2018

Universität Bremen

Benefits and risks of using native parasitoids for augmentative biological control of the invasive pest *Halyomorpha halys* in Europe

THESIS SUBMITTED FOR THE DEGREE OF DR. RER. NAT.
AT THE UNIVERSITY OF BREMEN - FACHBEREICH 2

JUDITH M. STAHL MSC.

Supervisors: Dr. Tim Haye, Dr. Dirk Babendreier and Prof. Dr. Thomas Hoffmeister

Examiners: Prof. Dr. Eric Conti and Dr. Tracy Leskey

Defended on the 17. December 2018, committee chaired by Prof. Dr. Marko Rohlfs

CABI

B I N G O

This copy contains a shortened version of the PhD dissertation thesis presented to the PhD evaluation committee, in accordance to the “Promotionsordnung (Dr. rer. nat.) der Universität Bremen für den Fachbereich 2 (Biologie/Chemie)” of the 08.07.2015, § 12.

In particular, for chapter 2, 3, and 7, only the abstracts are presented as the content of these chapters has already been published. Indication on the scientific journals where to find the full content of these chapters is given at the beginning of the respective chapter.

One man could watch about 50 of these vials with comparative ease, for the adults work rather slowly.

– D. L. Parker, 1933, about working with an *Anastatus* species

Abstract

The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), is an herbivorous insect that originates from China, Japan and Korea. It has invaded both the American continent and Europe. In the USA, *H. halys* has been causing massive damage to various crops, e.g. apples, peaches, sweet corn and beans. In Europe and Eurasia, including northern Italy and western Georgia, severe damage has been observed in pear and hazelnut orchards. Frequent applications of broad-spectrum insecticides against *H. halys* have led to increased mortality of natural enemies and ensuing outbreaks of secondary pests in the USA.

In its native range, *H. halys* populations are well regulated by natural enemies, including a number of egg parasitoids in the genera *Trissolcus* (Hymenoptera: Platygastridae) and *Anastatus* (Hymenoptera: Eupelmidae), with *Trissolcus japonicus* (Ashmead) being the most abundant species. However, with the restricted access to exotic biological control agents due to the Nagoya protocol and growing concern about non-target effects of exotic biological control agents, in the recent years, research is focusing more on augmentative biological control using native natural enemies against invasive pests.

In Europe, native scelionid species typically associated with other pentatomids attempt to attack *H. halys* eggs, but are not able to complete development. As such, European *Trissolcus* and *Telenomus* species are not promising candidate agents for biological control of *H. halys*. In contrast, another European egg parasitoid, *Anastatus bifasciatus* (Geoffroy), is the species most commonly found parasitizing *H. halys* eggs, and can complete its development on live *H. halys* eggs. This polyphagous species has been reared from sentinel and natural field-collected *H. halys* eggs in several European countries and is the most promising European biological control agent for *H. halys*. In this PhD thesis I evaluated the potential of *A. bifasciatus* for augmentative biological control against the brown marmorated stinkbug in Europe.

Investigations on the life history of *A. bifasciatus*, described in **chapter 2**, characterized the parasitoid as a synovigenic species with a lifetime fecundity of fifty-two eggs over an average female lifespan of three months. In northwestern Switzerland, it completes three generations and its oviposition period overlaps with that of the pest. Development is possible on viable *H. halys* eggs of all ages and eggs stored at -80°C for up to two years, which are promising results for a potential mass rearing strategy.

In **chapter 3**, the host range of the polyphagous *A. bifasciatus* was examined, focusing on the suitability of *H. halys* as a host in comparison to non-target host species. In no-choice experiments all nine heteropteran and 14 out of 19 tested lepidopteran non-target species produced viable *A. bifasciatus* offspring. The proportion of *A. bifasciatus* females producing offspring did not differ between non-target and target for 19 out of the 28 non-target species. The results of this study confirmed the polyphagous nature of *A. bifasciatus* and suggest that a number of non-target species, including Lepidoptera of conservation interest, may be attacked in the field. Thus, non-target effects following inundative releases of *A. bifasciatus* cannot entirely be ruled out.

Interspecific interactions with a second potential European biological control agent of *H. halys*, *Ooencyrtus telenomicida* (Vassiliev) (Hymenoptera: Encyrtidae) were evaluated in **chapter 4**. Both species readily oviposited in eggs containing eggs and early instar larvae of the competitor. *Ooencyrtus telenomicida* offspring emergence from multiparasitized eggs was significantly lower than from rearing controls, independent of the order of parasitization. *Anastatus bifasciatus* offspring emergence was only influenced by the presence of *O. telenomicida* when it parasitized as the second species. These results suggest that *A. bifasciatus* is the superior intrinsic competitor and no negative implications for inundative biological control with *A. bifasciatus* are expected.

To evaluate the efficacy against *H. halys* and non-target parasitism in the field, experimental releases of *A. bifasciatus* were conducted over a period of three years in Swiss and Italian fruit orchards, described in **chapter 5**. Parasitism of sentinel *H. halys* eggs, measured by offspring emergence, was recorded after all eight release events, but parasitism levels were low, averaging 7%. Eggs of exemplary non-target species were parasitized similarly, confirming laboratoy results from chapter 3. Impact on the target is assumed to be underestimated, because mortality induced by host feeding and unemerged *A. bifasciatus* were not taken into account.

Addressing that issue in **chapter 6**, a PCR-based screening tool for *Anastatus* species was developed and tested in terms of specificity and sensitivity, as well as application to field samples. Only DNA of *Anastatus* species was amplified successfully, and 1 h old *A. bifasciatus* eggs could be detected with 95% success. Screening sentinel field-exposed eggs from chapter 5 revealed that 14% of unemerged *H. halys* eggs from parasitized egg masses contained *A. bifasciatus*, thereby confirming the underestimation of parasitism.

With additional sentinel *H. halys* egg masses exposed in the southeast of Switzerland in 2017 and 2018, the first adventive populations of *T. japonicus* in Europe could be recorded in **chapter 7**. The establishment of this promising classical biological control agent of *H. halys* might lead to a significant decrease in pest populations in coming years. Until *T. japonicus* populations are high enough, an integrated strategy to control *H. halys* in Europe might be most promising. This could combine attract-and-kill methods, selective spraying of border rows, and if cost effective, inundative *A. bifasciatus* releases during *H. halys* oviposition peaks.

Zusammenfassung

Die marmorierte Baumwanze, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), ist ein herbivores Insekt aus China, Japan und Korea, das sowohl auf dem amerikanischen Kontinent als auch in Europa invasiv vorkommt. In den USA hat *H. halys* massiven Schaden an einer Vielzahl von Kulturpflanzen wie Apfel, Pfirsich, Mais und Bohnen verursacht. In Europa und Eurasien, insbesondere in Norditalien und Westgeorgien, sind schwere Schäden im Birnenanbau und in Haselnussplantagen aufgetreten. Die häufigen Applikationen von Breitbandinsektiziden zur Bekämpfung der Wanze in den USA haben dort zu erhöhter Mortalität von natürlichen Feinden und, darauf folgend, Ausbrüchen von Sekundärschädlingen geführt.

In Asien werden die Eier der Wanze von Parasitoiden der Gattungen *Trissolcus* (Hymenoptera: Scelionidae) und *Anastatus* (Hymenoptera: Eupelmidae) attackiert, was eine natürliche Populationskontrolle darstellt. *Trissolcus japonicus* (Ashmead) ist die am häufigsten auf *H. halys*-Eiern anzutreffende Art und demzufolge der vielversprechendste Kandidat für klassische biologische Kontrolle. Da allerdings der Zugang zu exotischen natürlichen Feinden dank des Nagoya-Protokolles zunehmend erschwert ist und Bedenken bezüglich Nichtzieleffekte durch die Einführung exotischer natürlicher Feinde in den letzten Jahren deutlich gestiegen sind, geht die Forschung mittlerweile in die Richtung augmentiver biologischer Kontrolle mit einheimischen natürlichen Feinden gegen invasive Arten.

In Europa attackieren mehrere Scelioniden-Arten *H. halys*-Eier, sind aber nicht in der Lage ihre Larvalentwicklung auf lebensfähigen Wanzeneiern abzuschließen. Dementsprechend sind europäische *Trissolcus*- und *Telenomus*-Arten für die biologische Kontrolle von *H. halys* ungeeignet. Im Gegensatz dazu ist *Anastatus bifasciatus* (Geoffroy), die am häufigsten auf europäischen *H. halys*-Eiern zu findende Art. Sie wurde aus künstlich ausgebrachten und natürlichen *H. halys*-Eigelegen in mehreren europäischen Ländern gezüchtet und ist in der Lage sich auf frischen Wanzeneiern zu entwickeln. *Anastatus bifasciatus* ist demnach die vielversprechendste Art für den biologischen Pflanzenschutz von *H. halys* in Europa. Für die vorliegende Doktorarbeit habe ich das Potential dieses Parasitoiden für die augmentative biologische Kontrolle der marmorierten Baumwanze in Europa untersucht.

Untersuchungen zur Biologie und Phänologie von *A. bifasciatus* sind in **Kapitel 2** beschrieben und charakterisieren *A. bifasciatus* als synovigenen Parasitoiden, der im Durchschnitt über seine dreieinhalb Monate Lebenszeit 52 Eier legt. In der Nordwestschweiz kann *A. bifasciatus* drei Generationen vollenden und seine Ovipositionsperiode überlappt mit der von *H. halys*. Der Parasitoid kann sich auf *H. halys*-Eiern jedweden Alters – bis zum Schlupf der Nymphen, und auf Eiern, die für bis zu zwei Jahre bei -80°C gelagert wurden entwickeln, was vielversprechend für eine Massenzucht ist.

