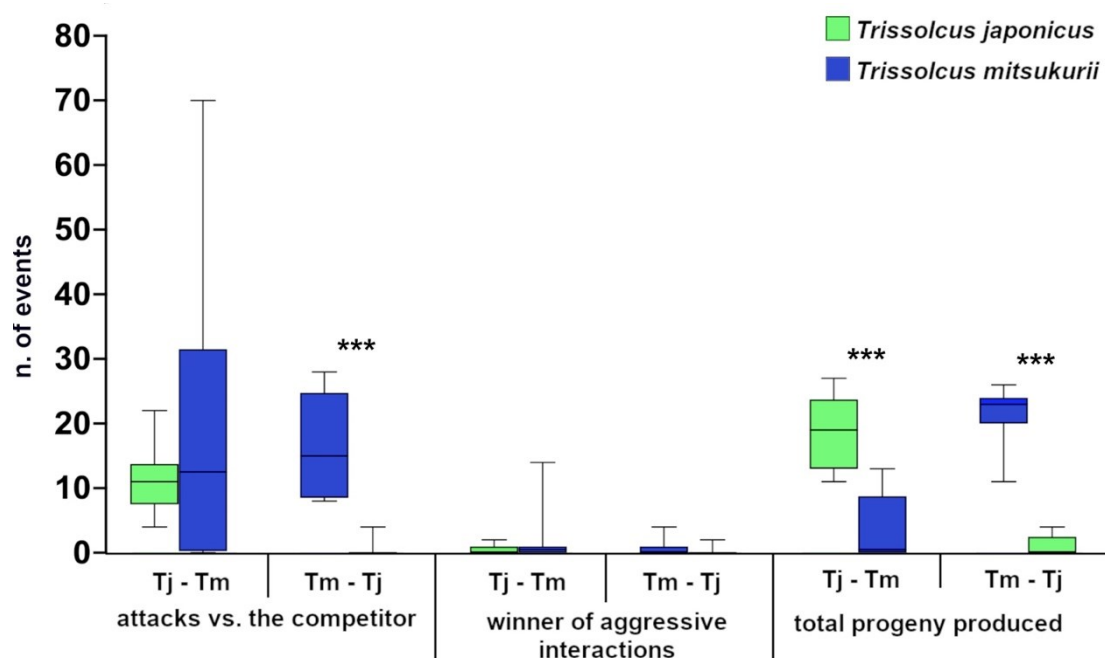
Figure 4. Behaviors of *Trissolcus japonicus* and *Trissolcus mitsukurii* in direct competition: first access to the *Halyomorpha halys* egg parasitization was given, alternatively, to one species followed by the other. Time spent in each behavior is shown as a percentage of the total time elapsed in the test (1.5h). Asterisks indicate significant differences (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) within each behaviour trait between the two species. In the boxplots the boxes indicate the first and third quartile, the thick line in between shows the median, whiskers indicate $1.5\times$ the interquartile range.



The number of attacks was not significantly different between resident-intruder combinations when *T. japonicus* female was the resident ($F_{1,22}=0.466$, $p=0.502$; $H=0.120$, $p=0.729$), whereas when *T. mitsukurii* was the resident female, it had a greater number of attacks versus *T. japonicus* ($F_{1,22}=145.900$, $p<0.001$; $H=17.280$, $p<0.001$) (Fig. 5). No difference between species was observed

in resolution of the aggressive interactions (combination: *T. japonicus* resident species: $F_{1,22}=0.447$, $p=0.511$; $H=0.120$, $p=0.701$; combination: *T. mitsukurii* resident species: $F_{1,22}=1.239$, $p=0.278$; $H=0.853$, $p=0.249$). The first-arriving females, regardless of species, produced the greatest number of progeny (combination: *T. japonicus* resident species: $F_{1,22}=31.450$, $p<0.001$; $H=15.870$, $p<0.001$; combination: *T. mitsukurii* resident species: $F_{1,22}=124.100$, $p<0.001$; $H=17.280$, $p<0.001$). No dead or hatched *H. halys* eggs were observed in any of the replicates.

Figure 5. Aggression behavior of *Trissolcus japonicus* and *Trissolcus mitsukurii* by progeny production in direct competition with first access, alternatively for the two parasitoid species, to the *Halyomorpha halys* egg parasitization. During the test time (1.5h), the number of events are recorded with Tj-Tm (*T. japonicus* is the first species in the arena (resident), *T. mitsukurii* the species introduced (intruder)) and Tm (resident)-Tj (intruder) sequences. Asterisks indicate significant differences (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) within each behaviour trait between the two species. In the boxplots the boxes indicate the first and third quartile, the thick line in between shows the median, whiskers indicate 1.5× the interquartile range.



Discussion

Under these laboratory experimental conditions, *T. japonicus* and *T. mitsukurii* showed that when in indirect competition for *H. halys* eggs, females of both species seem unable to recognize signs of previous parasitization when encountering egg masses recently parasitized by the other species. This was confirmed by the high percentage of eggs parasitized by the second female arrival in most of the combinations tested. Only when 7 days had elapsed since *H. halys* eggs were first parasitized did late-arriving females change their behavior to attack fewer host eggs than in absence of competition. In this case, the second arriving female was probably able to perceive the presence of the pupa inside the egg (e.g. by detecting chemical cues).

In the field a female may reject a previously parasitized egg mass at any stage in order to search for a more suitable host, but this scenario cannot be investigated with our experimental design. To obtain information about this potential situation, we evaluated the parasitization ability of the female within the time period from the acceptance of the host and the first abandonment of the egg mass. In this case, we found no differences after five days from initial parasitization compared to the absence of competition, but differences emerged in the T7 combination, in which the second arriving female parasitized a lower number of eggs. The ability to multiparasitize was also observed by Haye et al. (2021) with *T. japonicus* when in competition with *T. cultratus*.

In general *T. mitsukurii* females spend more time in evaluating the egg mass for oviposition suitability than *T. japonicus* (host acceptance time). Unfortunately, we cannot compare this parameter from our study with the data obtained by Costi et al. (2022), since their measurement also included the host searching time.

Overall, the competition between the two species did not lead to higher host egg survival, and the number of hatched eggs recorded was extremely low in all the scenarios tested. Instead, the species interactions led to a higher number of dead eggs.

Although females of *T. japonicus* and *T. mitsukurii* in most cases (T0 – T5) parasitized host egg masses at an equal rate, independently of the order of species introduction and the interval before discovery of host eggs by the second species, the eventual production of progeny was, on the whole, significantly affected: in most cases progeny produced by the first arriving female was similar to when the competition was absent, with the only exception being when the second species arrived immediately after the first one (T0). In this case, the first arrived female produced significantly fewer progeny. In general, the first arrived species has the highest reproductive success, as was observed by Costi et al. (2022) for the same species and in other *Trissolcus* species (Weber et al., 1996). The outcome of intrinsic competition might be different if the success of the second *Trissolcus* arriving species is subject to a temporal trophic shift as observed by Haye et al. (2021), where *T. cultratus* was found to be a superior intrinsic competitor of *T. japonicus* when arriving as the second species at zero and three days after initial *T. japonicus* parasitization, but not after 1, 2, 4 or 5 days. Haye et al. (2021) showed that *T. cultratus* is also able to act as a hyperparasitoid (at least under their experimental conditions), but this was not observed in *T. japonicus*. Hyperparasitism was not observed in *T. japonicus* and *T. mitsukurii* also (Costi et al. 2022). In our tests, the second-arriving species was able to produce at least a minimal number of progeny when the elapsed time between initial parasitization and the arrival of the second parasitoid female was short (not more than three days after the primary parasitization) and exceptionally *T. japonicus* also after 5 days. This behavior is explained by Haye et al. (2021) for *T. cultratus* which is also potentially able to exploit a competitor parasitoid larva for its development in addition to the host pentatomid egg.

The parasitizing behavior of both *Trissolcus* species tested appeared very similar under our test conditions, and indirect interactions among females showed that the first female had a greater success in progeny production and did not suffer severely in competition with the second-arriving species. In contrast, the second-arriving species had less success in progeny production, with a decreasing trend as the elapsed time from first parasitization increased. Moreover, in contrast to the high level of *H. halys* eggs parasitized by the first-arriving females (in which parasitoid eggs were successfully laid), as might be expected, the second-arriving females parasitized host eggs at a rate that decreased slightly over time, although rates of total parasitized host eggs remained at high levels even at 7 days. The same trend was also observed by Costi et al. (2022) although they concluded that *T. mitsukurii* has a greater intrinsic (larval) competition ability, something that we did not observe. No conclusion can be made about possible differences among haplotypes between our studies since Costi et al. (2022) did not report the haplotype profile of their tested parasitoid populations. The success of first-arriving species is not always the case: when *Trissolcus*-parasitized host eggs are followed by multiparasitism with *Psix* (also Scelionidae) or *Anastatus* (Eupelmidae), in most cases it is the second-arriving species that is most successful in leaving progeny (Weber et al., 1996; Konopka et al., 2017a; Mi et al., 2021). Data collected by Konopka et al. (2017) on competition between *T. japonicus* and *A. bifasciatus* clearly explain the hyperparasitism ability of the tested eupelmid species when it is the second arriving female, which is able to develop successfully in *H. halys* host eggs initially parasitized by *T. japonicus*, at any stage of development of the first arriving parasitoid. Collectively, our data suggests the inability of *T. japonicus* and *T. mitsukurii* to act as hyperparasitoids, while at the same time, both parasitoids are not able to clearly recognize the unsuitability of a previously parasitized host since they will proceed to re-parasitize it.

With respect to biological control of *H. halys*, the experimental laboratory results obtained under our competitive scenarios highlight a potentially important issue when *T. japonicus* and *T. mitsukurii* occur in sympatry: interspecific competition can result in a waste of search time and eggs when the late-arriving species encounters already parasitized host eggs. These results agree with those of Bovin and Brodeur (2006), who described multiparasitism in biological control as having a negative impact if the late-arriving species is intrinsically a weaker species. However, maladaptive behavior of *T. japonicus* described by Haye et al. (2021) (observed in our study by both *T. japonicus* and *T. mitsukurii*) resulting in a waste of progeny due to the oviposition in an unsuitable developing substrate can be overestimated.

When the two *Trissolcus* egg parasitoid females were introduced simultaneously in the arena with a single *H. halys* host egg mass (extrinsic competition), both species proved able to oviposit and produce progeny, though with differing success. We observed no significant differences between *T. japonicus* and *T. mitsukurii* in locating the host egg mass in the arena after their release, which

occurred within a few minutes. As soon as both females came into contact with each other in the presence of the host egg mass, guarding behavior and mutual aggression rapidly occurred. Whenever a female first arrived on the egg mass, parasitization started quickly after a short egg inspection. In the presence of the competitor, the foraging female's behavioral pattern changed: parasitization was suspended and guarding behavior of the host egg mass was adopted, followed by attempts to repel the competitor when it approached. This sometimes escalated to a physical contest with fighting if the intruder female did not move away from the egg mass, as observed by Field et al. (1998). In our experiments, *T. mitsukurii* was found to be the dominant species of the two, spending a large part of the time guarding the egg mass. It was also more aggressive, initiating a significantly greater number of attacks and winning fights than *T. japonicus* females. Costi et al. (2022) observed a rare exhibition of contest behavior when *T. japonicus* was the first introduced female in the arena, while a greater number of contests was observed when *T. mitsukurii* was introduced as first female. The aggressivity of *T. mitsukurii* appears to be unrelated to the egg mass size (as suspected by Costi et al. 2022). Our results are in line with Costi et al. (2022), although they used an artificially reduced host egg mass size (14 eggs/mass vs. the standard egg mass size of 28 eggs, as also used by Haye et al. 2022). A similar case was observed when *T. mitsukurii* was faced with *T. basalis* or with *T. nakagawai* (Hokyo and Kiritani, 1966; Nishimoto et al., 2015). The fighting behavior between *T. japonicus* and *T. mitsukurii* lasted for a very short time (just a few seconds per event), and always resulted in a retreat from the egg mass by the beaten female. After retreating, the loser moved some distance away from the host egg mass, waiting for an opportunity to return. Of course, in a confined experimental arena the competition between contestants may be exaggerated, engaging several times in a “waiting-game” described by Field et al. (1998), where the dominant female on the egg mass aggressively defends the host resource, and the intruder female waits for an opportunity to have access to the host. Similar strategies for host exploitation of an already guarded egg mass were observed by Haye et al. (2021) during interactions of *T. cultratus* with *T. japonicus*, where the “stalking” strategy (slow movements) of the first and the “ambush” strategy (fast moves toward the guarding female) of the second species may produce different output in aggressive interactions and progeny production. In our tests we never observed signs of body injuries after aggressive interactions. Similarly, in *T. basalis* interspecific competition, body injuries were rare (Field et al. 1998). Guarding and fighting are risky and expensive in terms of time for parasitization, as well as being costly in energy; however, these behaviors permit the most aggressive females to defend the host patch from a competitor and assure successful progeny production (Field et al. 1998). Results of our work and that of Costi et al. (2022) suggest *T. mitsukurii* to be a superior extrinsic competitor, but despite displaying less aggressive behavior *T. japonicus* females were able to exploit opportunities for parasitization; these events generally occurred when *T. mitsukurii* females were engaged in oviposition. In female competition, *T. mitsukurii* spent nearly

twice as much time in parasitization activity than *T. japonicus*, and produced on the whole a higher number of progeny, which partially contrasts with Costi et al (2021). However, *T. japonicus* displayed superior competitive ability and aggression in host patch defense when in contests with congeneric (*T. cultratus*) and non-congeneric (*A. bifasciatus* and *A. japonicus*) species that attack *H. halys* (Konopka et al., 2017a, 2017b; Haye et al. 2021; Mi et al., 2021). Strong aggressive host patch defense was observed also in other Scelionidae, *T. basalis* and *T. nigripedius* (Wilson 1961; Cumber, 1964; Mahmoud and Lim, 2008). Interactions could also be less aggressive in some scelionids, for example *Gryon* sp. nr. *gonikopalense* Sharma and *Trissolcus hyalinipennis* Rajmohana & Narendran, where both species appear to be more tolerant of the presence of interspecific foraging females (Hougardy and Hogg, 2021).

In our tests of extrinsic competition with asynchronously released *Trissolcus* females, parasitizations in progress by the resident female abruptly stopped upon arrival of a second female to the host egg mass, and the resident behavior changed from foraging to guarding and defense. In this situation, the time spent in parasitization by both females during the contest was not significantly different. Female *T. mitsukurii* were also able to access and dominate the host egg mass when it arrived first, and in some cases also when it arrived second. However, guarding behavior was strongly influenced by species arrival sequence, and *T. mitsukurii* spent the most time guarding eggs when it was the resident species, while no difference was detected between *T. japonicus* as the resident species and *T. mitsukurii* as the intruder. When *T. japonicus* was the intruder, it spent most of the time away from the egg mass. Guarding behavior in both species frequently led to an attack on the competitor, and *T. japonicus* was less successful when it was the intruder. No differences in number of attacks were detected when *T. japonicus* was the resident species and *T. mitsukurii* the intruder. However, contests with asynchronous arrival on the host egg mass by the two *H. halys* parasitoids resulted in greater success of the first-arriving species in term of total progeny produced. This was quite clear since there was less success in emergence when multiparasitism occurred and more of the half of egg mass was already parasitized by the first-arriving species. Similar results were observed by Field and Calbert (1999) during intraspecific competition by *T. basalis* with differences in arrival time of greater than 30 minutes.

Conclusions

Indirect competition between females of the two *Trissolcus* species led to more progeny produced by the first-arriving species, even though in most cases neither species recognized previously parasitized host eggs, resulting in multiple parasitism.

Although interspecific competition resulted in the death of the host close to 100%, both in direct and indirect competition, multiparasitism within *T. japonicus* and *T. mitsukurii* resulted in significant

waste of eggs and potential progeny of the second-arriving species. When simultaneously present, *T. mitsukurii* was more aggressive than *T. japonicus*, dominating the host resources and producing more progeny. When the arrival of females of the two species was asynchronous, although *T. mitsukurii* is also aggressive as the intruder, success in progeny production was biased in favor of the resident female. The interactions among the two species, despite the simplified arenas which cannot account for various environmental factors, may suggest that the reduced progeny production by the second arriving female could have negative impact on its dynamics population. However, conditions in the field and factors such as environment adaptations, host searching abilities and interaction with other species, are important factors that need to be further evaluated (Denno and Finke, 2006; Ode et al., 2022). While considering that data obtained in this study derive from simplified laboratory experimental conditions, results obtained may nevertheless be useful in designing plans for field releases within biological control programs, and may contribute to understanding evolution of competition in the field (Boivin and Brodeur, 2006; Collier et al., 2002; Denno and Finke, 2006). Indeed, outcomes of laboratory studies in simplified experimental microcosms need to be carefully evaluated since, as shown by Haye et al. (2021), increased oviposition pressure in confining test arenas can affect parasitization behavior and successful emergence of progeny, leading to an overestimation of effects that occur in the field. Thus, studies using more complex microcosms should be adopted for further studies of competition, and supported where possible by actual field data where the species of interest occur in sympatry.