In **Kapitel 3** wird das Wirtsspektrum des polyphagen Parasitoiden näher untersucht, mit Fokus auf der Eignung von *H. halys* als Wirt im Vergleich zu Nichtzielarten. In Laborexperimenten war *A. bifasciatus* in der Lage, in Eier von allen neun Wanzen- und 14 von 19 getesteten Lepidopterenarten Nachwuchs zu produzieren. Die Proportion von *A. bifasciatus*-Weibchen, die Nachwuchs produzierten war gleich in Nichtzielarten und der jeweiligen *H. halys*-Kontrolle für 19 der 28 getesteten Nichtzielarten. Diese Ergebnisse bestätigen die polyphage Veranlagung des Parasitoiden und geben Hinweise darauf, dass Nichtzielarten, u.a. auch Lepidopteren, welche unter Naturschutz stehen, im Feld attackiert werden. Dementsprechend können Nichtzieleffekte von *A. bifasciatus*-Massenfreisetzungen nicht ausgeschlossen werden.

Interspezifische Interaktionen zwischen *A. bifasciatus* und einem weiteren potentiellen europäischen biologischen Pflanzenschutzkandidaten, *Ooencyrtus telenomicida* (Vassiliev) (Hymenoptera: Encyrtidae) wurden in **Kapitel 4** näher betrachtet. Beide Arten parasitierten *H. halys*-Eier, die bereits Eier und Junglarven der jeweils anderen Art enthielten. Die Nachwuchsproduktion von *Ooencyrtus telenomicida* war signifikant niedriger aus multiparasitierten *H. halys*-Eiern als aus den

Zuchtkontrollen mit unparasitierten Wirtseiern, unabhängig von der Reihenfolge der Parasitierung. *Anastatus bifasciatus* hingegen war nur negativ durch die Präsenz des Konkurrenten beeinträchtigt, wenn sie als zweite Art parasitiert hatte. Diese Ergebnisse legen nahe, dass es sich bei *A. biasciatus* um den überlegenen intrinsischen Konkurrenten handelt. Für die biologische Kontrolle mit *A. bifasciatus* werden keine negativen Effekte erwartet.

Um die Effizienz von *A. bifasciatus* gegen *H. halys* zu testen und potentielle Parasitierung von Nichtzielarten im Feld festzustellen, wurden über drei Jahre experimentelle Massenfreisetzungen in Schweizer und Italienischen Obstanlagen durchgeführt, welche in **Kapitel 5** näher beschrieben sind. Parasitierung von künstlich ausgebrachten *H. halys*-Eiern wurde mittels Parasitoidennachwuchs ermittelt und konnte nach allen acht Freilassungen erfasst werden. Allerdings waren Parasitierungswerte mit durchschnittlich 7% gering, und die verwendeten Beispiel-Nichtzielarten waren ähnlich hoch parasitiert, was die Laborergebnisse aus Kapitel 3 bestätigt. Es wird vermutet, dass die Kontrolleffizienz von *H. halys*-Eiern durch *A. bifasciatus* unterschätzt wird, da die Mortalität, welche durch das häufige Aufnehmen von Wirtsmaterial durch das adulte Parasitoidenweibchen auftritt, und die Mortalität durch Parasitoidennachwuchs, welcher vor dem Schlupf verendet ist, nicht mit in die Berechnungen miteinbezogen worden sind.

Dieser Aspekt wurde in **Kapitel 6** aufgegriffen, in dem eine PCR-basierte Methode zur Identifizierung von *Anastatus*-Arten entwickelt und bezüglich Spezifität, Sensitivität und Anwendung auf Feldproben getested wurde. Nur DNS von drei *Anastatus*-Arten wurde erfolgreich amplifiziert. Eine Stunde alte *A. biasciatus*-Eier wurden mit 95% Erfolg nachgewiesen. In 14% der *H. halys*-Eier aus parasitierten Eigelegen eines Freisetzungsversuchs aus Kapitel 5 konnte *A. bifasciatus*-DNS detektiert werden, was die Hypothese, dass die durch *A. bifasciatus* induzierte Wirtsmortalität unterschätzt wurde bestätigt.

Zusätzlich 2017 und 2018 in der Südostschweiz ausgebrachte *H. halys*-Eigelege konnten den ersten Nachweis von *T. japonicus*-Populationen in Europa erbringen (**Kapitel 7**). Dass der asiatische Parasitoid bereits in der Schweiz etabliert ist, lässt hoffen, dass die *H. halys*-Populationen in den kommenden Jahren schrumpfen werden. Bis *T. japonicus* die nötigen Populationsdichten erreicht hat, könnte eine integrierte Strategie am sinnvollsten sein. Diese könnte „attract-and-kill“-Methoden, selektive Insektizidapplikationen von Randreihen und, falls es sich als kosteneffektiv herausstellt, Massenfreisetzungen von *A. bifasciatus* zu Spitzeneiablagezeiten von *H. halys* beinhalten.

Declaration by author

Versicherung an Eides Statt

Ich, Judith Stahl, Rue de la Préfecture 2, CH-2800 Delémont, Switzerland

versichere an Eides Statt durch meine Unterschrift, dass ich die vorstehende Arbeit selbständig und ohne fremde Hilfe angefertigt und alle Stellen, die ich wörtlich dem Sinne nach aus Veröffentlichungen entnommen habe, als solche kenntlich gemacht habe, mich auch keiner anderen als der angegebenen Literatur oder sonstiger Hilfsmittel bedient habe.
Ich versichere an Eides Statt, dass ich die vorgenannten Angaben nach bestem Wissen und Gewissen gemacht habe und dass die Angaben der Wahrheit entsprechen und ich nichts verschwiegen habe.
Die Strafbarkeit einer falschen eidesstattlichen Versicherung ist mir bekannt, namentlich die Strafandrohung gemäß § 156 StGB bis zu drei Jahren Freiheitsstrafe oder Geldstrafe bei vorsätzlicher Begehung der Tat bzw. gemäß § 161 Abs. 1 StGB bis zu einem Jahr Freiheitsstrafe oder Geldstrafe bei fahrlässiger Begehung.

Delémont, 26.10.2018
____
Ort, Datum Unterschrift

Publications

Published:

Stahl, J.M., Babendreier, D., & Haye, T. (2018) Using the egg parasitoid *Anastatus bifasciatus* against the invasive brown marmorated stink bug in Europe: can non-target effects be ruled out? *Journal of Pest Science*, 1-13.

- A reformatted version has been incorporated in this thesis as chapter 3
- Author Contribution Statement: DB, JS and TH conceived and designed research. JS and TH conducted experiments. JS analysed data. JS wrote the manuscript, DB and TH edited it. All authors read and approved the manuscript.

Stahl, J.M., Babendreier, D., & Haye, T. (2018) Life history of *Anastatus bifasciatus*, a potential biological control agent of the brown marmorated stink bug in Europe. *Biological Control.*

- A reformatted version has been incorporated in this thesis as chapter 2
- Author Contribution Statement: DB, JS and TH conceived and designed research. JS and TH conducted experiments. JS analysed data. JS wrote the manuscript, DB and TH edited it. All authors read and approved the manuscript.

Stahl, J.M., Tortorici, F., Pontini, M., Bon, M.-C., Hoelmer, K., Marazzi, C., Tavella, L. & Haye, T. (2018) First discovery of adventive populations of *Trissolcus japonicus* (Ashmead) in Europe. *Journal of Pest Science.*

- A reformatted version has been incorporated in this thesis as chapter 7
- Author Contribution Statement: JS, CM and TH conceived and designed research. LT, FT, MP and MCB identified the parasitoids. KH provided parasitoids from Asia for molecular analysis. All authors contributed to writing the manuscript and approved the final version.

Submitted:

Stahl, J.M., Gariepy, T.D., Beukeboom, L.W. & Haye, T. (2018) Developing a molecular tool to identify *Anastatus* parasitoids of the brown marmorated stink bug. *Entomologia Experimentalis et Applicata.*

- A reformatted version has been incorporated in this thesis as chapter 6
- Author Contribution Statement: TH, TG, JS and LB conceived and designed research. JS and TG conducted experiments. JS and TG analysed data. JS and TG wrote the manuscript, LB and TH edited it. All authors read and approved the manuscript.

Table of content

1 General introduction

1.1 Invasive species

The number of invasive species, measured by the annual first records of alien species in a region in which the species has later become established, has been increasing in the last 200 years and this trend seems to continue (Seebens et al. 2017). For this thesis, the term *invasive species* shall be defined as non-native or alien species whose high reproductive success and/or spread have negative implications for the functioning of ecosystems (Simberloff et al. 2013), human health (Pysek and Richardson 2006), or the economy (Kettunen et al. 2009). The increase of invasive species can be attributed to growing trade and human mobility (Hulme 2009). In addition, changes in land use facilitate the establishment of alien species (Pauchard and Alaback 2004). Even though the number of potential invasive species is limited, new source pools become available by a change in trade partners and routes as well as a change in the environment by land use and climatic conditions and a large number of species may still become invasive in the future (Seebens et al. 2018).

For future predictions, identifying factors that facilitate invasion success is crucial, but these seem to be dependent on the combination of invading species and invaded community (Sakai et al. 2001, Richardson and Pysek 2006). Some traits of invading species such as phenotypic plasticity, short generation time and high growth rates, and some traits of the invaded community such as community immaturity, low relative competitive abilities of native species and niche opportunity, have been shown to be advantageous for invasion (Lonsdale 1999, Kolar and Lodge 2001, Roy et al. 2011). Regulations to slow the spread of introduced alien species are implemented increasingly but face the problem that many of these species were either not known as pests in their area of origin or not even known to science (Seebens et al. 2017). The immense impact of invasive species in their invaded areas might be explained by the ‘enemy release hypothesis’ (ERH) (Williamson 1996). This theory explains the invasion success of a species with the reduced impact of natural enemies in the invaded area, leading to a shift of energy allocation from defense to reproduction and growth, giving the invaders a competitive advantage against native species with their respective intact natural enemy complexes, and consequently resulting in an increase in distribution and abundance of the alien species (Keane and Crawley 2002, Roy et al. 2011). One of the key assumptions of the ERH is that in the native range of an invasive species its population size is regulated by natural enemies (Roy et al. 2011), which is also assumed for the invasive species of interest in this thesis, the brown marmorated stink bug.

1.2 The invasive brown marmorated stink bug: invasion history, biology and damage

The brown marmorated stink bug (BMSB), *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) (Figure 1), is an herbivorous insect that originates from China, Japan and Korea, where occasional outbreaks of *H. halys* cause damage to a variety of plants (Yanagi and Hagihara 1980). *Halyomorpha halys* has invaded both the American continent and Europe (Haye et al. 2015, Rice et al. 2014), and its impact in these regions has recently been reviewed by Leskey and Nielsen (2018). High haplotype diversity in Europe indicates several separate introductions from Asia as well as secondary introductions from the USA (Gariepy et al. 2015). Overwintering adults are easily transported with cargo or personal luggage (Hoebeke and Carter 2003) and have been frequently intercepted at the border of areas that have not yet been invaded, such as Australia (Walker 2009), New Zealand (Duthie 2012), and Great Britain (Tim Haye personal communication).

Figure 1 *Halyomorpha halys* adult (Photo: Tim Haye)

Before migrating to agricultural land, *H. halys* generally stays in the urban centers it arrived in, builds up its population and becomes noticeable as a nuisance pest due to its overwintering behavior. In autumn, males emit an aggregation pheromone that leads to clusters of *H. halys*, which then move to suitable overwintering sites, mainly human structures such as sheds or houses (Khrimian et al. 2014, Watanabe et al. 1994). In spring, the stink bugs leave their hibernations sites, start maturation feeding and mate a few weeks later (Haye et al. 2014a). Oviposition begins in May (Tim Haye observations 2018) or June, depending mainly on ambient temperature (Haye et al. 2014a). Egg masses of mostly 28 eggs are laid on the underside of host plants (Nielsen et al. 2008, Takahashi 1930). The hatching nymphs undergo five instars. While the first instar nymphs mainly stay around the empty egg mass to ingest the symbiotic microorganisms the mother transferred with the eggs, the successive instars are highly mobile and feed like the adults on leaves, shoots and especially maturing fruits of their host plants (Nielsen and Hamilton 2009, Lee et al. 2014, Taylor et al. 2014).

Halyomorpha halys is a highly polyphagous species that has been recorded from a total of 106 host plants in 45 families in its native range (reviewed in Lee at al. 2013). In the invaded regions in North America and Europe its host range includes more than 170 and 50 plant species, respectively (Bernon 2004, Haye et al. 2014b, Leskey and Nielsen 2018). In North America, where *H. halys* was first discovered in the mid-1990s (Hoebeke and Carter 2003), damage has been observed in fruit crops (peaches and apples), row crops (soybeans, corn) and vegetables (green beans, peppers, tomatoes) (Kuhar et al. 2012, Leskey et al. 2012a, b, Nielsen and Hamilton 2009) with economic losses of more than $37 Mio. alone in the apple production of the mid-Atlantic states in 2010 (Leskey et al. 2012a). In Europe, where *H. halys* is now reported from over ten countries and the Eurasian Georgia, especially pears, apples and hazelnuts have been attacked (Bosco et al. 2017, Maistrello et al. 2017).

1.3 Control methods for the brown marmorated stink bug

Current control efforts mainly include chemical control with insecticides, mass trapping using pheromones (‘attract and kill’) and biological control. Initially, insecticides registered for native stink bugs in the USA were applied, but applications increased manifold in infested apple and peach orchards in the mid-Atlantic region, interrupted established IPM programs and often failed to show the desired effects (Leskey et al. 2012a). Instead, ‘knockdown and recovery’ effects were observed when *H. halys* adults were treated with broad-spectrum insecticides such as pyrethroids and neonicotinoids (Lee et al. 2013a, Leskey et al. 2012c). Now, effective insecticides have been identified but the necessary frequent applications have led to increased mortality of natural enemies and ensuing outbreaks of secondary pests such as San Jose scale *Comstockaspis perniciosus* (Comstock), woolly apple aphid *Eriosoma lanigerum* Hausmann, and European red mite *Panonychus ulmi* (Koch) (Leskey et al. 2012b, Short et al. 2017).

Traps are mainly used as monitoring tools. A number of different trap designs with varying shapes, sizes and colours have been developed and are summarized in Rice et al. (2017) but black pyramid traps are considered most effective (Leskey et al. 2012d, Rice et al. 2017). Equipped with a blend of the aggregation pheromone of *H. halys* and the closely related oriental stink bug *Plautia stali* Scott (Hemiptera: Pentatomidae) (Tada et al. 2001a, b, Lee et al. 2002, Khrimian et al. 2014) as well as an insecticide strip, the traps are an efficient monitoring tool for the whole growing season (Leskey et al 2015, Morrison et al. 2015, Rice et al. 2017). Adding a light source to the pyramid trap can enhance trapping success mid-season (Rice et al. 2017).

Recently, effective ‘attract-and-kill’ strategies (Morrison et al. 2015, 2018) and insecticide-treated nets have been developed (Sabbatini Peverieri et al. 2018a, b). ‘Attract-and-kill’ for *H. halys* involves trees baited with pheromones that are treated regularly with insecticides (Morrison et al. 2015). It has been tested in North American apple orchards and shown to successfully reduce low to moderate *H. halys* populations with a reduction of the pesticide-treated area of 97% (Morrison et al. 2018). Consequently, neither natural enemies nor secondary pests were affected by the insecticide treatments (Morrison et al. 2018).

Control options for organic fruit production are still very limited and thus, there is an urgent need for environmentally friendly solutions, such as biological control.

1.4 The concept of biological control

Biological control is defined as a method to reduce pest populations and the resulting damage with natural enemies (Hoy 2008). For insect pests, these natural enemies can be microbials (nemadotes, fungi, bacteria) or macrobials (predators, parasitoids) (Hoy 2008). While predators are generally larger in size than their prey and consume more than one prey in their lifetime, parasitoids resemble predators in that they kill their host, and parasites in that they only need one host to complete their development (Godfray 1994). The majority of parasitoids belong to the Hymenoptera, but there are also dipteran and coleopteran parasitoids (Grenier 1988, Weber et al. 2008). Parasitoids can be subdivided into guilds (Mills 1994) that define the host stage the parasitoids attack (egg, larval, pupal, and adult parasitoids) and their developmental strategies: they either live in (endoparasitoids), or on (ectoparasitoids) their host, which is either killed or paralyzed immediately after parasitoid oviposition (idiobiont) or continues its growth and/or development (koinobiont). They are further divided into parasitoids can complete development on one host individually (solitary) or as a group (gregarious) (Ode and Rosenheim 1998).

Conservation biological control aims for natural pest control without interventions by manipulating habitats to favor natural enemies or reduce negative impacts of pesticides on natural enemy communities (Heimpel and Mills 2017). Interventions in the form of parasitoid releases are applied in classical and augmentative biological control. Classical biological control is the main biocontrol strategy for invasive species. It consists of the import, release and establishment of natural enemies normally from the native range of the invasive pest (Hoy 2008). Since classical biological control programs are planned introductions of exotic insects, concerns regarding non-target effects of the introduced biocontrol agents have led to the development of risk assessment procedures that are necessary to apply before any releases can take place (Bigler et al. 2006, Barratt et al. 2010, van Lenteren et al. 2006).

Augmentative biological control involves releases of natural enemies that can be inundative (parasitoids are effectively used like pesticides, with high numbers several times over the growing season; immediate pest control is achieved by the generation of natural enemies released) or inoculative (parasitoids are released usually once at the beginning of the season in comparatively low numbers and expected to increase their population within the season; multiplication is necessary for successful pest control) (Heimpel and Mills 2017). To date, augmentation has rarely been applied to control invasive species, since natural enemies that co-evolved with the pest, like those used in classical biological control, are considered more efficient and specific. The ‘new association’ theory questions this hypothesis, arguing that natural enemies that did not co-evolve with the pest are more likely to be successful due to lacking defensive strategies of their newly associated hosts (Hokkanen and Pimentel 1984). Generally, the term ‘new association’ biological control is used for controlling a native pest with an exotic biological control agent. With the implementations of the Nagoya protocol indigenous natural enemies are now increasingly being used for augmentative releases against invasive pests such as e.g., the generalist predators *Nesidiocoris tenuis* (Reuter) and *Macrolophos pygmaeus* Rambur (Hemiptera: Miridae) as well as native parasitoids against the South American tomato pinworm *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) in Southern European greenhouses (Molla et al. 2013, Zappala et al. 2012). Even in the field, augmentative biological control with native natural enemies is now considered against invasive species, such as the spotted wing drosophila *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) (Chabert et al. 2013, Stacconi et al. 2013).