Author Contributions:

Lucrezia Giovannini: Conceptualization, Investigation, Data curation, Writing – original draft, Writing – review & editing. Giuseppino Sabbatini-Peverieri: Conceptualization, Investigation, Data curation, Writing – original draft, Writing – review & editing. Sauro Simoni: Formal analysis, Writing – review & editing. Rita Cervo: Supervision, Writing – review & editing. Kim Alan Hoelmer: Supervision, Writing review & editing. Pio Federico Roversi: Funding acquisition, Resources, Supervision, Writing – review & editing.

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Chapter 4: Hyperparasitoid, an upper level in the trophic chain

4.1 Reproductive and developmental biology of *Acroclisoides sinicus*, a hyperparasitoid of scelionid parasitoids

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Abstract: *Acroclisoides sinicus* (Hymenoptera: Pteromalidae) was described in 1988 from China but recent findings in Europe and North America within the framework of *Halyomorpha halys* (Hemiptera: Pentatomidae) biological control indicate an Holarctic distribution. The few records and fragmented information on *A. sinicus* are derived from generic observations of other species belonging to the same genus, and its biological and ethological traits are still completely unexplored. It was suspected to be a facultative or obligate hyperparasitoid of many egg parasitoid species (e.g. Scelionidae and Eupelmidae), especially those parasitizing Pentatomidae eggs. Laboratory colonies of *A. sinicus* were established from specimens collected in the field in Europe and USA, which allowed us to investigate for the first time the life traits of this somewhat enigmatic species. Our studies confirmed the obligate hyperparasitoid hypothesis for species of Scelionidae, but not of Eupelmidae. Laboratory studies revealed that *A. sinicus* is extremely selective in its host recognition as only the pupal stage of its host species is exploited for parasitization. Taking into consideration its hyperparasitoid habit, the adventive *A. sinicus* populations in Europe and North America may potentially be severe threats to pentatomid natural control as new components in the trophic chain of pentatomids and their parasitoid guilds.

Keywords: *Halyomorpha halys*; *Trissolcus japonicus*; *Trissolcus mitsukurii*; Scelionidae; Eupelmidae; biological control

Introduction

Many members of the hymenopteran family Pteromalidae, one of the principal taxa of the superfamily Chalcidoidea, are difficult to identify taxonomically, and their biological and ethological traits are often poorly known (Bouček and Heydon, 1997). The members of this family are distributed

worldwide and most species are primary or secondary parasitoids of a great variety of insect species, and therefore play an important role in the natural control of pests (Bouček and Rasplus, 1991; Sureshan and Narendran, 2002; Grissell and Smith, 2006). In particular, the species of the genus *Acroclisoides* Girault and Dodd, which are distributed in Afrotropical, Australian, and South Asian regions, are thought to be facultative or obligate hyperparasitoids, principally of hemipteran eggs of the families Pentatomidae and Scutelleridae (Noyes, 2020; Rider, 2006). Primary parasitoids of the family Scelionidae appear to be the most frequent associations, but species of *Acroclisoides* also are associated with species of Eupelmidae (Sureshan and Narendran, 2002; Grissell and Smith, 2006; Clarke and Seymour, 1992). Despite attempts to study the biology of *Acroclisoides* species, the relationships with their hosts still remains largely unclear (Clarke and Seymour, 1992).

The Asian species *Acroclisoides sinicus* (Huang & Liao) (Hymenoptera: Pteromalidae) was recently discovered in central Europe (Northern Italy and Switzerland) and in North America (USA and Canada) during field surveys of *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) (Sabbatini Peverieri et al. 2019). These new findings show not only an Oriental (Huang and Liao, 1988; Xiao and Huang, 2000; Ko et al. 2018) but also a Holarctic distribution of this species.

In Europe, *Ac. sinicus* was found mainly associated with *H. halys* and its parasitoid guild, i.e. the native *Anastatus bifasciatus* (Geoffroy) (Hymenoptera: Eupelmidae), and the two adventive exotic species *Trissolcus japonicus* (Ashmead) (Hymenoptera: Scelionidae) and *Trissolcus mitsukurii* (Ashmead) (Hymenoptera: Scelionidae) (Sabbatini Peverieri et al. 2019) additional European records reveal associations also with the pentatomid *Palomena prasina* L. (Hemiptera: Pentatomidae) and the predatory pentatomid *Arma custos* (Fabricius) (Hemiptera: Pentatomidae) (Sabbatini Peverieri et al. 2019; Moraglio et al. 2019). In the USA (Georgia, Alabama and Maryland) and Canada, *Ac. sinicus* was found associated with at least 4 different pentatomid species (*H. halys*, *Chinavia hilaris* (Say), *Euschistus* sp. and *Brochymena* sp.) and in combination with three primary egg parasitoids, the eupelmid *Anastatus reduvii* (Howard) and the two scelionids, *Trissolcus edessae* Fouts and *Trissolcus euschisti* (Ashmead) (Sabbatini Peverieri et al. 2019). In addition, *Ac. sinicus* males and females have emerged from *Nezara viridula* (L.) (Hemiptera: Pentatomidae) egg masses parasitized by *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae) and *H. halys* egg masses parasitized by *Telenomus podisi* Ashmead (Hymenoptera: Scelionidae) collected from the field in the USA (PGT, unpublished data).

Biological and ethological traits of *Ac. sinicus* are still completely unknown and our work reports the first data on its biology under laboratory conditions. In this study we have hypothesized the possible hyperparasitoid habitus of this pteromalid species in relation to *T. japonicus* and *T. mitsukurii*, two Asian species which are now adventive in Europe, as primary parasitoids of *H. halys* (Sabbatini Peverieri et al. 2018; Stahl et al. 2019a). The European native species *An. bifasciatus*, a primary egg

parasitoid of pentatomids, was included in our study due to its frequent association with *Ac. sinicus* in the same egg masses. Additional observations were made of *Ac. sinicus* parasitizing a native European *Telenomus* sp. as a primary parasitoid of *Acrosternum heegeri* Fieber (Hemiptera: Pentatomidae); and of *Ac. sinicus* attacking *T. mitsukurii* as a primary parasitoid of the Palearctic pentatomid *Dolycoris baccarum* L. (Hemiptera: Pentatomidae) as a substitute for *H. halys* as primary host with *T. mitsukurii*. Moreover, the features of the immature stages of Pteromalidae are poorly known (Onagbola and Fadamiro, 2008; Tormos et al. 2009). We are not aware of any papers to date characterizing immature stages of any *Acroclisoides* species, therefore we schematically describe the juvenile development of *Ac. sinicus* in this work.

Material and Methods

Origin of the insects for studies in Europe

Acroclisoides sinicus were reared from *H. halys* egg masses collected during the summer of 2019 at infested sites in northeastern Italy (Sabbatini Peverieri et al. 2018; Sabbatini Peverieri et al. 2019). Field collected egg masses were reared in glass tubes (15 cm long and 2 cm wide, closed on both ends with a plastic net of 250µm mesh) and maintained in the climatic chambers at standard conditions of 26°C, 60% RH and 16:8 L:D until egg hatch or parasitoid emergence. Identification of adult parasitoids was based on recent taxonomic literature (Sabbatini Peverieri et al. 2019; Talamas et al 2017). The adults of *Ac. sinicus* were separated and placed in glass tubes, and reared in the climatic chambers under standard conditions using pure honey droplets as food. Voucher specimens of *Ac. sinicus* are deposited in CREA laboratory collections and are available upon request. *Trissolcus mitsukurii* and *An. bifasciatus* were obtained from laboratory colonies at CREA that originated from *H. halys* egg masses from infested field sites, while the *T. japonicus* colony was established with specimens supplied by the USDA-ARS Beneficial Insects Introduction Research Unit, in Newark, Delaware, USA (strain collected in Beijing, China) and imported into Italy under quarantine conditions and authorization number for laboratory studies DG/DISR/DISR05/0013647-19/04/2018. All colonies of the egg parasitoids were maintained on fresh *H. halys* egg masses (age < 24 h). Adult parasitoids were fed with pure honey droplets replenished twice a week. Adults and parasitized host eggs were reared in glass tubes in climatic chambers under the standard conditions mentioned previously.

Telenomus sp. (identified as *Telenomus* sp. 1, Francesco Tortorici collection, University of Turin, Italy) were obtained from egg masses of *A. heegeri* collected occasionally in 2020 during entomological field trips. A *Telenomus* sp. colony was reared and maintained under the same conditions as the other egg parasitoids, but using its natural host, *A. heegeri*.

The *H. halys* colony originated from individuals collected at infested sites in northern Italy in 2019 and 2020. Specimens were maintained in rearing cages (BugDorm® 4F4545 insect rearing cage, MegaView Science, Taiwan) using fresh fruits (apples, kiwi), vegetables (carrots, green beans), potted plants (soybean) and seeds (peanuts and soybean) as food; water was provided with wet cotton. The colony of *A. heegeri* and *D. baccarum* was established by collecting adults in the field during the 2019 and 2020 growing seasons, and reared in the lab under the same conditions as *H. halys*. Fresh pentatomid egg masses were collected daily from the colonies for the tests.

Establishment of Acroclisoides sinicus laboratory colonies in Europe

To test whether *A. sinicus* was a hyperparasitoid, we established lab-reared colonies of the primary parasitoids *T. japonicus*, *T. mitsukurii* and *An. bifasciatus*. Fresh *H. halys* egg masses were offered to females of the three parasitoids for primary parasitization. Considering the limited information available on *Ac. sinicus* reproductive biology, *H. halys* egg masses (n = 15 per species) were first parasitized by the primary parasitoids and after 24h offered to females of *Ac. sinicus* that had emerged from the egg masses collected in the field. To test the combination of *Ac. sinicus* with the two *Trissolcus* species, primary-parasitized egg masses were exposed to groups of three females of *Ac. sinicus* for 9 days (since under test conditions *Trissolcus* adults begin emerging from *H. halys* host eggs after 10 days of juvenile development in males and 11 days in females). To obtain an *Ac. sinicus* colony on *An. bifasciatus*, primary-parasitized egg masses were exposed for 20 days to females of *Ac. sinicus* (*Anastatus* adults begin emerging from *H. halys* eggs 22 days after oviposition under test conditions). In all combinations, after exposure to *Acroclisoides* the *H. halys* egg masses were maintained in climatic chambers under standard rearing conditions and checked daily for parasitoid emergence. Any newly emerged adults were removed as soon as possible to prevent intra- and interspecific interference. These F₁ generations of *Ac. sinicus* adults were used to produce the next five generations in order to obtain large rearing colonies maintained on the different primary parasitoids.

Rearing Acroclisoides sinicus in the USA under laboratory and field conditions

Acroclisoides sinicus was reared on *H. halys* egg masses parasitized by *T. euschisti*. Each of these species originated from individuals collected from infested sites in the Southeastern USA in 2019 and 2020 (parasitoids only). The colony of *H. halys* were maintained in rearing cages (27.9 cm long × 26.7 cm wide × 20.3 cm tall) on whole bean pods, apple slices, and raw peanuts at the USDA, ARS Southeastern Watershed Unit in Tifton, GA; knit cloth (97% cotton, 3% spandex) (Jo-Ann Stores, LLC, Hudson, OH) was used as a substrate for oviposition [18]. A *T. euschisti* colony was maintained in individual 25 x 150 mm culture tubes (Kimble®, Rockwood, TN) containing honey

and raisins. Refrigerated egg masses (≤ 12 h old when placed in a refrigerator with a temperature range of 2.8-3.3 degrees °C for 24 h) were used for hosts. Eggs were refrigerated to eliminate emergence of 1st instar *H. halys*, thus avoiding possible feeding by them on parasitized eggs. Individual female *T. euschisti* were provided with 2-3 *H. halys* egg masses for oviposition. After exposure to females, egg masses were held in a walk-in environmental chamber at 25°C $\pm$ 2.0°C, 70 $\pm$ 10%RH, and 16:8 L:D) until adult emergence. The first step in the process towards rearing *A. sinicus* was to offer a *H. halys* egg mass to two *T. euschisti* females for 2d in a culture tube. In 2019, 2, 3, 4, 6, and 8d old *T. euschisti* parasitized egg masses (n=10 for 2, 3, and 4d; n=25 for 6 and 8d) were exposed to a single *A. sinicus* female for 72h in a culture tube. Only one female was placed in a culture tube because females killed additional females. To start a new colony in 2020, individual 5d old *T. euschisti*-parasitized egg masses (n=30) were offered single *A. sinicus* females for 72h in a culture tube. After exposure to females, egg masses were held in a walk-in environmental chamber at the same settings above until adult emergence. These *A. sinicus* adults were used to produce four generations. In a preliminary timed exposure test in the field in 2020, lab-reared refrigerated *H. halys* egg masses were used as sentinels to provide hosts for detection of resident parasitoids in sassafras (*Sassafras albidum* (Nutt.) Nees) for 3, 4, 5, and 6d. In the laboratory, a portion of the knit cloth with an egg mass was cut from the cloth. Any damaged eggs were gently removed from an egg mass when eggs were counted. Each sentinel egg mass was placed on the underside of a sassafras leaf using a small hair clip. Three egg masses were deployed per treatment on 5 Sept. in Prattville, AL. Retrieved egg masses were held in a walk-in environmental chamber at the same settings above until adult emergence.

Acroclisoides sinicus reproductive biology studies in Europe

Fresh *H. halys* egg masses were offered for 24 h to females from laboratory colonies of the two primary egg parasitoids, *T. japonicus* and *T. mitsukurii*, to ensure complete parasitization. *Anastatus bifasciatus* was not tested further due to the failure to establish a colony (see Results). After primary parasitization, *H. halys* egg masses were reared in a climatic chamber under standard conditions to obtain a range of development stages of the primary egg parasitoid (see Table. 1 for a schematic representation of experimental designs). Preliminary studies of immature development of both *Trissolcus* species revealed that egg hatch and larval development from the first instar larva to the mature third instar larva occurred over 5 days (see Results, Tables 2 and 3). Morphological differences among these transitional stages are readily recognizable and correspond with the descriptions of the four scelionid species *Telenomus remus* (Nixon), *Telenomus solitus* Johnson, *Typhodites gerriphagus* (Marchal) and *Trissolcus basalis* (Woll.), respectively (see also Results, Fig.1) (Gerling, 1989; Navasero and Oatman, 1989; Sousa, 1999; Volkoff and Colazza, 1992).

Table 1. Schematic representation of the experimental studies of *Acroclisoides sinicus* reproductive biology. *Halyomorpha halys* egg masses were first parasitized by *Trissolcus* spp. (*T. japonicus* / *T. mitsukurii*) and subsequently exposed to females of *A. sinicus* in different combinations of ageing and exposure intervals. As a control, unparasitized fresh (< 24h) egg masses of *H. halys* were offered to females up to egg hatching (not shown here).

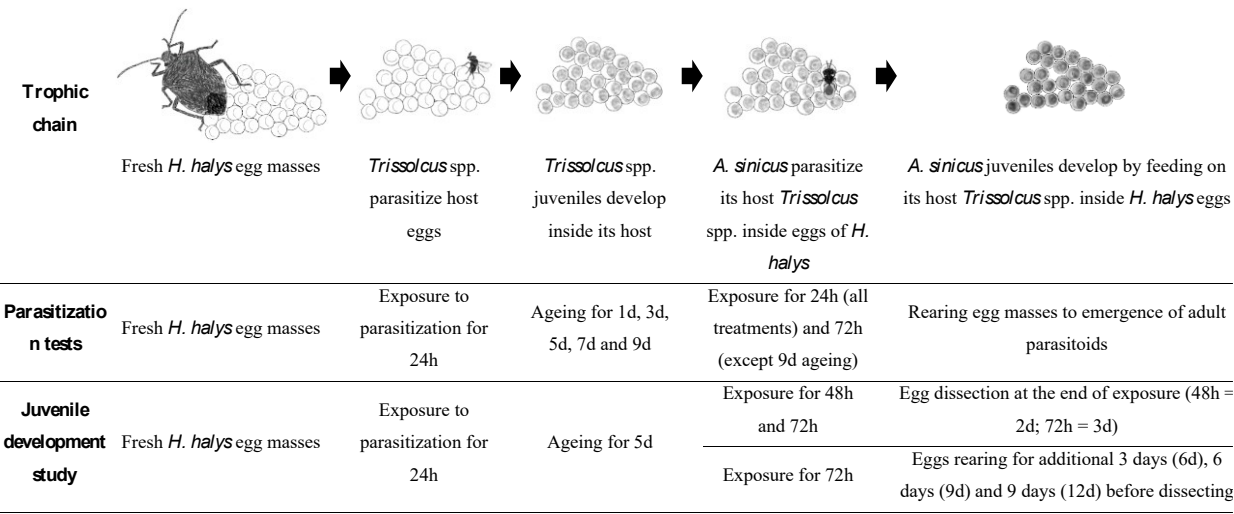
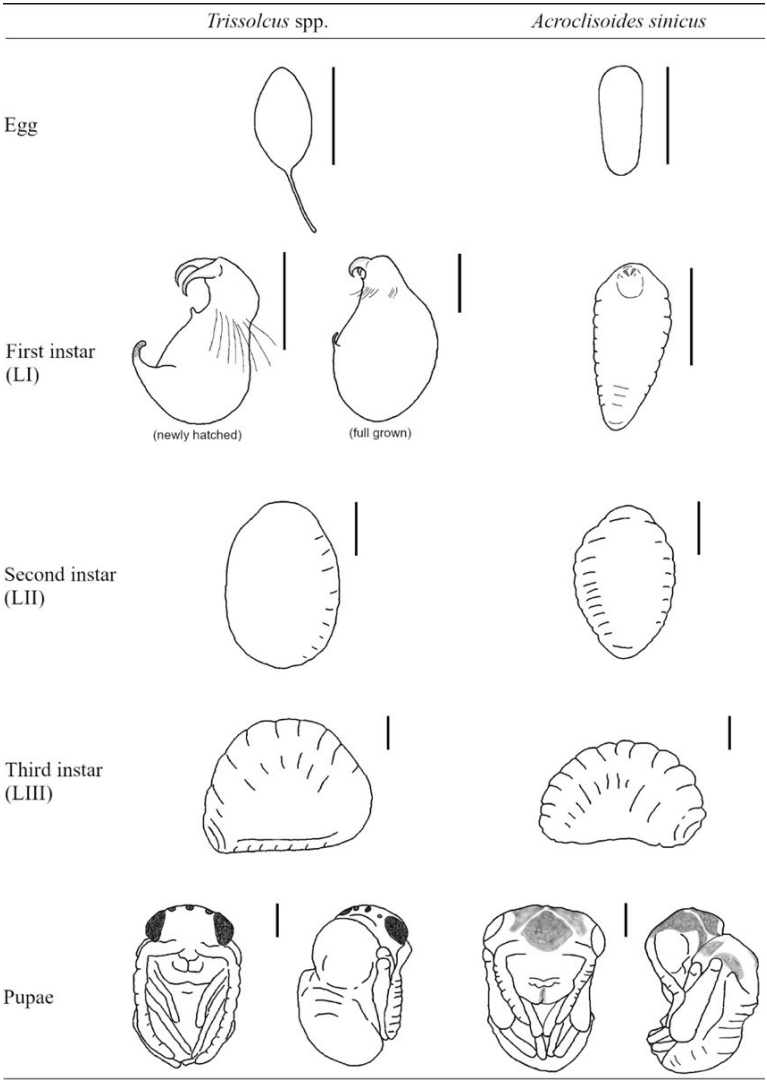


Figure 1. Schematic representation of juvenile developing stages of *Trissolcus* spp. (*T. japonicus* and *T. mitsukurii*) and *Acroclisoides sinicus*. Bars correspond to 200µm; drawings based on eggs and early larval stages mounted on microslides.



The pupal stage occurs approximately from 7 to 9 days at which time the parasite adult is recognizable (see Results, Tables 2 and 3). Our pre-liminary studies revealed that around day 5 following primary parasitization, the meconium is expelled and is often visible through the cuticle of the host egg (in *T. mitsukurii* an additional black ring appears below the operculum of the host egg) (Sabbatini Peverieri et al. 2019; Sabbatini-Peverieri et al. 2020). When this occurs the immature parasitoid enters, transitionally, to the pupal stage. Tests were conducted in a climatic chamber under standard conditions using single *Ac. sinicus* females 7–10 days old with no previous ovipositional experience. We assumed females to be mated if males were present in the rearing units prior to collecting females. Females were identified by the presence of a black spot on their fore wing (Sabbatini Peverieri et al. 2019). The black spot is not immediately apparent at female emergence but is clearly visible after 48h (GL and GSP, personal observations). Additionally, females have a physogastric abdomen enlarged with ovary growth. Egg masses containing primary parasitoids at various developmental stages (1, 3, 5, 7, and 9d after parasitization) were offered to *Ac. sinicus* females. *Ac. sinicus* females were exposed to each “age” class for 24h and 72h, except for the 9d group which was exposed only for 24h or 216h (9d).

Each treatment of parasitoid age/exposure time was replicated 5 times with a single female exposed to an egg mass in each replicate. Three weeks after exposure to *Ac. sinicus* females, *H. halys* eggs from which neither a nymph or parasitoid emerged, and recorded as pentatomid “dead eggs”, were dissected to detect the cause (dead primary parasitoid, dead *Ac. sinicus*, dead hemipteran, or undetermined content). To clarify whether *Ac. sinicus* could develop within an egg mass as a primary parasitoid (rebutting in this case our hypothesis), fresh egg masses of *H. halys* (n=20) were offered to single females in the same manner as described above and checked daily until eggs hatched or parasitoids emerged. To determine whether *Ac. sinicus* exhibits typical haplodiploid sex determination, a common trait in hymenopteran parasitoids, a fresh egg mass (n=28 eggs) of *H. halys* previously parasitized by *T. mitsukurii* was offered to an unmated female of *Ac. sinicus* for 9 consecutive days in the same manner as described above. Unmated *Ac. sinicus* females were obtained from rearing units from which males were removed shortly after emergence and prior to emergence of females, so that females that emerged had no opportunity to copulate.

Table 2. *Acroclisoides sinicus* reproductive success on egg masses of *Halyomorpha halys* parasitized by *Trissolcus japonicus* (means±s.e.); LI = first instar larvae, LII = second instar larvae, LIII = third instar larvae.

exposure combination (egg mass age / exposure time)	<i>T. japonicus</i> development stage	n. of eggs/egg mass	Adult emergence rate (%)		Dead <i>H. halys</i> eggs	sex ratio (% female)	
			<i>T. japonicus</i>	<i>A. sinicus</i>		<i>T. japonicus</i>	<i>A. sinicus</i>
24h x 24h	Egg	27.00 ± 0.70	93.68 ± 4.22	0.00 ± 0.00	6.46 ± 4.23	89.84 ± 2.74	0.00 ± 0.00
24h x 3d	Egg-LI	27.5 ± 0.28	99.07 ± 0.92	0.00 ± 0.00	1.85 ± 0.00	92.65 ± 2.65	0.00 ± 0.00
3d x 24h	LI	27.50 ± 0.50	97.11 ± 2.88	0.00 ± 0.00	2.88 ± 0.00	92.27 ± 1.96	0.00 ± 0.00
3d x 3d	LI-LII-LIII	25.25 ± 0.85	91.65 ± 4.82	0.00 ± 0.00	8.34 ± 4.82	95.99 ± 1.53	0.00 ± 0.00
5d x 24h	LIII	27.75 ± 0.25	94.60 ± 3.08	0.00 ± 0.00	5.39 ± 3.08	94.32 ± 1.81	0.00 ± 0.00
5d x 3d	LIII – pupae	28.00 ± 0.00	59.82 ± 7.95	19.64 ± 5.15	20.24 ± 1.79	88.10 ± 4.76	80.44 ± 8.33
7d x 24h	Pupae	26.75 ± 0.75	98.07 ± 1.92	0.00 ± 0.00	1.92 ± 0.00	91.38 ± 2.56	0.00 ± 0.00
7d x 3d	Pupae - pharate adult	27.40 ± 0.60	89.74 ± 2.88	1.43 ± 1.43	8.11 ± 1.87	89.29 ± 1.24	50.00 ± 0.00
9d x 24	Pharate adult	27.5 ± 0.57	92.75 ± 3.73	0.00 ± 0.00	7.24 ± 3.73	95.19 ± 0.79	0.00 ± 0.00
1d x 9d	From egg to pharate adult	25.56 ± 1.50	46.48 ± 4.95	23.21 ± 11.08	30.30 ± 6.46	53.50 ± 8.53	66.43 ± 17.34

Table 3. *Acroclisoides sinicus* reproductive success on egg masses of *Halyomorpha halys* parasitized by *Trissolcus mitsukurii* (means±s.e.); LI = first instar larvae, LII = second instar larvae, LIII = third instar larvae.

exposure combination (egg mass age / exposure time)	<i>T. mitsukurii</i> development stage	n. of eggs/egg mass	Adult emergence rate (%)		Dead <i>H. halys</i> eggs	sex ratio (% female)	
			<i>T. mitsukurii</i>	<i>A. sinicus</i>		<i>T. mitsukurii</i>	<i>A. sinicus</i>
1d x 24h	Egg	27.5 ± 0.51	100.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	61.57 ± 13.43	0.00 ± 0.00
1d x 3d	Egg-LI	27.0 ± 0.00	100.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	66.07 ± 19.64	0.00 ± 0.00
3d x 24h	LI	27.5 ± 0.52	100.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	52.98 ± 13.69	0.00 ± 0.00
3d x 3d	LI-LII-LIII	26.80 ± 0.73	100.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	87.42 ± 5.07	0.00 ± 0.00
5d x 24h	LIII	27.5 ± 0.57	95.01 ± 1.35	0.00 ± 0.00	4.98 ± 2.88	79.62 ± 4.76	0.00 ± 0.00
5d x 3d	LIII –pupae	25.8 ± 0.66	16.9 ± 0.00	33.01 ± 9.14	50.06 ± 10.56	100.00 ± 0.00	83.79 ± 7.95
7d x 24h	Pupae	28.0 ± 0.57	77.90 ± 18.58	1.23 ± 0.00	22.09 ± 18.58	77.9 ± 18.58	0.00 ± 0.00
7d x 3d	Pupae - pharate adult	26.2 ± 1.11	47.21 ± 18.32	27.00 ± 10.51	26.25 ± 17.51	96.87 ± 1.99	61.79 ± 17.25
9d x 24	Pharate adult	27.5 ± 0.57	100.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	93.69 ± 1.13	0.00 ± 0.00
1d x 9d	From egg to pharate adult	25.40 ± 1.20	14.40 ± 0.00	85.60 ± 14.40	0.00 ± 0.00	94.44 ± 0.00	90.69 ± 1.76

Acroclisoides sinicus immature development study in Europe.

Single egg masses ($n = 15$) of *H. halys* previously parasitized by *T. mitsukurii* and aged for 5 days to permit immatures of the primary parasitoid to reach the mature larval stage and enter into the pupal stage (see Results), were offered to groups of three *Ac. sinicus* females (7–10 days old). Three egg masses were exposed for 48h (2d) and three egg masses for 72h (3d) and then dissected. The rest of the egg masses were exposed for 72h to *Ac. sinicus* females and then dissected after three (6d total time), six (9d total time) and nine (12d total time) days from removal of females. Eggs were dissected under a stereomicroscope and the contents carefully inspected. Microslides were prepared when needed for examination of eggs or early larvae. Careful characterization of the three larval instars is critical for assessing the developmental age in species of Pteromalidae. Previous works (Onagbola, and Fadamiro, 2008; Tormos et al. 2009) separated larval ages through measurements of morphological features (mandible length and head capsule width) obtained by scanning electron microscopy in conjunction with statistical analysis for *Pteromalus cerealellae* (Ashmead) (Hymenoptera: Pteromalidae) and *Spalangia cameroni* Perkins (Hymenoptera, Pteromalidae), respectively, and recognized three larval instars in both species. In *P. cerealellae* and *S. cameroni* the second larval instar was approximately 2–3 times bigger in size than the first instar larvae, and the third instar larva was twice as large as the second one. Being aware of the complexity of instar determination in pteromalids, a detailed description of larval instars of *Ac. sinicus* will be a forthcoming study, and in our preliminary work we followed a descriptive and practical approach in referring to three larval instars of *Ac. sinicus* based on body growth following observations reported in (Onagbola, and Fadamiro, 2008; Tormos et al. 2009) (see Results, Fig. 1). The immature development study was conducted in a climatic chamber under standard conditions.

Development of Acroclisoides sinicus in association with native European pentatomids

Fresh egg masses of *A. heegeri* ($n = 3$, 27 eggs in total) were exposed to females of *Telenomus* sp. for 24h for parasitization. Shortly after primary parasitization, egg masses were exposed for nine days to groups of three female *Ac. sinicus* (7–10 days old) obtained from the colony reared on *T. mitsukurii*. After exposure, egg masses were held individually in climatic chambers under standard conditions until parasitoids emerged. The same experimental design was used to test the suitability of *D. baccarum* replacing *H. halys* as the primary host in the trophic chain. A fresh egg mass of *D. baccarum* ($n=1$, 21 eggs) was exposed to females of *T. mitsukurii* for 24h for parasitization. After primary parasitization, the egg mass was exposed for nine days to groups of three female *Ac. sinicus* (7–10 days old) obtained from the colony reared on *T. mitsukurii*. After exposure, the egg mass was held in a climatic chamber under standard conditions until parasitoids emerged.

Acroclisoides sinicus longevity tests in Europe

Newly emerged *Ac. sinicus* adults (males and females) reared on *T. mitsukurii* were isolated individually in glass tubes and provided either with droplets of pure honey or without honey (water was not provided in any case). Specimens were maintained under standard conditions in a climatic chamber without exposure to hosts and checked daily for mortality. Longevity was similarly assessed using *Ac. sinicus* from the *Telenomus* sp. colony that were fed with honey.

Polyphenism and female egg load of Acroclisoides sinicus study in Europe

To determine whether host size influenced body size of adult *Ac. sinicus*, we measured the thorax width of *Ac. sinicus* emerging from *A. heegeri* eggs parasitized by *Telenomus* sp., and from *T. mitsukurii* parasitized *H. halys* eggs from our laboratory colonies.

Morphological characteristics of the pentatomid eggs, including egg height (H), egg width ($R=D/2$) and width of the operculum ($r=d/2$), were also measured ($n = 12$ eggs per host species). Egg volume (V) was estimated using the parabolic barrel formula $V = \pi H(3r^2 + 4Rr + 8R^2)/15$ (Abram et al. 2015; Botch and Delfosse, 2018). The measurements of *Ac. sinicus* body size and the egg volume of pentatomid species were compared with Student's *t*-test.

To determine ovarian egg load, newly emerged *Ac. sinicus* females reared on *T. mitsukurii* from the *H. halys* laboratory colony were randomly chosen and placed individually in glass tubes provided with honey droplets and reared in a climatic chamber for 1, 7, 14 or 21d. After each interval, five *A. sinicus* females were dissected to assess the egg load in their ovaries. Eggs were stained with 1% toluidine blue and counted under a stereomicroscope. *Ac. sinicus* egg dimensions (length and width) were measured on 15 eggs randomly chosen from four females.

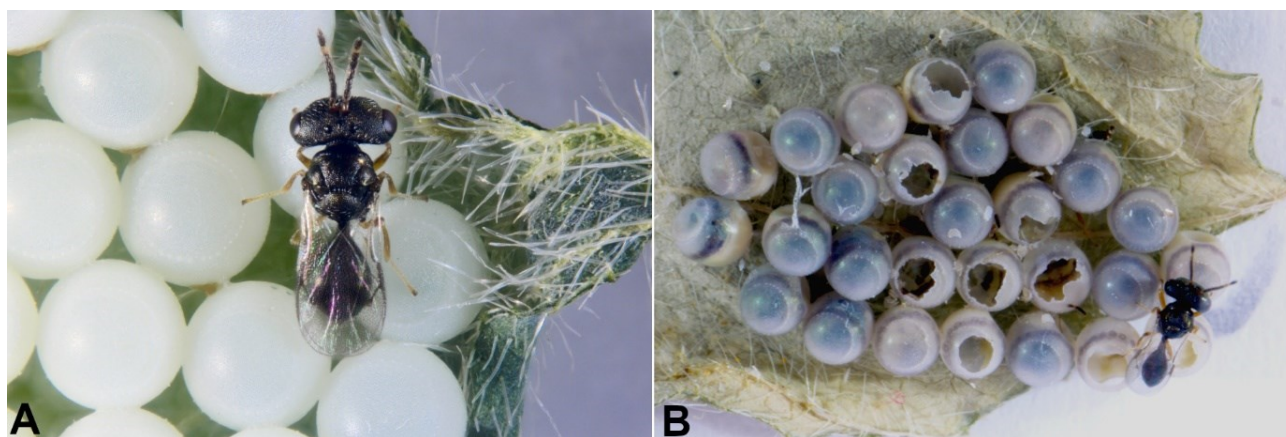
Results

Establishment of laboratory reared colonies in Europe

Starting from *Ac. sinicus* females obtained from field-collected *H. halys* egg masses, the establishment of lab-reared colonies was successful on *H. halys* eggs (Fig. 2A) parasitized by *T. japonicus* and *T. mitsukurii*, but not with those parasitized by *An. bifasciatus*. Despite several attempts to establish a colony on *H. halys* egg masses ($n=15$) first parasitized by *An. bifasciatus*, in no case was an *An. sinicus* F₁ generation obtained under laboratory conditions. From *H. halys* egg masses offered to *An. bifasciatus* for primary parasitization and then to *Ac. sinicus*, a mean of $56.4 \% \pm 9.58$ *An. bifasciatus* and $37.71 \% \pm 8.58$ *H. halys* nymphs emerged while $5.88 \% \pm 1.86$ of the eggs died. The contents of dissected dead eggs could not be determined and no *Ac. sinicus* mature larvae, pupae or pharate adults were observed.