1.5 Biological control of the brown marmorated stink bug

In its native range, *H. halys* populations are well regulated by natural enemies, including a number of egg parasitoids in the genera *Trissolcus* (Hymenoptera: Platygastridae) and *Anastatus* (Hymenoptera: Eupelmidae), with *Trissolcus japonicus* (Ashmead) being the most abundant species (Lee et al. 2013b, Yang et al. 2009, Zhang et al. 2017). Subsequently, *T. japonicus* has been considered for classical biological control in areas invaded by *H. halys*, but adventive populations were found in North America before risk assessments were completed, presenting regulatory agencies with a challenge (Talamas et al. 2015). In laboratory no-choice tests, *T. japonicus* attacked seven out of ten native North American Pentatomidae (Hedstrom et al. 2017), and in its native range in China, several non-target hosts were attacked not only in the laboratory but also in the field (Zhang et al. 2017). In Europe, host specificity tests are ongoing, but preliminary results confirm observations in North

America and China that *T. japonicus* has a broader host range and that non-target effects seem possible (Haye et al., unpublished data).

With the restricted access to exotic biological control agents due to the Nagoya protocol (Cock et al. 2010) and growing concern about non-target effects of exotic biological control agents, in the recent years, research is focusing more on augmentative biological control using native natural enemies against invasive pests. Indigenous parasitoids that attack an exotic host are typically generalist idiobionts, because their strategy to kill or paralyze the host immediately allows them to operate independently of the immune system of the host. Consequently, adopting new hosts poses fewer challenges for them than for koinobiont parasitoids (Askew and Shaw 1986, Zappala et. al 2012). Surveys looking for native egg parasitoids that have adopted *H. halys* as a new host have been conducted in Europe and North America (Abram et al. 2017). A number of *Trissolcus* species were found to attack *H. halys* but failed to develop in viable eggs (Haye et al. 2015). Thereby, the invasive stink bug serves as an ‘evolutionary trap’ for these European *Trissolcus* species (Abram et al. 2014). Only recently, a European species, *T. kozlovi*, capable of developing in viable *H. halys* eggs, was recorded from Italy (Moraglio et al. unpublished), but further tests are needed to investigate its potential for biological control of *H. halys*. In addition, a second species from another group, *Ooencyrtus telenomicida* (Vassiliev) (Hymenoptera: Encyrtidae) was found in Italy (Roversi et al. 2016) and is currently being tested in field release trials. The only species known at the beginning of the present study and still the most promising European candidate for augmentative biological control is, however, a third species: *Anastatus bifasciatus* (Geoffroy) (Hymenoptera: Eupelmidae) (Figure 2). To date, this widespread species is the only European egg parasitoid that has been collected from both sentinel and natural *H. halys* eggs in Italy and Switzerland (Haye et al. 2015, Costi et al. 2018).

1.6 *Anastatus bifasciatus* as a potential biological control agent

Despite being the most common natural enemy of *H. halys* in Europe, little is known about the Palearctic *A. bifasciatus* (Noyes 2014). Typical for many native parasitoids attacking exotic hosts (Zappala et al. 2012), parasitism of *H. halys* eggs by *A. bifasciatus* is generally low. In Southern Switzerland (Canton Valais) parasitism levels of sentinel frozen *H. hays* eggs did not exceed 0.5%, whereas in northern Italy parasitism levels ranged from 1 to 3% in agroecosystems and urban environments, respectively (Costi et al. 2018). However, the fact that *A. bifasciatus* is often the only species continuously recovered from *H. halys* eggs in Europe can be seen as an argument for a conservation or augmentative biological control approach (Costi et al. 2018). An augmentative biological control program using *A. bifasciatus* to reduce *H. halys* populations in European fruit orchards could follow either an inundative or an inoculative approach. Independent of the choice of approach, for a successful biological control program four aspects that need to be clarified have been postulated by Smith (1996) for the commonly used egg parasitoid Trichogramma (Hymenoptera: Trichogrammatidae): the most suitable species or strain must be selected, a mass rearing system developed, the parasitoids distributed, and a strategy for field releases implemented (e.g. Tang et al. 2017). Species or strain selection should follow the assessment of life history traits like fecundity, longevity, development, sex ratio, and host preference (Smith 1996). These parameters are highly relevant for the development of an appropriate biological control strategy for any specific natural enemy-pest system.

Figure 2 *Anastatus bifasciatus* female on *Dendrolimus pini* eggs (Photo: Tim Haye)

The safety of classical biological control using invertebrates, particularly potential negative impacts on non-target species, has been discussed primarily in the context of classical biological control (Bigler et al. 2006, Barratt et al. 2010), but an extensive literature study by Lynch et al. (2001) revealed that far more inundative than classical biological control releases have led to population level effects on non-target species. However, their focus was primarily on exotic biological control agents, whereas inundative releases typically involve native natural enemies. Using native natural enemies such as *A. bifasciatus* instead of exotic ones reduces the potential risks of biological control considerably, since the agents are already naturally present. However, continuous inundative releases of native generalist biological control agents can also lead to non-target effects due to overflow into adjacent habitats (van Lenteren and Loomans 2006) and thus, endangered and beneficial species within those habitats could be at risk. Inundative releases of native parasitoids could further facilitate apparent competition between the native and exotic host sharing the same natural enemies (Holt 1977, Holt and Bonsall 2017). Host specificity is considered key to safety in biological control (Nechols et al. 1992) and in the context of *A. bifasciatus*, this can refer to the species host range, broadly defined as the set of species that can support development of the parasitoid (Strand and Obrycki, 1996). *Anastatus bifasciatus* has mainly been reported as parasitoid of lepidopteran tree pests like pine and oak processionary moths (*Thametopoea pityocampa* Den. and Schiff and *T. processionea* L. (Lepidoptera: Notodontidae) (e.g. Avci 2000, Tiberi et al. 1990), but in total, its known host range comprises more than 30 species in the orders Lepidoptera and Hemiptera (compiled by Noyes 2014), characterizing *A. bifasciatus* as a generalist parasitoid.

If potential risks associated with mass releases of *A. bifasciatus* are considered acceptable, in a next step, a release strategy would need to be developed, addressing factors including the type (inundative or inoculative), timing and method of releases. Ideally, this strategy should be based on data from laboratory experiments, investigating the parasitoid's general biology, which is then tested under field conditions. Even though apple is not the best host plant for *H. halys* concerning nutritional status of adult stink bugs and number of produced eggs (Funayama 2004), damage in apple alone in the mid-Atlantic states of the USA in 2010 has led to more than 90% harvest loss for individual growers (American/Western Fruit Grower 2011, Leskey and Hamilton 2010). Since in Switzerland, fruit production is dominated by apple cultivation, which is grown on more than 4,000 ha (Bravin et al. 2012), and both *A. bifasciatus* and *H. halys* prefer tree habitats, Swiss apple orchards may be considered a good option for experimental field releases.

1.7 Thesis objectives

In this PhD thesis I evaluated the potential of the native European egg parasitoid *A. bifasciatus* for augmentative biological control against the invasive brown marmorated stinkbug, *H. halys,* in Europe. To address the objective of my thesis several aspects were examined: the life history of *A. bifasciatus* (chapter 2); the host range of *A. bifasciatus* and the suitability of *H. halys* in comparison to non-target species to assess potential risks associated with mass releases of *A. bifasciatus* (chapter 3); the intrinsic competition between *A. bifasciatus* and *O. telenomicida*, another potential European biological control considered for augmentation biological control of *H. halys* (chapter 4); the efficacy of *A. bifasciatus* against *H. halys* and potential non-target parasitism in Swiss and Italian fruit orchards after experimental field releases (chapter 5); and the development and evaluation of a molecular marker for *A. bifasciatus* as a tool to better assess field parasitism (chapter 6). In addition, I report on the first discovery of adventive *T. japonicus* populations in Europe (chapter 7).

1.8 References

Abram PK, Gariepy TD, Boivin G, Brodeur J (2014) An invasive stink bug as an evolutionary trap for an indigenous egg parasitoid. Biological Invasions 16, 1387–1395.

Abram PK, Hoelmer KA, Acebes-Doria A et al. (2017) Indigenous arthropod natural enemies of the invasive brown marmorated stink bug in North America and Europe. Journal of Pest Science 90, 1009-1020.

Abram PK, Moffat CE (2018) Rethinking biological control programs as planned invasions. Current Opinion in Insect Science 27, 9–15.

Aebi A, Schönrogge K, Melika G, Quacchia A, Alma A, Stone GN (2007) Native and introduced parasitoids attacking the invasive chestnut gall wasp *Dryocosmus kuriphilus*. EPPO bulletin 37, 166–171.

American/Western Fruit Grower (2011) Brown Marmorated Stink Bug Causes $37 Million in Losses to Mid-Atlantic Apple Growers. April 14, 2011. http://www.growingproduce.com/article/21057/brown-marmorated-stink-bug-causes-37-million-in-losses-to-mid-atlantic-apple-growers. Accessed September 15, 2018.

Askew RR, Shaw MR (1986) Parasitoid communities: their size, structure and development. In Waage, J and Greathead, D (eds), Insect Parasitoids, 13th Symposium of Royal Entomological Society of London (pp. 225-264). Academic Press, London: now Elsevier.