Several *Ac. sinicus* females that emerged from field-collected *H. halys* egg masses and held in the confined space of glass tubes were frequently observed to display aggressive behaviors such as biting the antennae, mouthparts and wings of other females. Aggressivity among females was observed within the rearing tubes, but was more frequent when primary parasitized egg masses were available, and host guarding behaviour was observed when potential competitors were present (see supplementary video S1). Mating was observed while handling colonies: males emerged (generally one per egg mass) ca. one day before females and lingered on the egg mass waiting for female emergence (Figure 2B); upon female emergence, mating occurred promptly and lasted just a few seconds.

Figure 2. *Acroclisoides sinicus*: (A) a female on egg mass of *Halyomorpha halys*; (B) a male is attending the egg mass waiting for newly emerged females to copulate.



Reproductive biological traits of Acroclisoides sinicus in Europe and USA

Acroclisoides sinicus emergence was obtained when egg masses previously parasitized by the two *Trissolcus* species were exposed to females of *Ac. sinicus* for nine consecutive days after parasitization by the primary scelionid, and when exposing 5d and 7d old primary-parasitized egg masses for 72h to the females (Tables 2 and 3). No other exposure combinations produced any *Ac. sinicus* progeny; from those others, only *T. japonicus* or *T. mitsukurii* adults emerged. The single exception occurred for the combination of 7d x 24h with *T. mitsukurii* as the primary parasitoid, from which only one male *A. sinicus* emerged (Table 3). In no combination did any *H. halys* eggs hatch. When fresh unparasitized egg masses of *H. halys* were exposed to females of *Ac. sinicus*, only nymphs of the pentatomid emerged ($96.42\% \pm 0.87$ hatched nymphs).

In tests with *T. japonicus* as the primary parasitoid, followed by dissection of dead pentatomid eggs from the 5, 7 and 9d age combinations with the 72h *Ac. sinicus* exposure, means ($\pm$ s.e.) of $7.14\% \pm 3.27$, $3.77\% \pm 1.47$ and $8.08\% \pm 1.83$ per egg mass, respectively, contained pupae and/or adults of *Ac. sinicus*, while means of $17.85\% \pm 4.00$, $4.37\% \pm 2.07$, and $13.82\% \pm 0.00$ per egg mass,

respectively, had undetermined contents. Only in the test with nine days of exposure (1d x 216h [9d]) was it possible to detect dead mature larvae and dead pupae of *T. japonicus* ($8.25 \% \pm 2.28$) within dead pentatomid eggs. In all combinations with parasitoid emergence, both sexes of the primary parasitoid *T. japonicus* and *Ac. sinicus* were represented (Table 2).

In tests with *T. mitsukurii* as the primary parasitoid, dissection of the dead pentatomid eggs in the 5 and 7d age combinations with the 72h *Ac. sinicus* exposure revealed that mean values ($\pm$ s.e.) of $6.23 \% \pm 1.95$ and $1.62 \% \pm 1.00$ per egg mass, respectively, contained pupae and adults of *Ac. sinicus*; a mean of $2.50 \% \pm 2.50$ per mass had undetermined contents for the 5d combination. In all combinations with parasitoid emergence, both sexes of the primary parasitoid *T. mitsukurii* and the mated hyperparasitoid were represented (Table 3). Unmated *Ac. sinicus* females that had been exposed to parasitized *T. mitsukurii* immatures produced only male progeny.

In the USA, *Acroclisoides sinicus* emergence in the laboratory in 2019 was obtained only when exposing 6d and 8d old *T. euschisti* parasitized egg masses for 72h to the females. For 6d old *T. euschisti* parasitized egg masses, *Ac. sinicus* males and females emerged from 25% of the egg masses while for 8d old parasitized egg masses, *Ac. sinicus* males and females emerged from 33.3 % of the egg masses. In 2020, both sexes of *Ac. sinicus* emerged from 100% of the egg masses previously parasitized by *T. euschisti* aged for 5d and exposed to hyperparasitism for 72h.

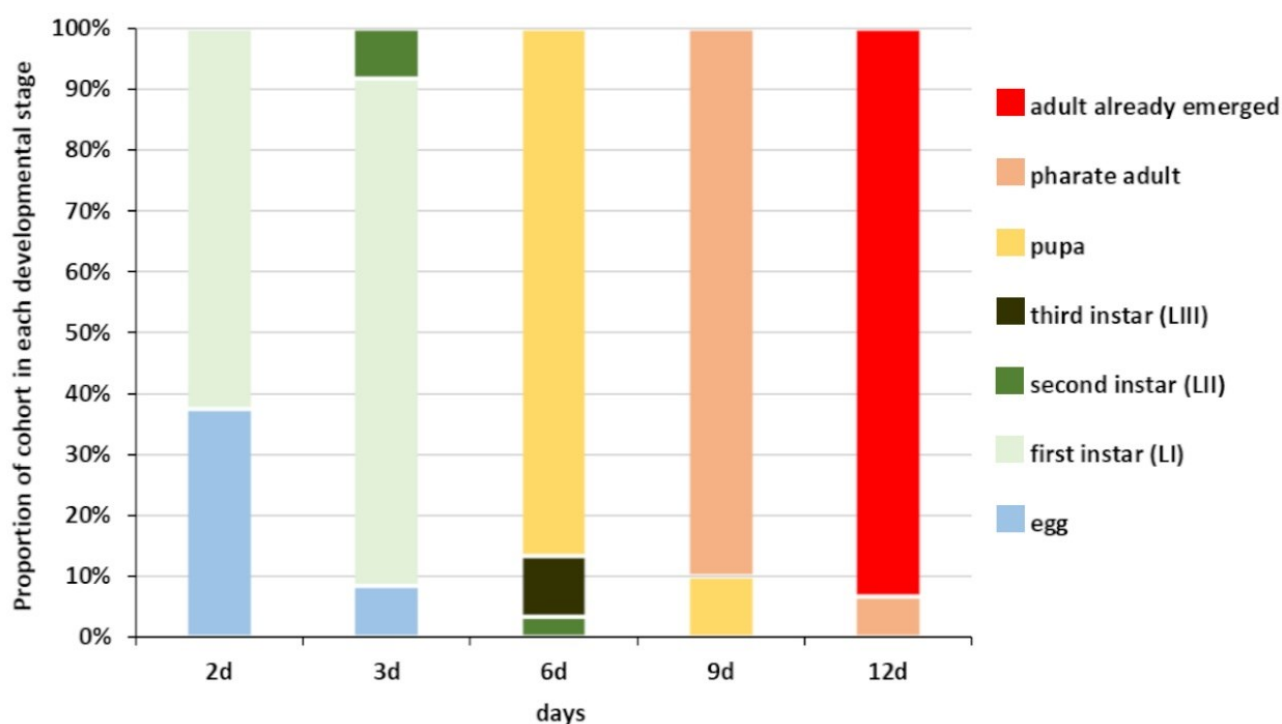
In the preliminary timed exposure test on sassafras in 2020, parasitism of scelionids by *Ac. sinicus* was significantly influenced by the duration of exposure time of *H. halys* eggs masses used as sentinels on leaves of this tree. Adult *Ac. sinicus* did not emerge from egg masses exposed in the field for 2d and 4d. However, a single *Ac. sinicus* male emerged from an egg mass exposed for 5days; 3 *Ac. sinicus* larvae and 14 larvae and one pupa of *T. podisi* died. Adult *Ac. sinicus* and *T. podisi* emerged from two egg masses exposed for 6d. From one egg mass, 6 *Ac. sinicus* adults (5 females, 1 male) and 10 *T. podisi* adults (6 females, 4 males) emerged; 2 *Ac. sinicus* larvae and 4 larvae and 1 pupa of *T. podisi* died. From the second egg mass, 4 females of both *Ac. sinicus* and *T. podisi* emerged; 2 *Ac. sinicus* larvae and 7 larvae and 1 pupa of *T. podisi* died; one overwintering 3rd instar of *Ac. redivii* was detected. Because *T. podisi* adults emerged from egg masses deployed in the field for 6d, it was likely the primary scelionid host species parasitized by *Ac. sinicus*. The results of the field exposure test agreed with those of the laboratory study.

Acroclisoides sinicus immature development studies in Europe

Using *H. halys* egg masses previously parasitized by *T. mitsukurii* and aged for 5 days (permitting *T. mitsukurii* specimens to reach the transition stage from mature larva to pupa), considerably less of 40% of *H. halys* eggs contained hyperparasitoid eggs after a 48h initial exposure to *Ac. sinicus* females (“2d”), and the rest contained immatures of the first larval instar (LI) (Figures 3 and 4A,B).

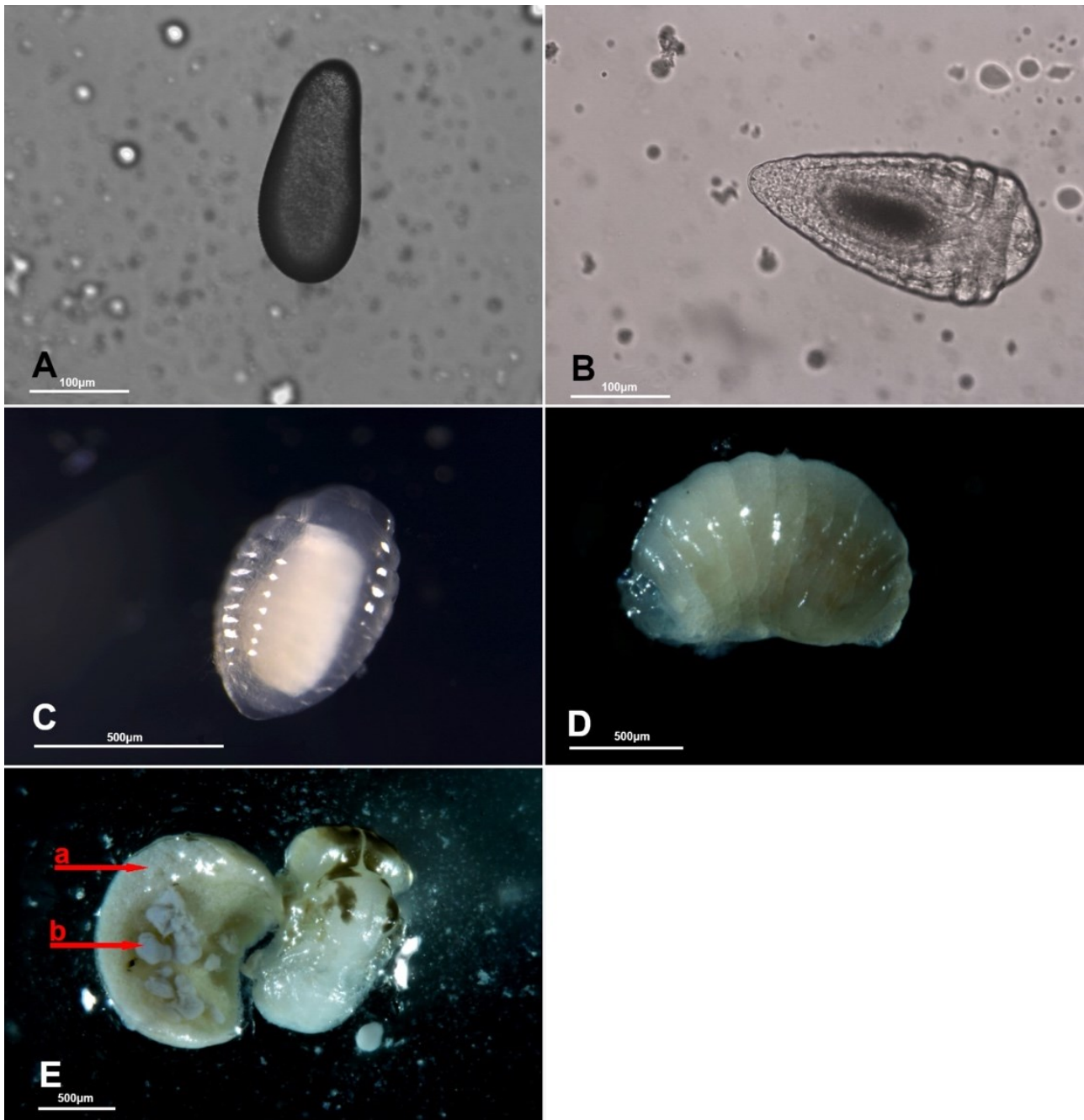
At the end of the 72h exposure to *Ac. sinicus* females (“3d”), most of the immatures were in the first larval stage (LI). After a 72h exposure to *Ac. sinicus* females and successive rearing of the hyperparasitized *H. halys* egg masses, the following development stages were observed: after three days of rearing (“6d”) most of the immatures were pupae with a few remaining as second and third larval instars (LII and LIII, respectively) (Figures 3, and 4C,D,E). After 6 days of rearing (“9d”) most of the specimens were in transition between dark pupae and pharate adults; after 9 days of rearing (“12d”), most of the adults had already emerged. A schematic representation of the immature stages of parasitoids is shown in Fig. 1.

Figure 3. Phenology of *Acroclisoides sinicus* immature development in *Halyomorpha halys* egg masses previously parasitized by *Trissolcus mitsukurii*. Primary-parasitized egg masses were aged for 5 days before exposing to *A. sinicus* females. Hyperparasitized *H. halys* eggs were dissected after the following ageing periods: 2d = after 48h of continuous exposure to females; 3d = after 72h of continuous exposure to females; 6d=72h exposure + 3 days ageing, 9d=72h exposure + 6 days ageing; 12d=72h exposure + 9 days ageing.



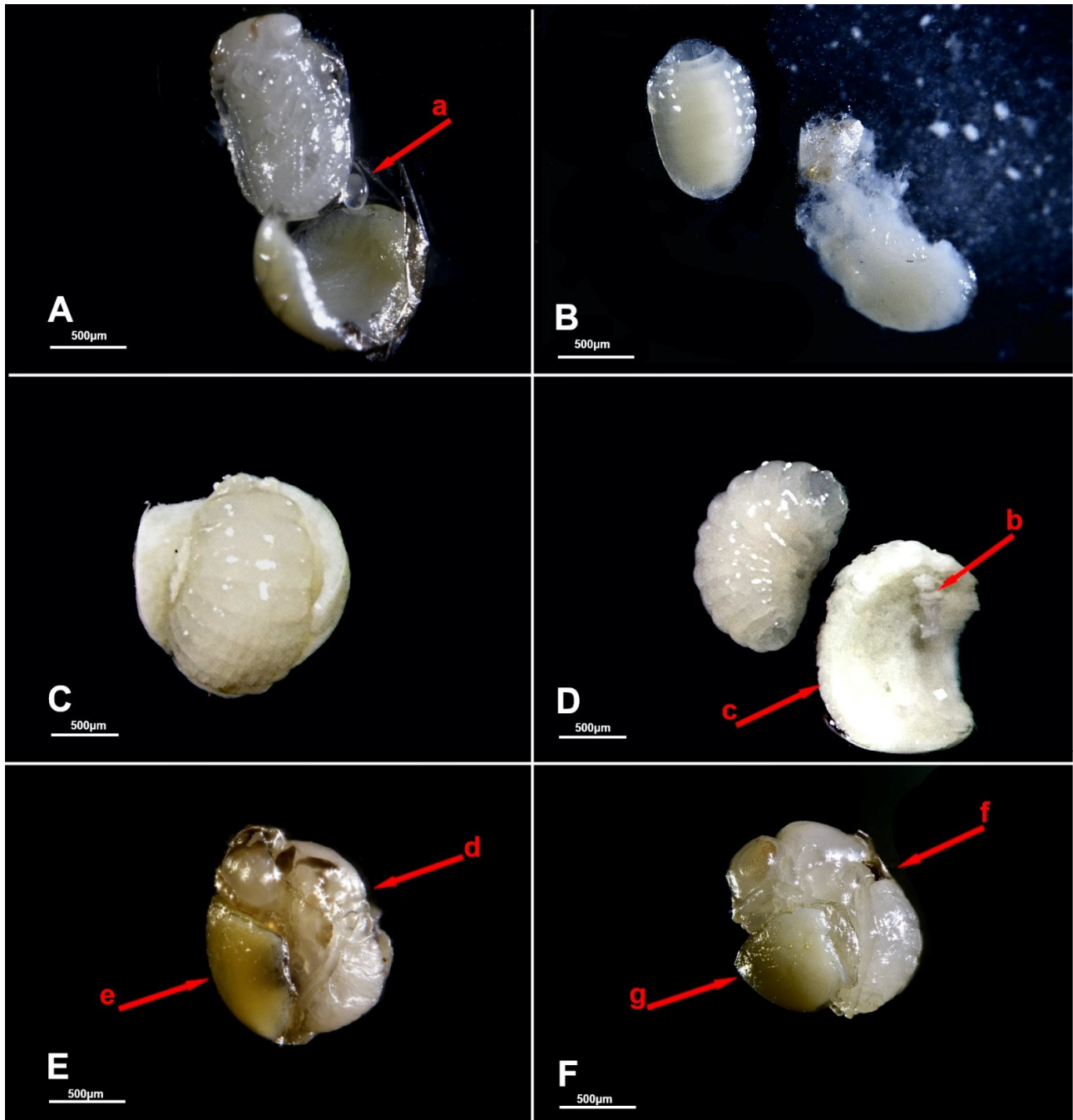
Eggs of *Ac. sinicus* are typically hymenopteriform (spindle-shaped) with a smooth surface, while those of *Trissolcus* species are stalked (with a tube-like extension at one end) (Clausen, 1940). All larval instars of *Ac. sinicus* are typically hymenopteriform (spindle-shaped, body segmented) with two horn-like mandibles, whereas in scelionids the first instar larva is teleaform (body segmentation not visible, cephalothorax separated from abdomen by a constriction, strong mandibles very large and curved, sclerotized caudal process) and the older instars are hymenopteriform (Clausen, 1940) (Fig. 1). Dissections of *T. mitsukurii* hyperparasitized by *Ac. sinicus* in *H. halys* eggs revealed that this hyperparasitoid is an ectoparasitoid, with the first larval stage starting to feed on the body of its host and eventually completely consuming it (Figure 5A,B).

Figure 4. *Acroclisoides sinicus*: (A) egg and (B) first instar larvae (LI); (C) second instar larvae (LI), (D) third instar larvae (LI) and pupae (E) ; arrow a = large, semicircular meconium of the host species (*Trissolcus* sp.), arrow b = meconium of *A. sinicus* appear as dark-grey grains above the creamy-white meconium of its host.



Eventually the *Ac. sinicus* mature larvae (and successively the pupae) completely occupies the space within the *H. halys* egg (Figure 5C,D,E) which would have been occupied otherwise by the pupae of its *Trissolcus* host (Figure 5F). Traces of meconium of *Ac. sinicus* expelled by the mature larvae before pupating are clearly visible close to the meconium of its host (Figures 4E, 5D).

Figure 5. *Acroclisoides sinicus* dissected from *Halyomorpha halys* eggs (eggs dissected and chori-on removed): (A) first instar larvae (LI, arrow a) adhering externally to its host, the pupa of *Trissolcus japonicus* and its meconium (partially separated for viewing); (B) third instar larvae (LIII) and residues of its host after complete feeding; (C) third instar larvae (LIII) adhering to meconium of its host *Trissolcus mitsukurii*; (D) meconium of the host (arrow c) and first traces of *Ac. sinicus* meconium (arrow b) (partially separated for viewing); (E) pupa of *Ac. sinicus* (arrow d) adhering to the meconium of its host (arrow e) and displacing its volume within the *Halyomorpha halys* egg; (F) *Trissolcus mitsukurii* pupa (arrow f) and its meconium (arrow g) when not parasitized by *Ac. sinicus*.

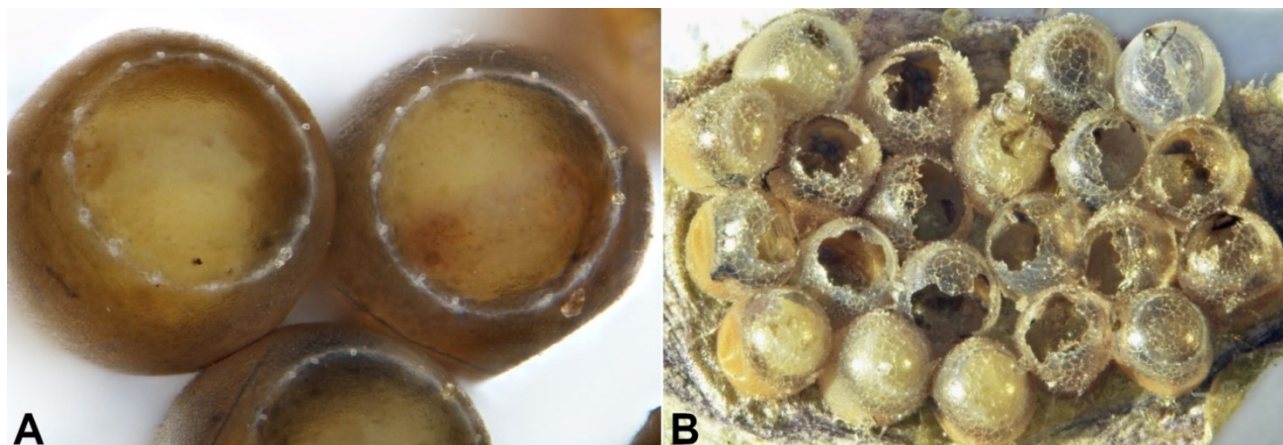


Development of Acroclisoides sinicus in combinations with European species

From the three egg masses of *A. heegeri* previously parasitized by *Telenomus* sp. and then exposed to *Ac. sinicus* females, 3 male and 7 female *Ac. sinicus* emerged from one egg mass (Figure 6); from the other two egg masses 21 specimens of *Telenomus* sp. emerged. From the single egg mass

of *D. baccarum* previously parasitized by *T. mitsukurii* and then exposed to females of *Ac. sinicus*, 2 male and 10 female *Ac. sinicus* emerged, one pentatomid egg hatched and 8 eggs were dead with no emergence.

Figure 6. *Acroclisoides sinicus*: pupae inside eggs of *Acrosternum heegeri* previously parasitized by *Telenomus* sp. (A), and adult emergence holes from eggs of *Dolycoris baccarum* primary-parasitized by *Trissolcus mitsukurii* (B).



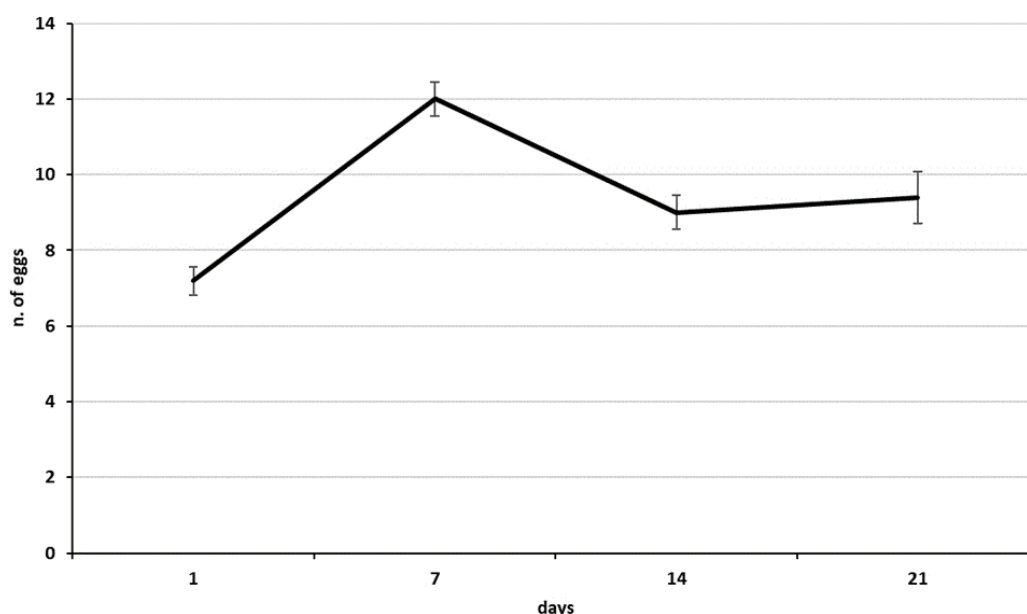
Acroclisoides sinicus longevity tests in Europe.

Honey-fed females of *A. sinicus* developing on *H. halys* egg masses previously parasitized by *T. mitsukurii* lived for a mean of 45.55 days (± 5.09 s.e., $n = 11$), and males survived for 22.5 days (± 3.28 s.e., $n = 10$). Starved females survived for only 5.4 days (± 0.16 s.e., $n = 10$). Honey-fed females of *Ac. sinicus* developing on *A. heegeri* egg masses previously parasitized by *Telenomus* sp. lived for 26.0 days (± 2.51 s.e., $n=6$), and males lived for 16.0 days (± 2.0 s.e., $n=2$).

Polyphenism and female egg load of Acroclisoides sinicus

Adults of both *Ac. sinicus* sexes that emerged from *H. halys* + *T. mitsukurii* were larger than those that emerged from *A. heegeri* + *Telenomus* sp. The thorax width of *A. sinicus* from both host species ($616.22 \mu\text{m} \pm 6.19$ s.e. from *H. halys*; $451.80 \mu\text{m} \pm 6.56$ s.e. from *A. heegeri*) was significantly different ($t=13.48$, $df=25$, $P<0.0001$). Eggs of the two pentatomid hosts also varied in volume: *H. halys* eggs were significantly larger ($t=27.95$, $df= 20$, $P<0.0001$,) than those of *A. heegeri* (1.48 mm^3 and 0.61 mm^3 , respectively). The egg load in ovaries of females that emerged from *H. halys* eggs first parasitized by *T. mitsukurii*, without ovipositional experience varied from 7 to 12 eggs (Figure 7). Mean length and width of *Ac. sinicus* eggs were $0.262 \text{ mm} \pm 0.003$ s.e. and $0.106 \text{ mm} \pm 0.002$ s.e., respectively.

Figure 7. Mean egg load ($\pm$ S.E) in ovaries of *Acroclisoides sinicus* from the first day after emergence throughout 3 weeks of adult life (from *Halyomorpha halys* egg masses first parasitized by *Trissocus mitsukurii*).



Discussion

The life cycle of *Ac. sinicus* is poorly known from literature, but our laboratory study provides important details about its biology that will be valuable for planning more detailed biological studies in the future, such as studies of trophic host-parasitoid chains. The frequent association with different species of field-collected pentatomid egg masses worldwide suggested that this species could be an obligate or facultative hyperparasitoid of primary egg parasitoid species belonging to the genera *Trissolcus*, *Telenomus* and *Anastatus* (Clarke and Seymour, 1992; Sabbatini Peverieri, 2019). Our results have confirmed the hypothesis of obligate hyperparasitism by *Ac. sinicus* of several *Trissolcus* and *Telenomus* species present in Europe and the USA, and it also explains several of the species associations reported in the literature. However, we were not able to rear *Ac. sinicus* using *An. bifasciatus* as a primary parasitoid, suggesting that this eupelmid species may not be a suitable host, at least under laboratory conditions. Since both these parasitoid species emerged from field-collected egg masses, their interactions are in need of further studies. The absence of parasitoid progeny after offering females unparasitized egg masses of *H. halys* confirmed that *Ac. sinicus* is unable to be a primary parasitoid of *H. halys*, at least under our test conditions. Along with several other parasitoid associations recently document-ed worldwide (Sabbatini Peverieri, 2019; Moraglio et al. 2019), and the results of our laboratory tests using different hosts with primary parasitoids, we conclude that *Ac. sinicus* is an obligate hyperparasitoid of scelionids, but not of eupelmids.

There is a very precise parasitization window for *Ac. sinicus* females to deposit their own eggs in their hosts to ensure their development, i.e. the time frame represented by the transition from mature

larva and meconium expelling to pupal stages, or perhaps only the pupal stage of its host. Production of *Ac. sinicus* offspring was observed only in primary parasitoid combinations with *T. japonicus* and *T. mitsukurii* pupal stages (*H. halys* egg dissections revealed *Ac. sinicus* eggs and early instar larvae only associated with the presence of scelionid pupae). This was also demonstrated by the co-presence of the meconium of the primary parasitoid and that of the hyperparasitoid within the *H. halys* egg; thus, the pentatomid egg shell provides a protected environment for developing *Ac. sinicus* immatures, which feeds on scelionid pupae. In all cases so far observed, the meconium of *Ac. sinicus* is deposited directly on the meconium of its host and not randomly within in the pentatomid egg (Sabbatini Peverieri, 2020). The olfactory cues in the meconium could assist female *Ac. sinicus* in their search for hosts and subsequent acceptance, as is known to be the case with many parasitoid and predator species (Steidle and vanLoon, 2002). Progeny production was not observed when larval instars or pharate adults of *Trissolcus* species were offered for parasitization.

Hyperparasitism can occur with different levels of specialization depending on the associations between different host parasitoid and hyperparasitoid species. For example, the eupelmid *An. bifasciatus* is known to be a broadly generalist primary parasitoid, able to exploit both pentatomid and lepidopteran eggs, but it is also a facultative hyperparasitoid of several primary parasitoids at different development stages, including *T. japonicus* (Viggiani, 1994; Konopka et al. 2017; Stahl et al. 2020). Many pteromalids are pupal parasitoids, and *Ac. sinicus* is not an exception.

It was not possible to clearly define developmental time for *Ac. sinicus* immature stages because progeny production was obtained only by exposing the host for 72h to parental females. Progeny production was not successful with only a 24h exposure time except in a single case. The parasitization process likely occurs between the second and third day of host exposure, thus the developmental time of *Ac. sinicus* should be between 10 or 11 days. The single exception of progeny production after a 24h host exposure resulted in a 10d developmental time and produced a male. A similar developmental time was reported for the pteromalid *P. cerealellae* (Onagbola and Fadamiro, 2008). Under the same rearing conditions, immature development of *Ac. sinicus* is similar to that of *Trissolcus* species attacking *H. halys* (Hokyo and Kiritani, 1966; Yang et al. 2009).

Males always emerge a day before the females and mating occurs immediately on the egg mass, evidence that sib-mating occurs frequently, as observed for *P. cerealellae* (Onagbola and Fadamiro, 2008). Sib-mating is also a common behaviour in egg parasitoids of pentatomids such as *T. japonicus* and *Gryon pennsylvanicum* (Ashmead) (Hymenoptera: Scelionidae) (Yang et al. 2009; , Sabbatini Peverieri et al. 2012).

As shown by our preliminary studies on immature development of *T. japonicus* and *T. mitsukurii*, the first larval stages are teleaform, which is typical for scelionids , and mandibles are abnormally developed compared to the last larval instar. Different types of mandibles may reflect different

behaviors by first instar larvae of these parasitoids. *Trissolcus japonicus* and *T. mitsukurii* may be adapted to develop in a more competitive environment, fighting for resources with competitors in the *H. halys* parasitoid guild. In contrast, *Ac. sinicus* larvae apparently develop in a less competitive environment, attacking a host that is generally less active, i.e. the pupa of its scelionid host. In all cases, the purpose of mandibles of such larvae may be to puncture the host integument and imbibe fluid food oozing from the host body, as described for *S. cameroni* (Tormos et al. 2009). A detailed study of immature development and morphology on *T. japonicus* and *Ac. sinicus* is currently in progress (Sabbatini Peverieri et al. in prep.).

When provided with carbohydrate in the form of honey, *Ac. sinicus* adults live a long time in the lab, similar to *T. japonicus* or *An. bifasciatus* (Sabbatini Peverieri et al. 2020; Stahl et al. 2019). Without food *Ac. sinicus* can survive for several days, much longer than *Trissolcus* (Sabbatini Peverieri, 2020). Longevity seems to be host dependant, for *Ac. sinicus* females developing on the *H. halys* - *T. mitsukurii* combination lived twice as long as those developing on *A. heegeri* - *Telenomus* combination. These differences could due to the quantity and/or quality of food resources of the different hosts: *Ac. sinicus* that developed on the *A. heegeri* - *Telenomus* sp. association are smaller than those that developed on *H. halys* - *T. mitsukurii* one, and *Ac. heegeri* eggs are smaller than those of *H. halys*. This was also seen in *T. mitsukurii*, whose adults live longer when they develop on *H. halys* eggs than on *N. viridula* eggs (Arakawa et al. 2004; Boyle and Weber, 2020).

The Asian *Ac. sinicus* attacks not only several exotic *Trissolcus* species that have become invasive in Europe, but also several European native egg parasitoids, developing in different host associations, such as *Telenomus* sp. developing on *Ac. heegeri* or *T. mitsukurii* developing on *D. baccarum*. Emergence of *Ac. sinicus* males and females from field-collected egg masses strongly suggests that that this hyperparasitoid might also attack at least some of the native *Trissolcus* and *Telenomus* species parasitizing a range of pentatomid host eggs in the USA (Sabbatini Peverieri et al. 2019), and development of *Ac. sinicus* in the laboratory confirms its hyperparasitism behavior of the North American scelionid *T. euschisti*. In addition to results reported in this paper, rearing records from other field surveys reported the development of *Acc. sinicus* on (unidentified) parasitoids within the pentatomid genera *Brochymena* sp., *C. hilaris*, *Euschistus* sp., *P. prasina* and *H. halys* [Sabbatini Peverieri et al. (2019) and KAH unpublished data]. The hyperparasitism of scelionids, including *T. japonicus* as shown in our study, could have important consequences for classical biological control of *H. halys* by release of exotic parasitoids (independently of *T. japonicus* or *T. mitsukurii*). A related question concerns the ecological impact of such hyperparasitoids on the global natural control provided by native egg parasitoid guilds and their role in the ecosystem. The recent discoveries of adventive *Ac. sinicus* in newly invaded areas of Europe and North America suggest that it is likely to become a permanent part of the local fauna, but the impact of this species to the ecosystem of the

American and European fauna is a matter to be assessed in the future (Nofemela, 2013). Biological traits presented in our work can explain many aspects of biological and ethological features under field conditions and can be helpful for future planning of research with such complicated parasitoid species, where highly specific, optimal timing of host suitability within the entire trophic chain is fundamental for the success of the parasitoid's reproduction.