Avcı M (2000) Investigations on structure of egg-batches, parasitism and egg laying habits of *Thaumetopoea pityocampa* (Den. & Schiff.) (Lep.: Thaumetopoeidae) in various regions of Turkey. Türkiye Entomoloji Dergisi 24, 167–178.

Barratt B, Howarth F, Withers T, Kean J, Ridley G (2010) Progress in risk assessment for classical biological control. Biological Control 52, 245–254.

Bernon G (2004) Biology of *Halyomorpha halys*, the brown marmorated stink bug (BMSB). United States Department of Agriculture, Animal and Plant Health Inspection Service, Center for Plant Health Science and Technology. Report No.: T3P01, 17.

Bigler F, Babendreier D, Kuhlmann U (2006) Environmental impact of invertebrates for biological control of arthropods: methods and risk assessment. CABI, Wallingford.

Bosco L, Moraglio ST, Tavella L (2017) *Halyomorpha halys*, a serious threat for hazelnut in newly invaded areas. Journal of Pest Science 91, 661-670.

Bravin E, Carint D, Hanhart J (2012) Economic Evaluation of the Swiss Apple and Pear Production. In II International Symposium on Horticulture in Europe 1099, pp. 541–548.

Chabert S, Allemand R, Poyet M, Eslin P, Gibert P (2013) Ability of European parasitoids (Hymenoptera) to control a new invasive Asiatic pest, *Drosophila suzukii*. Biological Control 63, 40–47.

Cock MJ, van Lenteren JC, Brodeur J, Barratt BI, Bigler F, Bolckmans K, Cônsoli FL, Haas F, Mason PG, Parra JRP (2010) Do new access and benefit sharing procedures under the convention on biological diversity threaten the future of biological control?. BioControl 55, 199–218.

Costi E, Haye T, Maistrello L (2018) Surveying native egg parasitoids and predators of the invasive *Halyomorpha halys* in Northern Italy. Submitted to Journal of Applied Entomology.

Duthie C (2012) Risk analysis of *Halyomorpha halys* (brown marmorated stink bug) on all pathways. Ministry for Primary Industries, Wellington.

Funayama K (2004) Importance of apple fruits as food for the brown-marmorated stink bug, *Halyomorpha halys* (Stål) (Heteroptera: Pentatomidae). Applied Entomology and Zoology 39, 617–623.

Gariepy TD, Bruin A, Haye T, Milonas P, Vétek G (2015) Occurrence and genetic diversity of new populations of *Halyomorpha halys* in Europe. Journal of Pest Science 88, 451–460.

Grenier S (1988) Applied biological control with tachinid flies (Diptera, Tachinidae): a review. Anzeiger für Schädlingskunde, Pflanzenschutz, Umweltschutz 61, 49–56.

Haye T, Abdallah S, Gariepy, T, Wyniger D (2014a) Phenology, life table analysis and temperature requirements of the invasive brown marmorated stink bug, *Halyomorpha halys*, in Europe. Journal of Pest Science 87, 407–418.

Haye T, Wyniger D, Gariepy T (2014b) Recent range expansion of brown marmorated stink bug in Europe. In Proceedings of the 8th International Conference on Urban Pests, 20–23 July 2014, Zurich, Switzerland (pp. 309–314). Executive Committee of the International Conference on Urban Pests.

Haye T, Fischer S, Zhang J, Gariepy T (2015) Can native egg parasitoids adopt the invasive brown marmorated stink bug, *Halyomorpha halys* (Heteroptera: Pentatomidae), in Europe? Journal of Pest Science 88, 693–705.

Hedstrom C, Lowenstein D, Andrews H, Bai B, Wiman N (2017) Pentatomid host suitability and the discovery of introduced populations of *Trissolcus japonicus* in Oregon. Journal of Pest Science 90, 1169–1179.

Hoebeke ER, Carter ME (2003) *Halyomorpha halys* (Stål) (Heteroptera: Pentatomidae): a polyphagous plant pest from Asia newly detected in North America. Proceedings of the Entomological Society of Washington, 105, 225–237.

Hokkanen H, Pimentel D (1984) New approach for selecting biological control agents. The Canadian Entomologist 116, 1109–1121.

Hoy MA (2008) Classical Biological Control. In: Capinera J.L. (eds) Encyclopedia of Entomology. Springer, Dordrecht, pp.906–923.

Hulme PE (2009) Trade, transport and trouble: managing invasive species pathways in an era of globalization. Journal of Applied Ecology 46, 10–18.

Keane RM, Crawley MJ (2002) Exotic plant invasions and the enemy release hypothesis. Trends in Ecology & Evolution 17, 164–170.

Kettunen M, Genovesi P, Gollasch S, Pagad S, Starfinger U, Brink P, Shine C (2009) Technical support to EU strategy on invasive alien species (IAS). Institute for European Environmental Policy (IEEP), Brussels.

Khrimian A, Zhang A, Weber DC, Ho HY, Aldrich JR, Vermillion KE, Siegler MA, Shirali S, Guzman F, Leskey TC (2014) Discovery of the aggregation pheromone of the brown marmorated stink bug (*Halyomorpha halys*) through the creation of stereoisomeric libraries of 1-bisabolen-3-ols. Journal of Natural Products 77, 1708–1717.

Kolar C, Lodge DM (2001) Progress in invasion biology: predicting invaders. Trends in Ecology & Evolution 16, 199–204.

Konopka JK, Haye T, Gariepy TD, McNeil JN (2017) Possible coexistence of native and exotic parasitoids and their impact on control of *Halyomorpha halys*. Journal of Pest Science 90, 1119–1125.

Kuhar TP, Kamminga KL, Whalen J, Dively GP, Brust G, Hooks CR, Hamilton G, Herbert DA (2012) The pest potential of brown marmorated stink bug on vegetable crops. Online. Plant Health Progress. https://doi:10.1094/PHP-2012-0523-01-BR.

Lee KC, Kang CH, Lee DW, Lee SM, Park CG, Choo HY (2002) Seasonal occurrence trends of hemipteran bug pests monitored by mercury light and aggregation pheromone traps in sweet persimmon orchards. Korean Journal of Applied Entomology 41, 233–238.

Lee D-H, Wright SE, Leskey TC (2013a) Impact of insecticide residue exposure on the invasive pest, *Halyomorpha halys* (Hemiptera: Pentatomidae): analysis of adult mobility. Journal of Economic Entomology 106, 150–158.

Lee D-H, Short BD, Joseph SV, Bergh JC, Leskey TC (2013b) Review of the biology, ecology, and management of *Halyomorpha halys* (Hemiptera: Pentatomidae) in China, Japan, and the Republic of Korea. Environmental Entomology 42, 627–641.

Lee D-H, Nielsen AL, Leskey TC (2014) Dispersal capacity and behavior of nymphal stages of *Halyomorpha halys* (Hemiptera: Pentatomidae) evaluated under laboratory and feld conditions. Journal of Insect Behavavior 27, 639–651.

Leskey T, Hamilton GC (2010) Brown Marmorated Stink Bug Working Group Meeting.

http://projects.ipmcenters.org/Northeastern/FundedProjects/ReportFiles/Pship2010/Pship2010-Leskey-ProgressReport-237195-Meeting-2010_11_17.pdf. Accessed September 15, 2018.

Leskey TC, Short BD, Butler BR, Wright SE (2012a) Impact of the invasive brown marmorated stink bug, *Halyomorpha halys* (Stål), in mid-Atlantic tree fruit orchards in the United States: case studies of commercial management. Psyche: A Journal of Entomology, 2012, 1–14.

Leskey TC, Hamilton GC, Nielsen AL et al. (2012b) Pest status of the brown marmorated stink bug, *Halyomorpha halys* in the USA. Outlooks on Pest Management 23, 218-226.

Leskey TC, Lee DH, Short BD, Wright SE (2012c) Impact of insecticides on the invasive *Halyomorpha halys* (Hemiptera: Pentatomidae): analysis of insecticide lethality. Journal of Economic Entomology 105, 1726–1735.

Leskey TC, Wright SE, Short, BD, A. Khrimian (2012d) Development of behaviorally-based monitoring tools for the brown marmorated stink bug (Heteroptera: Pentatomidae) in commercial tree fruit orchards. Journal of Entomological Science 47, 76–85.

Leskey, T.C., Agnello, A., Bergh, J.C., Dively, G.P., Hamilton, G.C., Jentsch, P., Khrimian, A., Krawczyk, G., Kuhar, G.T.P., Lee, D.-H., et al. 2015. Attraction of the invasive *Halyomorpha halys* (Hemiptera: Pentatomidae) to traps baited with semiochemical stimuli across the United States. Environmental Entomology, 44, 746–756.

Leskey TC, Nielsen AL (2018) Impact of the invasive brown marmorated stink bug in North America and Europe: history, biology, ecology, and management. Annual Review of Entomology 63, 599–618.

Lonsdale WM (1999) Global patterns of plant invasions and the concept of invasibility. Ecology 80, 1522–1536.

Lynch L, Hokkanen HMT, Babendreier D, Bigler F, Burgio G, Gao ZH, Kuske S, Loomans A, Menzler-Hokkanen I, Thomas, MB, Tommasini G, Waage JK, van Lenteren JC, Zeng QQ (2001) Insect biological control and non-target effects: perspective. In: Wajnberg E, Scott JK, Quimby PC (eds) Evaluating indirect ecological effects of biological control. CABI, Wallingford, p 99.