Conclusion:

We conclude that *Ac. sinicus* is an obligate hyperparasitoid of pupae of the scelionids *T. japonicus*, *T. mitsukurii* and *Telenomus* sp. but not the eupelmid *An. bifasciatus*. Adult emergence of the hyperparasitoid was obtained upon exposing 5d and 7d old primary-parasitized egg masses for 72h to *Ac. sinicus* females, suggesting that this pteromalid is a highly specific hyperparasitoid exploiting hosts at the pupal stage. The recent findings in Europe and North America of this Asian hyperparasitoid emerging from phytophagous pentatomids are concerning in that this pteromalid may interfere in the trophic chains which regulate population dynamics of stink bugs.

Author Contributions:

L.G. and G.S.-P. Conceptualization and Formal analysis; K.A.H. and P.F.R. Funding acquisition; P.F.R. Resources and Supervision; L.G., G.S.-P., P.G.T. and K.A.H. Investigation, Data curation and Writing—original draft; L.G., G.S.-P., P.G.T., K.A.H., P.F.R. Writing—review & editing. All authors have read and agreed to the published version of the manuscript.

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Discussion and Conclusion

The general aim of this thesis was to investigate the ecological and ethological traits of the egg parasitoids of the invasive species *Halyomorpha halys* and their tritrophic interactions to evaluate also the possibility of using them as natural enemies for the biological control of this pest in invaded areas. Nowadays, biological control is considered the most effective and sustainable solution for the long-term management of this pest.

The egg parasitoids are considered the most important natural enemies of *H. halys* both in the area of origin and in the invaded areas of pest. In the invaded areas of North America and Europe only some indigenous species of egg parasitoids have accepted *H. halys* as host and these include *Telenomus* spp., *Trissolcus* spp., *Gryon* spp., *Ooencyrtus* spp. - The first studies emphasized the possibility of using native natural enemies for biological control of *H. halys*, but their impact has been found to be overall irrelevant (Abram et al. 2017). So, the attention has been focused on that exotic species recently founded in the invaded areas of the pest, in particular in Europe and Northern Italy: *Trissolcus japonicus*, the main natural enemies of *H. halys* in the area of origin (Southern Asia), and *Trissolcus mitsukurii* which is widespread in Asia and its distribution extends to the southern limit of eastern Australia (Johnson, 1991).

The results of large-scale monitoring conducted in Northern Italy and in Switzerland, where *H. halys* caused important damage in agricultural crops, show that *T. japonicus* and *T. mitsukurii* rapidly spread into all types of habitats where *H. halys* has been surveyed. In particular, the monitoring evidenced the different patterns of occurrence of *Trissolcus* four years later their first detection, with *T. japonicus* that results more abundant in northwestern Italy and in Switzerland, with respect to *T. mitsukurii* which is widespread mostly in the northeastern. This different expansion could be explained by the timing of arrival of the two species and first locality of accidental introduction, since the earliest official record of *T. mitsukurii* for northern Italy was traced back to 2016 (Scaccini et al. 2020), two years before the earliest detection of *T. japonicus* (Sabbatini-Peverieri et al. 2018). Furthermore, the different abundance recorded in these areas may be responsible also for the dissimilar observed habitat prevalence (agricultural/urban). It also need to be considered that different habitats, but also different types of the crop with different economic practices could facilitate or further limit the spread of either species.

Based on the *H. halys* egg masses collected in the field, the emergence of native parasitoids, as aspected, was rarely observed and the study confirmed that among these ones, the generalist *A. bifasciatus* was the predominant species. These results, as others (Stahl et al. 2019a; Stahl et al. 2019b; Maistrello et al. 2020) initially supported the use of the indigenous of *A. bifasciatus* as an ideal candidate in biological control programs against *H. halys*. However, the results of the recent study by

Iacovone et al. (2022) conducted during a biological control program carried out in Trentino-Alto Adige, where both *A. bifasciatus* and the adventive *T. mitsukurii* are present, suggest that despite the augmentative release of the indigenous species (1000 individuals/ha), the exploitation efficiency, (number of emerged parasitoids out of the total number of eggs within the discovered egg masses), remained lower for *A. bifasciatus* (median among 53 egg masses = 53.6%) than for *T. mitsukurii* (median among 29 egg masses = 92.9%). This indicates that, despite the attempts to use an indigenous species for the control of *H. halys*, even releasing a large number of specimens, however, the exotic species shows to be more adapted for biological control of this pest, also in invaded area. Moreover, *A. bifasciatus* is a generalist species, with Pentatomidae but also Lepidoptera as hosts, and its augmentative release in the environment may in turn be detrimental to non-target species. This aspect however needs further investigations.

The monitoring also evidenced that both *T. japonicus* and *T. mitsukurii* showed a favorable degree of specificity towards *H. halys* also with notable levels of parasitism rate. Only in a relatively occasional event, they developed on other hosts, nevertheless, the data on native stink bugs were limited since the focus of the present study was on target host *H. halys*.

In the end, the field surveys also evidenced a widespread presence of the exotic hyperparasitoid *Ac. Sinicus* founded frequently associated with *H. halys* and its parasitoid guild whose biology was almost unknown until now. The study (discussed above) light on this aspect.

In conclusion, the present study (with shared survey protocols and analysis approaches) provides a baseline on parasitoid species composition and parasitism rate that may be used in the future to assess the impact of both adventive and released *Trissolcus* egg parasitoids populations and thus evaluate the output of classical biological control programs.

Future monitoring, infact, will be crucial to understand the dynamic populations and the progress of biological control programs, and, in this context the study on the characteristics of the meconia of *H. halys* could be a useful tool to use during field surveys. The meconium is produced by species in different ways and remains unchanged inside the egg, also for a long time after the parasitoid emergence. This can be clearly seen within the emptied host egg through the exit hole, by easy dissection if necessary, so, although for definitive identification of the species the molecular analysis of remnants is recommended, this approach allows recognizing quickly the genus or species of the egg parasitoid that emerged.

Due to the limited control action provided by native natural enemies and considering the presence of the adventive and widely distributed population of *T. japonicus* and *T. mitsukurii* in the newly invaded areas of *H. halys*, the future prospects of classical biological control against this pest have fallen on these parasitoids species. Proposals for the introduction of new exotic biocontrol agents are made by

international research institutions and experts and submitted for review and approval by regulatory agencies (van Lenteren et al. 2006a; EPPO 2014; FAO-IPPC 2017; Barratt et al. 2018).

Then, the next step was to evaluate the physiological host range of *T. japonicus* because it is considered the most important natural enemy of *H. halys* in its native area (Zhang et al. 2017). The results of the study, conducted under laboratory conditions, show that *T. japonicus* was able to parasitize both the target and nine of the 16 non-target species tested even if with differing degrees of success and this confirms the oligophagous habit of this species as reported in other studies conducted worldwide (Lara et al. 2019; Haye et al. 2020; Saunders et al. 2021) but also in its area of origin (Zhang et al. 2017). The developmental suitability of European non-target host species for *T. japonicus* was demonstrated by successful offspring emergence from most of species tested, other pentatomid species were less suitable while others reported a higher egg mortality (dead eggs) suggesting potentially unsuccessful parasitization attempts by *T. japonicus*. The host egg morphology and its characteristics and females behaviors during parasitization were correlated with the host acceptance rates and this aspect helps us to propose a simplified and new predictive approach for non-target studies.

The same approach, even if with differences in experimental design, was used to assess the physiological host range of the other exotic species, *T. mitsukurii*. Differently from *T. japonicus* and *A. bifasciatus*, *T. mitsukurii* had been poorly investigated till now as a candidate biological control agent of *H. halys*. Field data from Japan indicate for *T. mitsukurii* an oligophagous behavior (Yasumatsu and Watanabe 1964; Hokyo et al., 1966; Ryu and Hirashima, 1984; Arakawa and Namura 2002). The results of this study show that *T. mitsukurii* displayed in laboratory tests no specific host specialization and the ability to parasitize and develop successfully in eight out of 13 tested non-target pentatomid species as was previously assessed also for *T. japonicus*. However, it is well-known that testing conditions in the simplified laboratory environment can be misleading in non-target risk analysis; therefore, the results must be carefully evaluated. For this reason, more complex systems are needed to better simulate field environmental factors, and the experiments should adopt multidisciplinary and integrated approaches for a clear definition of *Trissolcus*, and in general of biological control agents, host range. One of these systems could be represented by Y-tube olfactometer bioassays. Through this approach we then investigated the ability of the Asian egg parasitoid *T. mitsukurii* (Hymenoptera: Scelionidae) to exploit odours associated with the target host *H. halys* (Hemiptera: Pentatomidae) and with some non-target stink bugs native to Southern Europe.

Among the different stink bug-associated odours tested in the olfactometer, only those from plants bearing an egg mass of *H. halys* or *N. viridula* elicited positive attraction in *T. mitsukurii* females. These results validated the hypothesis that a more in-depth evaluation of the parasitoid host specificity, for example through chemical ecology investigation, would further restrict the parasitoid

host range that was depicted by physiological host range assays. Infact, while previous host-acceptance investigations revealed a broad host range for *T. mitsukurii* in Europe, present results suggest that host location at a long distance would likely favor parasitization of *H. halys* (or *N. viridula*), rather than the other stink bugs tested, such as the pentatomid predator *Arma custos*. Possibly, the presence of such an ecological filter would have positive implications for preventing undesired impacts on non-targets in case of intentional release of the parasitoid in biological control programs. On the other hand, there are no physiological impediments for *T. mitsukurii* to develop in some non-targets (e.g. the pentatomid predator *A. custos*), hence when such species co-occur in the field with *H. halys*, they would likely be more exposed to parasitization due to occasional encounters during foraging. So, also in this case, only long-term field monitoring will provide information on the real impact of these species on non-target host, which in turn will contribute to the improvement of biological control programs.

Based on a risk analysis of *T. japonicus*, a petition for its release in the field in Italy was approved in 2020, with plans to rear and release over 120,000 females in five regions and two provinces in northern Italy (MiPAAF, 2020). Prior to release, a national action program has been developed to describe the measures to be implemented for the biological control of *H. halys* in these regions and the two provinces. The program includes activities such as 1) environmental risk assessment of *T. japonicus*, 2) organization of the release activities and strategies (including management, rearing, and production of this natural enemy), 3) identification of the priority areas (considering data of monitoring, amount of damages and future perspective), 4) number of specimens to be released, number of release sites and when released in the field 5) monitoring strategies after the release (both of target and non-target hosts). Also communication with institutions, citizens, public and private companies, and representatives of the productive world also play an important role in the success of the program.

Considering the sympatry of *T. japonicus* and *T. mitsukurii*, as reported by the previous data, both in Italy and Europe in general, and the potential opportunity to use these species in biological control programs against *H. halys*, a study about the potential interspecific competition was assessed in order to evaluate dynamic population and pest control outcomes. Interspecific competition, in fact, can potentially disrupt biological control programs, or cause synergism, or it may have no influence at all (Boivin and Brodeur, 2006; Casula et al. 2006; Mills, 2006; Ode et al. 2022). The different scenarios simulated under laboratory conditions show that the adult females of both *Trissolcus* species seem unable to recognize signs of the previous parasitization when encountering egg masses recently parasitized by the other species. In the context of indirect competition, the second female arrives, in fact, parasitized the egg of *H. halys* while inside there was already the egg or the larva of the other species (multiparasitization). However, in terms of progeny, the first arrived female had greater

success in progeny production and did not suffer severely in competition with the second arriving species, regardless of species. In contrast, the second-arriving species had less success in progeny production, with a decreasing trend as the elapsed time from first parasitization increased. In this case multiparasitism within *T. japonicus* and *T. mitsukurii* resulted in a significant waste of eggs and potential progeny of the second-arriving species. When simultaneously present (direct competition) *T. mitsukurii* shows a more aggressive behavior than *T. japonicus*, dominating the host resources and producing more progeny. While, in the last case, when the arrival of females of the two species was asynchronous, although *T. mitsukurii* was also aggressive as the intruder, but success in progeny production was biased in favor of the resident female, regardless of species. The results may suggest that the reduced progeny production by the second arriving female could have, over time, impacts on its population dynamics and from a biological control point of view, on our results, a single-species release appears to be the best strategy since multiparasitism behavior by both species displayed resulted in wasted searching time and energy and lower reproductive potential.

However, although the experiment attempted to reproduce all of the possible scenarios that could occur in the field, the interactions between these two species cannot account for various environmental factors such as environment adaptations, host searching abilities, and interaction with other species that need to be further evaluated (Denno and Finke, 2006; Ode et al. 2022). Also in this case studies with more complex microcosms should be adopted for further investigation even if only field data will provide informations on populations dynamimcs and the effectiveness of biological control programs..

T. japonicus and *T. mitsukurii* are not the only species that have followed *H. halys* in its worldwide expansion With them, also the Asian species *A. sinicus* was recently found in Europe (Northern Italy and Switzerland) and in North America (USA and Canada) mainly associated with *H. halys* and its parasitoid guild, including *Trissolcus* species. The biological and ethological traits of *A. sinicus* were still completely unknown but the study about its biology confirmed the hypothesis of an obligate hyperparasitoid, in particular of Scelionidae species, such as *T. japonicus* and *T. mitsukurii*. In addition, it seems to attack at least some of the native *Trissolcus* and *Telenomus* species that in turn parasitize a range of pentatomid host eggs such as the native *A. heegeri* or *D. baccarum*. *A. Sinicus* seems to be a very specialist parasitoid, in its reproductive biology, there is a very precise parasitization window for adult females to deposit their own eggs in their hosts to ensure their development. This time frame is represented by the transition from mature larva and the pupal stage of its host, in this case the primary parasitoid. Probably, the olfactory cues in the meconium expelled by the mature larvaassist female *A. sinicus* in their search for hosts and subsequent acceptance, as it is known to be the case with many parasitoid and predator species (Steidle and vanLoon, 2002). Thus, obligate hyperparasitoid occupies the highest level in multitrophic systems and may interfere the

populations densities of primary parasitoid species at lower trophic level. Host-parasitoid-hyperparasitoid relationships may generate a variety of unexpected trophic pathways. With a variety of life cycle strategies and host ranges, hyperparasitoids often transcend trophic levels in their search for hosts (Brodeur et al. 2000).

So, now, a related question concerns the ecological impact of *A. sinicus* on the ecosystem of North America and European fauna and in general on the global natural control provided by native egg parasitoid guilds.

Future perspective

These studies opened further research questions that need to be implemented by focused future studies. Our studies highlighted the importance of field monitoring to investigate the distribution of the invasive pest *H. halys* and the species composition of its parasitoid guild. According to the biological control program, the inoculative releases of *T. japonicus* in the regions of Northern Italy aim to create a stable population of natural enemy that over time can self-regulate with the population of pest *H. halys*. Indeed, once the host-parasitoid populations are balanced, the population levels of *H. halys* will be such that will not cause further damage to the crops. Following the augmentative release of *T. japonicus*, post-release monitoring will be crucial to learn more about the potential direct and indirect impact that this egg parasitoid can cause on native hosts. It will be important to understand how the populations of both indigenous, exotic egg-parasitoids and hyperparasitoid will evolve and also how to improve the biological control program.

Since most of the studies about natural enemies and their hosts are carried out under laboratory conditions, specifically designed field experiments that should take into account hidden trophic interactions but also environmental factors are necessary to improve information about what could happen in field conditions.

In addition, climate change could affect population dynamics in the future in different ways. First, changes in environmental conditions could directly impact the physiology and behavior of biological control agents and/or the host. Second, the life history of these organisms is strongly linked to the season so changes in the timing of important seasonal events could have strong impacts. Third, changing the climate could contribute to asymmetrical outcomes in host-parasitoid interactions resulting, for example, in reduced parasitoid efficacy or the possible exclusion of parasitoids, but not their host. So, climate feedbacks are likely to be complex and/or difficult to predict but climate models could contribute to understanding these trends.

Furthermore, one of Europe's 2030 goals as part of its latest plan on sustainability and biodiversity, is to halve the use of pesticides, favoring greener practices to make food systems healthier and

environmental friendly. So, also in this context, biological control certainly plays an important role as it offers effective long-term solutions and sustainable approaches, particularly in the case of invasive species such as *H.halys*.

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Annexes

Supplementary materials for: “ Assessing the Distribution of Exotic Egg Parasitoids of *Halyomorpha halys* in Europe with a Large-Scale Monitoring Program

Figure S1. Distribution of the collected *Halyomorpha halys* egg masses in Italy (IT) and Switzerland (CH), according to the different surveyed contexts: cropland, semi-natural habitats and urban areas.

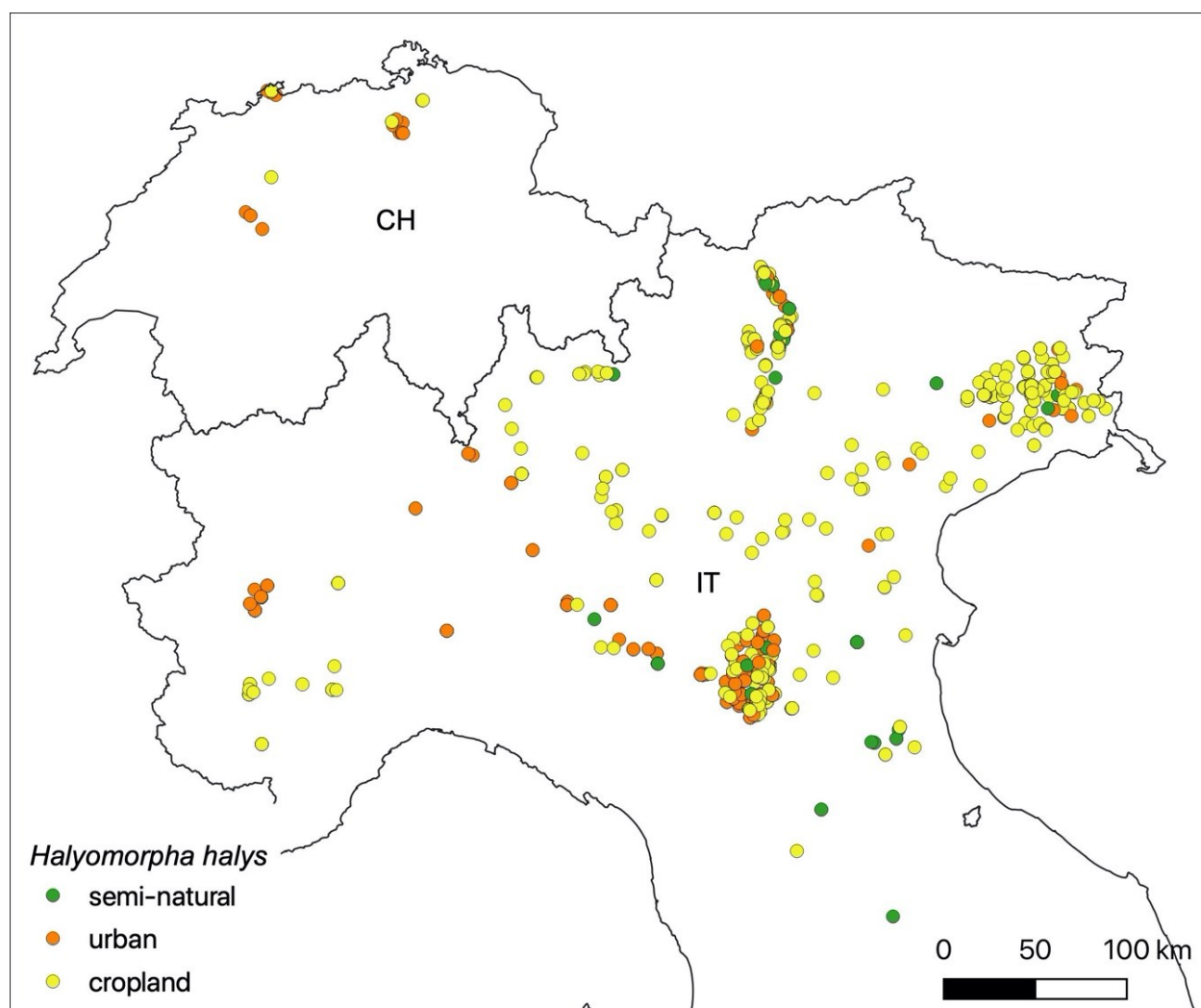


Table S1. Survey methods applied in the different areas: a) visual inspection in fixed locations (FV), repeated at least three times during the sampling season; b) visual inspection in non-fixed locations (V) and c) deployment of sentinel egg masses (S).

Country	Region/Canton	Survey
Italy	Emilia Romagna	FV, V
	Friuli-Venezia Giulia	V
	Lombardy	V
	Piedmont	FV
	Trentino-Alto Adige ¹	FV, V, S ¹
	Veneto	FV, V, S
Switzerland	Basel	V
	Bern	V
	Ticino	S ²
	Zürich	V

(¹Zapponi et al., 2020; ²Stahl et al., 2019b)

Table S2. Host plant for the collected *Halyomorpha halys* egg masses.

Host plant species		
<i>Acer campestre</i>	<i>Geranium sp.</i>	<i>Prunus sp.</i>
<i>Acer negundo</i>	<i>Helianthus annuus</i>	<i>Prunus spinosa</i>
<i>Acer sp.</i>	<i>Hedera helix</i>	<i>Punica granatum</i>
<i>Actinidia sp.</i>	<i>Juglans sp.</i>	<i>Sambucus nigra</i>
<i>Ailanthus altissima</i>	<i>Laburnum anagyroides</i>	<i>Sorbus aria</i>
<i>Ocimum basilicum</i>	<i>Lactuga sp.</i>	<i>Syringa sp.</i>
<i>Cannabis sp.</i>	<i>Liquidambar sp.</i>	<i>Tilia platyphyllos</i>
<i>Carpinus betulus</i>	<i>Malus domestica</i>	<i>Tilia sp.</i>
<i>Cornus mas</i>	<i>Paulownia tomentosa</i>	<i>Viburnum lantana</i>
<i>Cornus sanguinea</i>	<i>Solanum lycopersici</i>	<i>Vitis vinifera</i>
<i>Corylus avellana</i>	<i>Prunus armeniaca</i>	<i>Ziziphus jujuba</i>
<i>Crataegus monogyna</i>	<i>Prunus avium</i>	
<i>Diospyros kaki</i>	<i>Prunus cerasus</i>	
<i>Phaseolus vulgaris</i>	<i>Prunus domestica</i>	
<i>Ficus carica</i>	<i>Prunus padus</i>	
<i>Fraxinus sp.</i>	<i>Prunus persica</i>	

Supplementary materials for: “Combining physiological host range, behavior and host characteristics for predictive risk analysis of *Trissolcus japonicus*”

Supplementary material 1 Statistics of hatchability of manipulated and unmanipulated hemipteran eggs, dead eggs, *Trissolcus japonicus* parasitization success and females’ (part 1) behavior during the video recorded parasitization opportunity (2 h), and pairwise comparison of 2 h parasitization event vs. 24 h; “n.a.” not assessed due to the low number of replicates; “-“ no parasitization (part 2).

X= χ

Table Part 1

Species	Hatchability rate of hemipteran eggs		Hemipteran egg fate offered for oviposition		
	unmanipulated eggs	manipulated eggs	% of eggs with no parasitoid and no hemiptera emerged (dead eggs)	% of nymphs emerged (n.)	% of successful parasitized eggs (n.)
<i>H. halys</i> (control)	=3.66; p=0.0558				
<i>Pi. lituratus</i>	=0.04; p=0.8426		X=3.10; p=0.0785	X=26.28; p=0.0000	X=16.24; p=0.0001
<i>D. baccarum</i>	=2.33; p=0.1272		X=9.15; p=0.0025	X=25.57; p=0.0000	X=14.66; p=0.0001
<i>Ca. purpeiripennis</i>	=0.00; p=0.9820		X=6.25; p=0.0107	X=12.42; p=0.0004	X=1.80; p=0.1801
<i>Ca. mediterraneus</i>	=0.91; p=0.3408		X=15.84; p=0.0001	X=16.71; p=0.0000	X=2.67; p=0.0969
<i>E. ventralis</i>	=0.76; p=0.3846		X=1.07; p=0.3000	X=27.10; p=0.0000	X=11.41; p=0.0007
<i>Ra. nebulosa</i>	=0.05; p=0.8181		X=1.77; p=0.1838	X=1.10; p=0.2953	X=0.01; p=0.9312
<i>St. lunata</i>	-		X=0.32; p=0.5745	X=24.91; p=0.0000	X=11.41; p=0.0007
<i>Pa. prasina</i>	=3.25; p=0.0712		X=0.10; p=0.7492	X=1.50; p=0.2201	X=0.01; p=0.9104
<i>Pe. strictus</i>	-		X=0.44; p=0.5091	X= 25.12; p=0.0000	X=12.66; p=0.0004
<i>Sc. sidertis</i>	-		X= 0.19; p=0.6593	X=10.55; p=0.0012	X=7.33; p=0.0068
<i>A. heegeri</i>	=0.38; p=0.5385		X=1.35; p=0.2460	X=14.82; p=0.0001	X=2.06; p=0.1508
<i>N. viridula</i>	=6.61; p=0.0131		X=4.66; p=0.0308	X=29.33; p=0.0000	X=26.83; p=0.0000
<i>Co. marginatus</i>	=2.17; p=0.1338		X=0.65; p=0.4193	X=28.06; p=0.0000	X=21.56; p=0.0000
<i>G. juniperi</i>	=0.63; p=0.4274		X=0.58; p=0.4462	X=27.50; p=0.0000	X=19.50; p=0.0000
<i>Rh. iracundus</i>	=3.80; p=0.0513		X=0.93; p=0.3352	X=27.50; p=0.0000	X=19.50; p=0.0000

Table Part 2

	Behavior and handling time							Parasitization 2h vs. 24h
	first egg contacting ($\pm$ S.E.)	duration of first egg inspection ($\pm$ S.E.) (s)	Egg inspection before acceptance ($\pm$ S.E.) (s)	oviposition ($\pm$ S.E.) (s)	marking ($\pm$ S.E.) (s)	time span from test start to acceptance for oviposition ($\pm$ S.E.) (s)	A/C ratio	
<i>H. halys</i> (control)								X=0.00; p=1.0000
<i>Pi. lituratus</i>	t=0.99 p=0.325	t=0.82 p=0.413	n.a.	n.a.	n.a.	n.a.	n.a.	X=0.00; p=1.0000
<i>D. baccarum</i>	t=0.51 p=0.612	t=1.99 p=0.051	t=1.16 p=0.252	t=1.35 p=0.18	t=0.62 p=0.54	t=1.68 p=0.099	n.a.	X=0.00; p=1.0000
<i>Ca. purpeiripennis</i>	t=2.24 p=0.003	t=3.24 p=0.002	t=1.31 p=0.196	t=0.982 p=0.33	t=0.67 p=0.507	t=0.15 p=0.883	X=8.43; p=0.0037	X=0.00; p=1.0000
<i>Ca. mediterraneus</i>	t=1.58 p=0.12	t=0.19 p=0.85	n.a.	n.a.	n.a.	n.a.	n.a.	X=1.23; p=0.2674
<i>E. ventralis</i>	t=2.47 p=0.016	t=0.89 p=0.379	-	-	-	-	-	-
<i>Ra. nebulosa</i>	t=0.07 p=0.95	t=1.57 p=0.119	t=0.08 p=0.937	t=5.40 p=8.28*10 ⁻⁷	t=6.62 p=5.67*10 ⁻⁹	t=2.32 p=0.023*	X=5.46; p=0.0194	X=0.03; p=0.8619
<i>St. lunata</i>	t=0.96 p=0.341	t=1.92 p=0.059	-	-	-	-	-	-
<i>Pa. prasina</i>	t=2.03 p=0.047	t=1.09 p=0.281	t=1.98 p=0.052	t=1.56 p=0.124	t=2.95 p=0.004	t=2.31 p=0.024	X=2.75 p=0.0972	X=0.11; p=0.7455
<i>Pe. strictus</i>	t=0.583 p=0.562	t=0.74 p=0.459	n.a.	n.a.	n.a.	n.a.	n.a.	X=0.00; p=1.0000
<i>Sc. sidertis</i>	t=6.494 p=0.0000	t=1.57 p=0.124	-	-	-	-	-	-
<i>A. heegeri</i>	t=2.52 p=0.014	t=1.72 p=0.09	t=0.409 p=0.682	t=0.58 p=0.563	t=3.33 p=0.017	t=0.44 p=0.024	X=0.06; p=0.9957	X=0.71; p=0.4002
<i>N. viridula</i>	t=0.18 p=0.857	t=1.19 p=0.237	-	-	-	-	-	-
<i>Co. marginatus</i>	t=2.43 p=0.018	t=2.62 p=0.011	-	-	-	-	-	-
<i>G. juniperi</i>	t=2.46 p=0.165	t=3.03 p=0.003	-	-	-	-	-	-
<i>Rh. iracundus</i>	t=1.08 p=0.286	t=3.76 p=0.0003	-	-	-	-	-	-

Supplementary material 2 Measures of the volume of target and non-target eggs and emerged *Trissolcus japonicus* female body size (among brackets n. of replicates).

Hemipteran		<i>Trissolcus japonicus</i> females		
species	Egg volume (mm ³)	head within (mm ³)	thorax within (mm ³)	femur III length (mm ³)
<i>H. halys</i>	1.079 ± 0.025 (n=20)	0.648 ± 0.017 (n=15)	0.616 ± 0.025 (n=15)	0.395 ± 0.256 (n=15)
<i>Ra. nebulosa</i>	1.641 ± 0.033 (n=20)	0.671 ± 0.033 (n=13)	0.628 ± 0.034 (n=13)	0.462 ± 0.026 (n=13)
<i>Pa. prasina</i>	1.021 ± 0.029 (n=20)	0.635 ± 0.021 (n=15)	0.592 ± 0.016 (n=15)	0.384 ± 0.025 (n=15)
<i>A. heegeri</i>	1.252 ± 0.046 (n=20)	0.543 ± 0.021 (n=7)	0.503 ± 0.021 (n=7)	0.320 ± 0.023 (n=7)
<i>Ca. purpeiripennis</i>	0.653 ± 0.019 (n=20)	0.560 ± 0.028 (n=9)	0.520 ± 0.028 (n=9)	0.302 ± 0.021 (n=9)
<i>Ca. mediterraneus</i>	0.636 ± 0.013 (n=20)	0.560 ± 0.033 (n=4)	0.520 ± 0.033 (n=4)	0.360 ± 0.033 (n=4)
<i>D. baccarum</i>	0.313 ± 0.007 (n=20)	0.467 ± 0.023 (n=3)	0.427 ± 0.023 (n=3)	0.261 ± 0.02 (n=3)
<i>St. lunata</i>	0.327 ± 0.093 (n=13)	n.a.	n.a.	n.a.
<i>Pi. lituratus</i>	0.489 ± 0.013 (n=20)	n.a.	n.a.	n.a.
<i>Pe. strictus</i>	0.367 ± 0.010 (n=20)	n.a.	n.a.	n.a.
<i>Sc. sideris</i>	0.152 ± 0.006 (n=20)	n.a.	n.a.	n.a.
<i>E. ventralis</i>	0.481 ± 0.016 (n=20)	n.a.	n.a.	n.a.
<i>N. viridula</i>	0.533 ± 0.011 (n=20)	n.a.	n.a.	n.a.
<i>Co. marginatus</i>	0.911 ± 0.027 (n=20)	n.a.	n.a.	n.a.
<i>G. juniperi</i>	0.924 ± 0.071 (n=20)	n.a.	n.a.	n.a.
<i>Rh. iracundus</i>	0.625 ± 0.024 (n=20)	n.a.	n.a.	n.a.

Supplementary material 3. Egg load in *Trissolcus japonicus* females ovary (among brackets S.E.) and statistics of Student's *t*-test.

Host			
days	<i>Halyomorpha halys</i>	<i>Dolycoris baccarmu</i>	Statistics
1	18.15 (±1.28)	8.50 (±0.97)	<i>t</i> = 5.937; df = 38; <i>P</i> < 0.001
3	29.80 (±0.90)	21.65 (±0.57)	<i>t</i> = 7.714; df = 38; <i>P</i> < 0.001
7	41.95 (±1.35)	26.25 (±1.13)	<i>t</i> = 8.680; df = 38; <i>P</i> < 0.001
14	43.60 (±1.42)	27.85 (±0.96)	<i>t</i> = 9.2.16; df = 38; <i>P</i> < 0.001
21	43.85 (±1.36)	33.50 (±0.66)	<i>t</i> = 6.902; df = 38; <i>P</i> < 0.001

Supplementary materials for: “Physiological host range of *Trissolcus mitsukurii*, a candidate biological control agent of *Halyomorpha halys* in Europe”

Table S1 Statistics (Kruskal-Wallis test followed by Dunn’s multiple comparison post-hoc test) for comparisons of emerged nymphs, *Trissolcus mitsukurii* parasitization success (emerged parasitoids) and hemipteran dead eggs (not hatched and no emergence of parasitoids) between non-target species and the target *Halyomorpha halys* during tests conducted for 24h and 2h time exposure; n.a. = not assessed due to the low number of replicates; number of replicates n. = 12, if not otherwise indicated among brackets.