Maistrello L, Vaccari G, Caruso S, Costi E, Bortolini S, Macavei L, Foca G, Ulrici A, Bortolotti PP, Nannini R, Casoli L, Fornaciari M, Mazzoli G, Dioli P (2017) Monitoring of the invasive *Halyomorpha halys*, a new key pest of fruit orchards in northern Italy. Journal of Pest Science 90, 1231–1244.

Matošević D, Melika G (2013) Recruitment of native parasitoids to a new invasive host: first results of *Dryocosmus kuriphilus* parasitoid assemblage in Croatia. Bulletin of Insectology 66, 231–238.

Mollá O, Biondi A, Alonso-Valiente M, Urbaneja A (2014) A comparative life history study of two mirid bugs preying on *Tuta absoluta* and *Ephestia kuehniella* eggs on tomato crops: implications for biological control. BioControl 59, 175–183.

Morrison WR, Lee DH, Short BD, Khrimian A, Leskey TC (2016) Establishing the behavioral basis for an attract-and-kill strategy to manage the invasive *Halyomorpha halys* in apple orchards. Journal of Pest Science 89, 81–96.

Nielsen AL, Hamilton GC, Matadha D (2008) Developmental rate estimation and life table analysis for *Halyomorpha halys* (Hemiptera: Pentatomidae). Environmental Entomology 37, 348–355.

Nielsen AL, Hamilton GC (2009) Seasonal occurrence and impact of *Halyomorpha halys* (Hemiptera: Pentatomidae) in tree fruit. Journal of Economic Entomology 102, 1133–1140.

Noyes J (2014) Universal Chalcidoidea Database–World Wide Web electronic publication. Natural History Museum Update August. http://www.nhm.ac.uk/our-science/data/chalcidoids/. Accessed 03 Jan 2018.

Os N, Varshney R (2018) Utilization of Trichogrammatid egg parasitoids in pest management. Acta Scientific Agriculture 2, 49–53.

Panzavolta T, Bernardo U, Bracalini M, Cascone P, Croci F, Gebiola M, Iodice L, Tiberi R, Guerrieri E (2013) Native parasitoids associated with *Dryocosmus kuriphilus* in Tuscany, Italy. Bulletin of Insectology 66, 195–201.

Pyšek P, Richardson DM (2010) Invasive species, environmental change and management, and health. Annual Review of Environment and Resources 35, 25–55.

Rice KB, Bergh CJ, Bergmann EJ, Biddinger DJ, Dieckhoff C, Dively G, Fraser H, Gariepy T, Hamilton G, Haye T, Herbert A, Hoelmer K, Hooks CR, Jones A, Krawczyk G, Kuhar T, Martinson H, Mitchell W, Nielsen AL, Pfeiffer DG, Raupp MJ, Rodrigues-Saona C, Shearer P, Shrewsbury P, Venugopal PD, Whalen J, Wiman NG, Leskey TC, Tooker JF (2014) Biology, ecology, and management of brown marmorated stink bug (Hemiptera: Pentatomidae). Journal of Integrated Pest Management 3, A1–A13. https://doi.org/10.1603/IPM14002.

Rice KB, Cullum JP, Wiman NG, Hilton R, Leskey TC (2017) *Halyomorpha halys* (Hemiptera: Pentatomidae) response to pyramid traps baited with attractive light and pheromonal stimuli. Florida Entomologist 100, 449–453.

Rice KB, Morrison WR, Short BD, Acebes-Doria A, Bergh JC, Leskey TC (2018) Improved trap designs and retention mechanisms for *Halyomorpha halys* (Hemiptera: Pentatomidae). Journal of Economic Entomology.

Richardson DM, Pyšek P (2006) Plant invasions: merging the concepts of species invasiveness and community invasibility. Progress in Physical Geography 30, 409–431.

Rondoni G, Bertoldi V, Malek R, Foti MC, Peri E, Maistrello L, Haye T, Conti E (2017) Native egg parasitoids recorded from the invasive *Halyomorpha halys* successfully exploit volatiles emitted by the plant–herbivore complex. Journal of Pest Science 90, 1087–1095.

Roversi PF, Marianelli L, Costi E, Maistrello L, Sabbatini PG (2016) Searching for native egg-parasitoids of the invasive alien species *Halyomorpha halys* Stål (Heteroptera Pentatomidae) in Southern Europe. Redia 99, 63–70.

Roy HE, Handley LJL, Schönrogge K, Poland RL, Purse BV (2011) Can the enemy release hypothesis explain the success of invasive alien predators and parasitoids? BioControl 56, 451–468.

Sabbatini PG, Binazzi F, Marianelli L, Roversi PF (2018a) Lethal and sublethal effects of long-lasting insecticide-treated nets on the invasive bug *Halyomorpha halys*. Journal of Applied Entomology 142, 141–148.

Sabbatini PG, Paolo BP, Roberta N, Leonardo M, Federico RP (2018b) Efficacy of long-lasting insecticide nets in killing *Halyomorpha halys* in pear orchards. Outlooks on Pest Management 29, 70–74.

Sakai AK, Allendorf FW, Holt JS, Lodge DM, Molofsky J, With KA, Baughman S, Cabin RJ, Cohen JE, Ellstrand NC, McCauley DE (2001) The population biology of invasive species. Annual Review of Ecology and Systematics 32, 305–332.

Seebens H, Blackburn TM, Dyer EE, Genovesi P, Hulme PE, Jeschke JM, Pagad S, Pyšek P, Winter M, Arianoutsou M, Bacher S (2017) No saturation in the accumulation of alien species worldwide. Nature Communications 8, 14435.

Seebens H, Blackburn TM, Dyer EE, Genovesi P, Hulme PE, Jeschke JM, Pagad S, Pyšek P, van Kleunen M, Winter M, Ansong M (2018) Global rise in emerging alien species results from increased accessibility of new source pools. Proceedings of the National Academy of Sciences, 2017 Jan 31, 19429.

Short BD, Khrimian A, Leskey TC (2017) Pheromone-based decision support tools for management of *Halyomorpha halys* in apple orchards: development of a trap-based treatment threshold. Journal of Pest Science 90, 1191–1204.

Simberloff D, Martin JL, Genovesi P, Maris V, Wardle DA, Aronson J, Courchamp F, Galil B, García-Berthou E, Pascal M, Pyšek P (2013) Impacts of biological invasions: what's what and the way forward. Trends in Ecology & Evolution 28, 58–66.

Smith SM (1996) Biological control with Trichogramma: advances, successes, and potential of their use. Annual Review of Entomology 41, 375–406.

Stacconi RM, Grassi A, Dalton D, Miller B, Ouantar M, Loni A, Ioriatti C, Walton V, Anfora

G (2013) First field records of *Pachycrepoideus vindemiae* as a parasitoid of *Drosophila suzukii* in European and Oregon small fruit production areas. Entomologia 1, 11–16.

Tada N, Yoshida M, Sato, Y (2001a) Monitoring of forecasting for stink bugs in apple 1. Characteristics of attraction to aggregation pheromone in Iwate Prefecture. Annual Report of Plant Protection of North Japan 52, 224–226.

Tada N, Yoshida, M, Sato, Y (2001b) Monitoring of forecasting for stink bugs in apple 2. The possibility of forecasting with aggregation pheromone. Annual Report of Plant Protection of North Japan 52, 227–229.

Takahashi S (1930) *Halyomorpha picus* Fab., pp. 617Ð 620. Insect Pests on Fruit Trees.

Talamas EJ, Herlihy MV, Dieckhoff C, Hoelmer KA, Buffington M, Bon MC, Weber DC, (2015) *Trissolcus japonicus* (Ashmead) (Hymenoptera, Scelionidae) emerges in North America. Journal of Hymenoptera Research 43, 119–128.

Tang, R., Babendreier, D., Zhang, F., Kang, M., Song, K. and Hou, M.L., 2017. Assessment of *Trichogramma japonicum* and *T. chilonis* as potential biological control agents of yellow stem borer in rice. Insects 8, 1–12.

Taylor CM, Coffey PL, DeLay BD, Dively GP (2014) The importance of gut symbionts in the development of the brown marmorated stink bug, *Halyomorpha halys* (Stål). PLoS One 9, 1–4.

Tiberi R (1990) Egg parasitoids of the pine processionary caterpillar, *Thaumetopoea pityocampa* (Den. & Schiff.) (Lep., Thaumetopoeidae) in Italy: distribution and activity in different areas 1. Journal of Applied Entomology 110, 14–18.

van Lenteren JC, Bale J, Bigler F, Hokkanen H, Loomans A (2006) Assessing risks of releasing exotic biological control agents of arthropod pests. Annual Review of Entomology 51, 609–634.

Walker K (2009) Brown marmorated stink bug (*Halyomorpha halys*). Updated on 11/25/2011. Available online: http://www.padil.gov.au.

Watanabe M, Arakawa R, Shinagawa Y, Okazawa T (1994) Overwintering flight of brown-marmorated stink bug, *Halyomorpha mista* to the buildings. Medical Entomology and Zoology 45, 25–31 (in Japanese with English summary).

Weber DC, Saska P, Chaboo CS (2008) Carabid Beetles (Coleoptera: Carabidae) as Parasitoids. In: Capinera J.L. (eds) Encyclopedia of Entomology. Springer, Dordrecht.

Yanagi T, Hagihara, Y (1980) Ecology of the brown marmorated stink bug. Plant Protection 34, 315–321.