Species	host egg fate after 24h of exposure			host egg fate after 2h of exposure		
	% of eggs with parasitoid emergence/egg mass	% of eggs hatched/ egg mass	% of dead eggs	% of eggs with parasitoid emergence/egg mass	% of eggs hatched/ egg mass	% of dead eggs
<i>Acrosternum heegeri</i>	Z=0.1642 P>0.9999	Z=0.2533 P>0.9999	Z=0.2592 P>0.9999	Z=1.956 P=0.4546	Z=2.441 P=0.1320	Z=0.000 P>0.9999
<i>Aelia accuminata</i>	Z=4.429 P=0.0001	Z=1.186 P>0.9999	Z=4.389 P=0.0002	Z=3.064 P=0.0196	Z=1.586 P>0.9999	Z=5.233 P<0.0001
<i>Ancyrosoma leucogrammes</i>	Z=5.051 P<0.0001 (n=10)	Z=4.699 P<0.0001 (n=10)	Z= 0.5988 P>0.9999 (n=10)	n.a.	n.a.	n.a.
<i>Arma custos</i>	Z=2.041 P=0.5772	Z=1.186 P>0.9999	Z=1.360 P>0.9999	Z=1.148 P>0.9999	Z=0.3972 P>0.9999	Z=0.5368 P>0.9999
<i>Carpocoris pudicus</i>	Z=1.193 P>0.9999	Z=0.4384 P>0.9999	Z=1.281 P>0.9999	Z=1.758 P=0.7093	Z=2.425 P=0.1379	Z=0.3984 P>0.9999
<i>Dolycoris baccarum</i>	Z=0.3330 P>0.9999	Z=0.0073 P>0.9999	Z=0.2789 P>0.9999	Z=0.2772 P>0.9999	Z=2.113 P=0.3112	Z=2.255 P=0.2171
<i>Eurydema oleracea</i>	Z=3.890 P=0.0014 (n=10)	Z=1.350 P>0.9999 (n=10)	Z= 4.406 P=0.0001 (n=10)	n.a.	n.a.	n.a.
<i>Eurydema ventralis</i>	Z=5.298 P=<0.0001	Z=3.872 P=0.0015	Z=2.997 P=0.0382	Z=3.351 P=0.0073	Z=2.396 P=0.491	Z=2.130 P=0.2983
<i>Graphosoma italicum</i>	Z=1.909 P=0.7878	Z=1.700 P>0.9999	Z=1.582 P>0.9999	Z=0.1432 P>0.9999	Z=0.4068 P>0.9999	Z=0.7765 P>0.9999
<i>Nezara viridula</i>	Z=4.960 P=<0.0001	Z=2.155 P=0.4358	Z=5.013 P=<0.0001	Z=3.351 P=0.0073	Z=1.052 P>0.9999	Z=5.500 P<0.0001
<i>Palomena prasina</i> (n=1)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
<i>Piezodorus lituratus</i>	Z=1.557 P>0.9999 (n=5)	Z=1.635 P>0.9999 (n=5)	Z=1.318 P>0.9999 (n=5)	n.a.	n.a.	n.a.
<i>Sciocoris</i> sp.	Z=1.049 P>0.9999 (n=8)	Z=0.6263 P>0.9999 (n=8)	Z=1.325 P>0.9999 (n=8)	n.a.	n.a.	n.a.
<i>Eurygaster maura</i>	Z=0.9009 P>0.9999	Z=0.4920 P>0.9999	Z=0.7109 P>0.9999	Z=1.340 P>0.9999	Z=2.654 P=0.0717	Z=1.401 P>0.9999
<i>Gonocerus juniperi</i>	Z=5.298 P<0.0001	Z=5.227 P<0.0001	Z=0.8961 P>0.9999	n.a.	n.a.	n.a.
<i>Rhynocoris iracundus</i> (n=3)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Table S2 Statistics (Chi-square test with Yates' correction) for comparisons of emerged parasitoids between non-target species and the target *Halyomorpha halys* during tests conducted for 2h single egg exposure test.

species	% of emerged parasitoids
<i>Acrosternum heegeri</i>	$\chi^2 = 19.74$ $P < 0.0001$
<i>Arma custos</i>	$\chi^2 = 27.33$ $P < 0.0001$
<i>Dolycoris baccarum</i>	$\chi^2 = 37.60$ $P < 0.0001$
<i>Graphosoma italicum</i>	$\chi^2 = 32.45$ $P < 0.0001$
<i>Nezara viridula</i>	$\chi^2 = 57.06$ $P < 0.0001$
<i>Palomena prasina</i>	-
<i>Eurygaster</i> sp.	$\chi^2 = 57.30$ $P < 0.0001$

Table S3 Egg size of suitable hemipteran host eggs and dimension parameters of emerged *Trissolcus mitsukurii*, females (all mean $\pm$ SE). The number of replicates is reported within parentheses

Host species	Hemiptera egg size				<i>Trissolcus mitsukurii</i> female dimension		
	Egg volume (mm ³)	R (mm)	r (mm)	H (mm)	head width (mm)	thorax width (mm)	hind tibia length (mm)
<i>Halyomorpha halys</i>	1.20 $\pm$ 0.09 (n=15)	0.58 $\pm$ 0.01	0.44 $\pm$ 0.00	1.33 $\pm$ 0.01	0.69 $\pm$ 0.01 (n=20)	0.65 $\pm$ 0.01 (n=20)	0.37 $\pm$ 0.01 (n=20)
<i>Acrosternum heegeri</i>	0.63 $\pm$ 0.01 (n=15)	0.47 $\pm$ 0.00	0.33 $\pm$ 0.00	1.01 $\pm$ 0.00	0.59 $\pm$ 0.00 (n=15)	0.54 $\pm$ 0.00 (n=15)	0.32 $\pm$ 0.00 (n=15)
<i>Adia acuminata</i>	0.33 $\pm$ 0.01 (n=13)	0.37 $\pm$ 0.00	0.27 $\pm$ 0.01	0.91 $\pm$ 0.01	0.47 $\pm$ 0.01 (n=8)	0.44 $\pm$ 0.01 (n=8)	0.27 $\pm$ 0.00 (n=8)
<i>Arma custos</i>	0.46 $\pm$ 0.02 (n=15)	0.41 $\pm$ 0.00	0.33 $\pm$ 0.00	0.97 $\pm$ 0.00	0.58 $\pm$ 0.00 (n=20)	0.54 $\pm$ 0.00 (n=20)	0.39 $\pm$ 0.00 (n=20)
<i>Ancyrosoma leucogrammes</i>	0.28 $\pm$ 0.02 (n=5)	0.34 $\pm$ 0.00	0.26 $\pm$ 0.00	0.89 $\pm$ 0.01	-	-	-
<i>Carpocoris pudicus</i>	1.00 $\pm$ 0.06 (n=15)	0.53 $\pm$ 0.01	0.38 $\pm$ 0.01	1.34 $\pm$ 0.01	0.70 $\pm$ 0.00 (n=10)	0.65 $\pm$ 0.01 (n=10)	0.44 $\pm$ 0.01 (n=10)
<i>Dolycoris baccarum</i>	0.38 $\pm$ 0.02 (n=15)	0.42 $\pm$ 0.00	0.30 $\pm$ 0.00	1.08 $\pm$ 0.00	0.54 $\pm$ 0.00 (n=20)	0.49 $\pm$ 0.01 (n=20)	0.30 $\pm$ 0.01 (n=20)
<i>Eurydema oleracea</i>	0.27 $\pm$ 0.05 (n=8)	0.33 $\pm$ 0.01	0.24 $\pm$ 0.01	0.93 $\pm$ 0.01	0.37 $\pm$ 0.01 (n=4)	0.30 $\pm$ 0.01 (n=4)	0.17 $\pm$ 0.01 (n=4)
<i>Eurydema ventralis</i>	0.54 $\pm$ 0.01 (n=15)	0.43 $\pm$ 0.00	0.32 $\pm$ 0.01	1.00 $\pm$ 0.01	-	-	-
<i>Graphosoma italicum</i>	0.63 $\pm$ 0.04 (n=15)	0.46 $\pm$ 0.00	0.33 $\pm$ 0.00	1.14 $\pm$ 0.01	0.59 $\pm$ 0.00 (n=20)	0.55 $\pm$ 0.00 (n=20)	0.35 $\pm$ 0.01 (n=20)
<i>Nezara viridula</i>	0.56 $\pm$ 0.01 (n=15)	0.42 $\pm$ 0.00	0.31 $\pm$ 0.00	1.19 $\pm$ 0.01	0.56 $\pm$ 0.00 (n=5)	0.52 $\pm$ 0.01 (n=5)	0.34 $\pm$ 0.01 (n=5)
<i>Palomena prasina</i> ¹	1.05 $\pm$ 0.01 (n=10)	0.66 $\pm$ 0.00	0.64 $\pm$ 0.01	0.66 $\pm$ 0.00	0.66 $\pm$ 0.00 (n=10)	0.64 $\pm$ 0.01 (n=10)	0.42 $\pm$ 0.00 (n=10)
<i>Piezodorus lituratus</i>	0.41 $\pm$ 0.01 (n=10)	0.39 $\pm$ 0.00	0.31 $\pm$ 0.00	1.01 $\pm$ 0.01	0.53 $\pm$ 0.01 (n=7)	0.48 $\pm$ 0.01 (n=7)	0.32 $\pm$ 0.00 (n=7)
<i>Sciocoris</i> sp.	0.45 $\pm$ 0.02 (n=10)	0.42 $\pm$ 0.01	0.30 $\pm$ 0.00	0.99 $\pm$ 0.01	0.54 $\pm$ 0.02 (n=15)	0.49 $\pm$ 0.02 (n=15)	0.28 $\pm$ 0.02 (n=15)
<i>Eurygaster maura</i>	0.70 $\pm$ 0.02 (n=15)	0.50 $\pm$ 0.01	0.33 $\pm$ 0.00	1.12 $\pm$ 0.01	0.62 $\pm$ 0.00 (n=16)	0.59 $\pm$ 0.00 (n=16)	0.37 $\pm$ 0.01 (n=16)

¹the measures of the pentatomid egg size and of the related emerged parasitoid females refer to a single egg mass

Table S4 Egg load (mean $\pm$ SE) of emerged *Trissolcus mitsukurii* females and statistic comparisons (Kruskal-Wallis test followed by Dunn's multiple comparison post-hoc test) between non-target species and the target *Halyomorpha halys*. The number of replicates is reported within parentheses.

Species	Egg load (7day)	Statistics
<i>Halyomorpha halys</i>	44.10 $\pm$ 0.67 (n=10)	-
<i>Acrosternum. heegeri</i>	32.90 $\pm$ 0.88 (n=10)	Z=2.464; <i>P</i> =0.1098
<i>Aelia acuminata</i>	16.25 $\pm$ 1.31 (n=4)	n.a.
<i>Arma custos</i>	28.60 $\pm$ 0.74 (n=10)	Z=4.423; <i>P</i> <0.0001
<i>Ancyrosoma leucogrammes</i>	-	-
<i>Carpocoris pudicus</i>	41.30 $\pm$ 2.13 (n=10)	Z=0.6086; <i>P</i> >0.9999
<i>Dolycoris baccarum</i>	29.50 $\pm$ 1.07 (n=10)	Z=3.981; <i>P</i> =0.0005
<i>Eurydema oleracea</i>	17.00 $\pm$ 1.52(n=3)	n.a.
<i>Eurydema ventralis</i>	-	-
<i>Graphosoma italicum</i>	28.00 $\pm$ 1.05 (n=10)	Z=4.586; <i>P</i> <0.0001
<i>Nezara viridula</i>	23.75 $\pm$ 0.76 (n=4)	n.a.
<i>Palomena prasina</i>	37.62 $\pm$ 0.16 (n=8)	n.a. ¹
<i>Piezodorus lituratus</i>	23.60 $\pm$ 1.36 (n=10)	Z=5.884; <i>P</i> <0.0001
<i>Sciocoris</i> sp.	28.20 $\pm$ 1.18 (n=10)	Z=4.470; <i>P</i> <0.0001
<i>Eurygaster maura</i>	34.90 $\pm$ 0.56 (n=10)	Z=1.586; <i>P</i> =0.9025

¹ statistic is not assessed due to females belonging to a single cohort (only one egg mass)

Supplementary materials for: “Olfactory responses of *Trissolcus mitsukurii* to plants attacked by target and non-target stink bugs suggest low risk for biological control”

Table S1: Summary of the hypotheses tested within Gaussian GLM on the residence time of *Trissolcus mitsukurii* exposed to odours from plant with an eggs mass of *Arma custos* (AC), *Dolycoris baccarum* (DB), *Eurydema ventralis* (EV), *Halyomorpha halys* (HH), or *Nezara viridula* (NV). Control consisted of a clean soybean or cauliflower plant (CP). For each comparison, the estimated coefficient (the log ratio between the treatment and the control residence time), SE, t-value and P-value are reported.

Planned comparisons	Estimate	SE	t value	P	
AC vs CP	-1.71	0.77	-2.21	0.0275	*
DB vs CP	-0.79	0.70	-1.13	0.2596	ns
EV vs CP	0.12	0.77	0.15	0.8793	ns
HH vs CP	1.82	0.71	2.54	0.0115	*
NV vs CP	1.80	0.82	2.20	0.0288	*

Significance: ‘*’ $P \leq 0.05$, ‘ns’ $P > 0.05$

Table S2 Summary of the hypotheses tested within binomial GLM on the first choice of *Trissolcus mitsukurii* exposed to odours from plant with an eggs mass of *Arma custos* (AC), *Dolycoris baccarum* (DB), *Eurydema ventralis* (EV), *Halyomorpha halys* (HH), or *Nezara viridula* (NV). Control consisted of a clean soybean or cauliflower plant (CP). For each comparison, the estimate coefficient (log odd), SE, z-value and P-value are reported.

Planned comparisons	Estimate	SE	z value	P	
AC vs CP	-0.35	0.26	-1.38	0.16798	ns
DB vs CP	0.078	0.23	0.34	0.73251	ns
EV vs CP	-0.063	0.25	-0.25	0.80262	ns
HH vs CP	0.67	0.25	2.74	0.00618	**
NV vs CP	0.22	0.27	0.80	0.42357	ns

Significance: ‘***’ $P \leq 0.00$, ‘ns’ $P > 0.05$

Table S3: Summary of the hypotheses tested within Gaussian GLM on the residence time of *Trissolcus mitsukurii* exposed to odours from stink bug females of *Arma custos* (AC), *Dolycoris baccarum* (DB), *Eurydema ventralis* (EV), *Halyomorpha halys* (HH), or *Nezara viridula* (NV). Control consisted of clean air (AIR). For each comparison, the estimated coefficient (the log ratio between the treatment and the control residence time), SE, t-value and P-value are reported.

Planned comparisons	Estimate	SE	t value	P	
AC vs AIR	-1.031	1.087	-0.95	0.344	ns
DB vs AIR	-0.68	1.02	-0.67	0.505	ns
EV vs AIR	-0.12	0.86	-0.14	0.892	ns
HH vs AIR	0.058	0.92	0.063	0.950	ns
NV vs AIR	-0.16	1.052	-0.15	0.877	ns

Significance: 'ns' $P > 0.05$

Table S4: Summary of the hypotheses tested within the binomial GLM on the first choice of *Trissolcus mitsukurii* exposed to odours from stink bug females of *Arma custos* (AC), *Dolycoris baccarum* (DB), *Eurydema ventralis* (EV), *Halyomorpha halys* (HH), or *Nezara viridula* (NV). Control consisted of clean air (AIR). For each comparison, the estimate coefficient (log odd), SE, z-value and P-value are reported.

Planned comparisons	Estimate	SE	z value	P	
AC vs AIR	0.00	0.30	0.00	1.000	ns
DB vs AIR	-0.24	0.29	-0.85	0.397	ns
EV vs AIR	-0.17	0.24	-0.72	0.474	ns
HH vs AIR	0.033	0.26	0.13	0.898	ns
NV vs AIR	-0.13	0.29	-0.44	0.662	ns

Significance: 'ns' $P > 0.05$

Table S5: Summary of the hypotheses tested within Gaussian GLM on the residence time of *Trissolcus mitsukurii* exposed to odours from stink bug eggs of *Arma custos* (AC), *Dolycoris baccarum* (DB), *Eurydema ventralis* (EV), *Halyomorpha halys* (HH), or *Nezara viridula* (NV). Control consisted of clean air (AIR). For each comparison, the estimated coefficient (the log ratio between the treatment and the control residence time), SE, t-value and P-value are reported.

Planned comparisons	Estimate	SE	t value	P	
AC vs AIR	0.66	0.99	0.66	0.509	ns
DB vs AIR	-1.3	0.81	-1.61	0.110	ns
EV vs AIR	1.10	1.14	0.97	0.332	ns
HH vs AIR	-0.17	0.92	-0.18	0.855	ns
NV vs AIR	-0.035	1.19	-0.030	0.976	ns

Significance: 'ns' $P > 0.05$

Table S6: Summary of the hypotheses tested within the binomial GLM on the first choice of *Trissolcus mitsukurii* exposed to odours from stink bug eggs of *Arma custos* (AC), *Dolycoris baccarum* (DB), *Eurydema ventralis* (EV), *Halyomorpha halys* (HH), or *Nezara viridula* (NV). Control consisted of clean air (AIR). For each comparison, the estimate coefficient (log odd), SE, z-value and P-value are reported.

Planned comparisons	Estimate	SE	z value	P	
AC vs AIR	0.00	0.30	0.00	1.000	ns
DB vs AIR	-0.26	0.24	-1.080	0.280	ns
EV vs AIR	0.53	0.35	1.50	0.133	ns
HH vs AIR	0.0074	0.27	0.27	0.786	ns
NV vs AIR	0.00	0.35	0.00	1.000	ns

Significance: 'ns' $P > 0.05$