Yang ZQ, Yao YX, Qiu LF, Li ZX (2009) A new species of Trissolcus (Hymenoptera: Scelionidae) parasitizing eggs of *Halyomorpha halys* (Heteroptera: Pentatomidae) in China with comments on its biology. Annals of the Entomological Society of America 102, 39–47.

Zappala L, Bernardo U, Biondi A, Cocco A, Deliperi S, Delrio G, Giorgini M, Pedata P, Rapisarda C, Garzia GT, Siscaro G (2012) Recruitment of native parasitoids by the exotic pest *Tuta absoluta* in Southern Italy. Bulletin of Insectology 65, 51–61.

Zhang J, Zhang F, Gariepy T, Mason P, Gillespie D, Talamas E, Haye T (2017) Seasonal parasitism and host specificity of *Trissolcus japonicus* in northern China. Journal of Pest Science 90, 1127–1141.

2 Life history of *Anastatus bifasciatus*

Published in: Biological Control, “Life history of Anastatus bifasciatus, a potential biological control agent of the brown mamorated stink bug in Europe”,
https://doi.org/10.1016/j.biocontrol.2018.10.016

Judith Maria Stahl[1,2]*, Dirk Babendreier[1], Tim Haye[1]

[1] CABI, Rue des Grillons 1, 2800 Delémont, Switzerland,

[2] Institute of Ecology and Evolutionary Biology, University of Bremen, Leobener Str. NW2, 28359 Bremen, Germany

Abstract

Anastatus bifasciatus (Geoffroy) (Hymenoptera: Eupelmidae) is the most widespread native egg parasitoid of the invasive agricultural pest *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) in Europe and considered as promising candidate for augmentative biological control. In this context, the parasitoid’s reproductive parameters, longevity, phenology, and temperature requirements for development were studied. In addition, the potential of using stored frozen *H. halys* eggs for mass production was investigated. In northwestern Switzerland, *A. bifasciatus* was able to complete three overlapping generations between June and October, which covers the entire oviposition period of its host. After an average pre-oviposition period of 11.9 days the mean potential lifetime fecundity of *A. bifasciatus* females was 52 eggs over an average oviposition period of 46 days. The number of *H. halys* eggs killed by either oviposition or host feeding was nearly the same. Females provided only with honey water lived on average 97.5 days. Development from egg to adult was possible from 15°C to 32°C, the lowest developmental time (15.9 days) being at 30°C. *Halyomorpha halys* eggs of all ages yielded parasitoid offspring, but offspring emergence decreased with increasing age. Eggs stored up to 24 months at -80°C were still suitable for parasitoid development. The wealth of information obtained on the life history of *A. bifasciatus* should be highly relevant to develop a release strategy of this parasitoid against its host, *H. halys.*

3 Pre-release risk assessment of *Anastatus bifasciatus*

Published in: Journal of Pest Science, "Using the egg parasitoid *Anastatus bifasciatus* against the invasive brown marmorated stink bug in Europe – can non-target effects be ruled out?"
https://doi.org/10.1007/s10340-018-0969-x

Judith M. Stahl[1,2]*, Dirk Babendreier[1], Tim Haye[1]

[1] CABI, Rue des Grillons 1, 2800 Delémont, Switzerland

[2] Institute of Ecology and Evolutionary Biology, University of Bremen, Leobener Str. NW2,
28359 Bremen, Germany

Abstract

The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), has been causing massive damage to various fruit and vegetable crops after its arrival in the US, and more recently in Europe. To provide an alternative control measure to pesticides, the native egg parasitoid *Anastatus bifasciatus* (Geoffroy) (Hymenoptera: Eupelmidae) was considered as a candidate biological control agent for inundative releases in Europe. In the risk assessment study presented here, all nine heteropteran and 14 out of 19 tested lepidopteran non-target species produced viable *A. bifasciatus* offspring. The proportion of *A. bifasciatus* females producing offspring did not differ between non-target and target for 19 out of the 28 non-target species. Larger host eggs corresponded to increased female biased sex ratio of the offspring as well as an increase in size, particularly for females, with hind tibia lengths varying from 645.5 ± 46 µm to 1084 ± 28.5 µm. Larger females were also found to have higher offspring production and increased life expectancy. The results of this study confirmed the polyphagous nature of *A. bifasciatus* and suggest that a number of non-target species, including Lepidoptera of conservation interest, may be attacked in the field. Thus, non-target effects cannot entirely be ruled out, but more information is needed from semi-field and field studies to fully assess potential environmental risks due to inundative releases of this native parasitoid.

4 Intrinsic competition between two European egg parasitoids of the brown marmorated stink bug

Abstract

Competition amongst parasitoids can influence species coexistence and community structure. Following the accidental introduction and spread of the invasive polyphagous agricultural pest *Halyormopha halys* (Stål) (Hemiptera: Pentatomidae), the two European egg parasitoids *Anastatus bifasciatus* (Geoffroy) (Hymenoptera: Eupelmidae) and *Ooencyrtus telenomicida* (Vassiliev) (Hymenoptera: Encyrtidae) are being investigated for inundative biological control. Both are superior intrinsic (larval) competitors when competing with *Trissolcus* species (Hymenoptera: Scelionidae) and the outcome of intrinsic competition between the two biological control candidates is difficult to predict. The oviposition of one parasitoid species into *H. halys* eggs previously parasitized by the other species and emergence from multiparasitized eggs was investigated with a time-course development study. Both species readily oviposited in eggs containing eggs and early instar larvae of the competitor, but oviposition decreased when eggs contained late instar larvae and pupae. *Ooencyrtus telenomicida* offspring emergence from multiparasitized eggs was significantly lower than from rearing controls, independent of the order of parasitization. *Anastatus bifasciatus* offspring emergence was not influenced by the presence of *O. telenomicida* when it parasitized as the first species, but emergence was decreased after oviposition in eggs containing *O. telenomicida* larvae and pupae. There was no indication that *O. telenomicida* can act as a facultative hyperparasitoid of *A. bifasciatus*. These results suggest that *A. bifasciatus* is the superior intrinsic competitor and no negative implications for inundative biological control with *A. bifasciatus* are expected.

4.1 Introduction

Competition amongst parasitoids can influence species coexistence and community structure (Godfray 1994). Egg parasitoids sharing one host compete extrinsically, on an adult level, and intrinsically, as larvae within the same host egg (Zwölfer 1971, 1979, Cusumano et al. 2012a). Extrinsic competition can be indirect such as during host finding, or direct such as contesting female wasps (Cusumano et al. 2016). In intrinsic competition, a distinction is made between supernumeraries of the same parasitoid species (superparasitism; intraspecific competition) and of different species (multiparasitism; interspecific competition) (Fisher 1961). For biological control, increased diversity of natural enemies can have null, additive, antagonistic or synergistic effects (Casula et al. 2006).

Interspecific competition between parasitoid species is common and concerns about potential negative implications for biological control programs were raised as early as 1918 when four introduced parasitoid species for the biological control of the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) in Hawaii were being investigated (Pemberton and Willard 1918). On the other hand, if no antagonistic effects occur, combined releases can be an effective biological control strategy, as demonstrated for the silverleaf whitefly *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) (Bográn et al. 2002, Heinz and Nelson 1996).

Many egg parasitoids in the genera *Anastatus* Motschulsky (Hymenoptera: Eupelmidae) and *Ooencyrtus* Ashmead (Hymenoptera: Encyrtidae) are highly polyphagous, attacking both lepidopteran and hemipteran hosts, and sometimes even share the same hosts. As a result, species from both genera have been used as biological control agents against the same pests, e.g. *A. japonicus* Ashmead, *O. corbetti* Ferrière and *O. phongi* Trjapitzin, Myartseva & Kostjukov against the litchi stink bug, *Tessaratoma papillosa* Drury (Hemiptera: Pentatomidae) (Hirose 1964, Nanta 1988, Halperin 1990, Liu et al. 2000) or *A. japonicus* and *O. kuvanae* Howard against the gypsy moth, *Lymantria dispar* L. (Lepidoptera: Erebidae) (Parker 1933).

In Europe, species from both genera, *Anastatus bifasciatus* (Geoffroy) and *O. telenomicida* (Vassiliev), have been reared from sentinel eggs of the invasive Asian brown marmorated stinkbug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) (Haye et al. 2015, Roversi et al. 2016, Costi et al. 2018), which was first introduced into Switzerland in the early 2000s (Wermelinger et al. 2008) and has since spread into more than 10 European countries (Haye et al. 2014). In the past couple of years, the highly polyphagous *H. halys* has become a key pest of fruit crops and hazelnut in Italy and Georgia (Bosco et al. 2017, Maistrello et al., 2017). Consequently, investigation on inundative uses of *A. bifasciatus* and *O. telenomicida* against *H. halys* has been initiated in Europe. The outcome of inundative releases will highly depend on the host searching behavior (Rodondi et al. 2017) and parasitism capacity of both species, but also on their ability to succeed in interspecific competition. Competitive situations may arise between species if both parasitoids are mass released simultaneously against *H. halys* in European fruit orchards, or where both species naturally co-occur.

In earlier studies investigating the competition of *A. bifasciatus* and *O. telenomicida* with Asian and European *Trissolcus* Ashmead species (Hympenoptera: Platygastridae), it was demonstrated that both species were superior intrinsic (larval) competitors (Cusumano et al. 2011, Konopka et al. 2017).

Since both are strongly synovigenic and have a similar life history, it remains difficult to predict the outcome of competition between the two species (Cusumano et al. 2012b, Stahl et al. 2018a).

In this paper, we investigated the intrinsic competition between the two European egg parasitoids *A. bifasciatus* and *O. telenomicida* to address the following questions: (1) Do *A. bifasciatus* and *O. telenomicida* females accept eggs previously parasitized by the other species?; (2) Are previously parasitized eggs suitable for development of either species?; (3) Is the outcome of the larval competition dependent on the order of parasitization?; (4) What are the implications for inundative biological control?

4.2 Material and methods

4.2.1 Insect rearing

4.2.1.1 Hosts

A colony of *H. halys* was established in 2012 from individuals collected in Zurich and Basel, Switzerland. Groups of 50 adults were kept in plastic cages ('BugDorm-4090 Insect Rearing Cage 47.5 x 47.5 x 47.5 cm', MegaView Science Co. Ltd., Taichung, Taiwan) at 26°C, 70% RH, and a 16L:8D photoperiod. Adults and nymphs were fed twice a week with a diet of corn, beans, apples, peanuts and carrots. Fresh branches of cherry trees (*Prunus avium* L.), tree of heaven (*Ailanthus altissima* (Mill.) Swingle), common ash (*Sorbus aucuparia* L.), or common ivy (*Hedera helix* L.) were offered as additional food source and oviposition substrate. Folded pieces of black mesh (separation fleece for pots, Windhager AG, Hünenberg, Switzerland) placed at the bottom of the cages served as additional oviposition substrate. Egg masses were collected on a daily basis and used for the competition assays.

A colony of *Nezara viridula* L. (Hemiptera: Pentatomidae) was established from individuals collected at various places in the Canton of Ticino, Switzerland in 2017 and used for rearing *O. telenomicida*. Both nymphs and adults were reared on corn, beans and potted cabbages in the same type of cages and under the same conditions as *H. halys*.

A colony of *Dendrolimus pini* L. (Lepidoptera: Lasiocampidae) was established in 2016 from commercially obtained eggs collected at Slatiňany, Czech Republic. The caterpillars were reared in the same cages as described above and provided with fresh pine branches twice a week. Newly emerged moths were provided with pine branches as oviposition substrate and eggs were collected on a daily basis to maintain the *A. bifasciatus* colony.

4. 2.1.2 Parasitoids

A colony of *A. bifasciatus* was established in 2013 from sentinel *H. halys* egg masses exposed in the Canton of Valais, Switzerland (Haye et al. 2015) and specimens from the original colony were identified by Lucian Fusu (Alexandru Ioan Cuza University, Iași, Romania). Freshly emerged *A. bifasciatus* adults were collected daily and transferred to 100 x 115 mm mesh-top cylindrical plastic containers placed above a 90 x 20 mm Petri dish filled with honey water solution. The container and the Petri dish were connected through two cotton wicks and stored in an incubator set at a light / temperature cycle of L 16h / 20°C and D 8h / 15°C. Every second day the honey water solution was exchanged, and new drops of honey added to the mesh to of the container. Seven days after emergence, fresh (< 24 h) *D. pini* eggs glued onto cardboard pieces were placed on the bottom of the container and replaced every second day. Parasitized *D. pini* eggs were kept at 26°C, 70% RH, and a 16L:8D photoperiod.

A colony of *O. telenomicida* was originally established in 2008 from *N. viridula* sentinel egg masses placed in tomato fields around Palermo, Italy. Freshly emerged *O. telenomicida* adults were collected daily and transferred to plastic tubes (7 cm height and 2.5 cm diameter) plugged with a sponge and kept in an incubator set at a light / temperature cycle of L 16h / 20°C and D 8h / 15°C. Adults were fed with honey droplets and provided with fresh *N. viridula* egg masses every second day. Parasitized *N. viridula* eggs were kept at 26°C, 70% RH, and a 16L:8D photoperiod.

4.2.2 Time-course development study

Randomly selected *A. bifasciatus* females (naïve, mated, 7 days old) were offered single fresh (< 24 h) *H. halys* eggs, which were glued individually on 1 cm x 1 cm black cardboard pieces with 'Cementit' (merz+benteli AG, Niederwangen, Switzerland) and placed in plastic Petri dishes (5 x 1 cm). All assays were carried out at 26°C, 70% RH, and a 16L:8D photoperiod. Individual *H. halys* eggs were offered instead of egg masses to increase the probability of *A. bifasciatus* oviposition and to avoid that *A. bifasciatus* females use approximately half of the eggs exclusively for host feeding but not for oviposition (Konopka et al. 2017, Stahl et al. 2018a). Females were observed for a maximum of three hours until probing. Females that had probed the *H. halys* egg were removed once they had left the egg for at least fifteen minutes. An egg was counted as 'probed' when females had inserted their ovipositor into the egg. Since *A. bifasciatus* females do not mark parasitized host eggs, successful oviposition was measured indirectly by keeping a proportion of the probed eggs as rearing control (n = 65) or destructively by dissections (n = 180).

Ooencyrtus telenomicida females (naïve, mated, 7 days old) were provided with single eggs that were attacked by *A. bifasciatus* 0 h, 1, 2, 3, 4, 5, 9 or 14 days earlier. In preliminary tests, *O. telenomicida* showed a similar behavior as *A. bifasciatus* with alternating 'drilling' and 'host feeding', but very little oviposition within the first seven hours of observation. Consequently, instead of observing *O. telenomicida* females during egg exposure, *H. halys* eggs were checked for parasitism after 24 h. In contrast to *A. bifasciatus*, eggs parasitized by *O. telenomicida* can easily be recognized without dissections by the respiratory egg stalk (one per egg) protruding from the host egg chorion (Takasu and Hirose 1988). Single unparasitized *H. halys* eggs (< 24 h) were offered in parallel to the competition trials to individual *O. telenomicida* females (n = 210) as controls to test developmental outcomes in absence of competitors. In addition, fresh *H. halys* eggs (n = 125) were kept as quality control of the host eggs. Since the sequence of parasitoids attacking the eggs may influence the outcome of the intrinsic competition, the entire experiment was repeated in reverse order. For each time interval 20 replicates were targeted, but with increasing age of parasitized eggs (> 4 days) acceptance by both species was so low, that in the older treatments only a few replicates (n = 3–10) were completed. Multiparasitized *H. halys* eggs as well as rearing controls were kept at 26°C, 70% RH, and a 16L:8D photoperiod and checked for emergences daily. Number, sex and species of emerged parasitoids were recorded.

4.2.3 Black box competition experiment

To further predict the potential outcome of combined releases of both parasitoid species an experiment without observations (black box) was conducted at 26°C, 70% RH, and a 16L:8D photoperiod. Single *H. halys* egg masses (28 eggs each, < 24 h old) were assigned to one of the following treatments: 1) exposure to one *A. bifasciatus* and one *O. telenomicida* female, 2) exposure to two *A. bifasciatus* females, 3) exposure to two *O. telenomicida* females, and 4) no exposure to parasitoids (control). All assays were conducted in small plastic Petri dishes (5 x 1 cm). Parasitoids were naive and seven days old when used for experiments. After 24 h parasitoids were removed, and exposed egg masses were stored at 26°C, 70% RH, and a 16L:8D photoperiod. Parasitoid emergence was checked daily and emerged offspring was sexed and identified to species-level.

4.2.4 Statistical analyses

Host probing (only *A. bifasciatus*) and oviposition (*A. bifasciatus* and *O. telenomicida*), were compared within species with a Generalized Linear Model (GLM) (family = binomial) and a Tukey's all-pair comparisons post hoc test with the different treatments (developmental times) as independent variable. For both parasitoid species the effect of multiparasitism on adult emergence was compared using a GLM (family = binomial), followed by a post hoc Tukey's all-pair comparisons test with the different treatments (developmental times) as independent variable. The number of progenies was compared between parasitoid species with a Mann-Whitney U test. The influence of superparasitism by *O. telenomicida* on the probing and oviposition of *A. bifasciatus* as well as emergence of both parasitoid species was tested with a GLM (family = binomial) with the number of egg stalks as independent variable. The number of emerged individuals from each group (nymphs, *A. bifasciatus*, *O. telenomicida)* and dead eggs was compared with a GLM (family = Poisson) with treatments of the black box experiment (unparasitized control, two *O. telenomicida* females, two *A. bifasciatus* females, one *O. telenomicida* female and one *A. bifasciatus* female) as independent variable. The number of progenies was compared between parasitoid species with a Mann-Whitney U test. All statistics were carried out with R, version 3.2.3 (Team RC 2014) and the development environment RStudio (Team RS 2017). For the Tukey's post hoc tests, the package 'multcomp' (Hothorn et al. 2017) was employed.

4.3 Results

4.3.1 Time-course development study

4.3.1.1 Probing and oviposition

Ooencyrtus telenomicida oviposition occurred in *H. halys* eggs containing different developmental stages of *A. bifasciatus* (Figure 1). When females were provided with 0 h, 1, 2, or 3 day old parasitized *H. halys* eggs, oviposition did not differ from controls, but oviposition significantly decreased (< 20%) when eggs were five to fourteen days old (binomial GLM, df = 8, 294, $\chi^2 = 164.1$, $p < 0.001$) (Figure 1). No significant differences were found for *A. bifasciatus* females probing *H. halys* eggs of different ages previously parasitized by *O. telenomicida* or unparasitized controls (binomial GLM, df = 8, 1292, $\chi^2 = 13.7$, $p = 0.091$) (Figure 2).

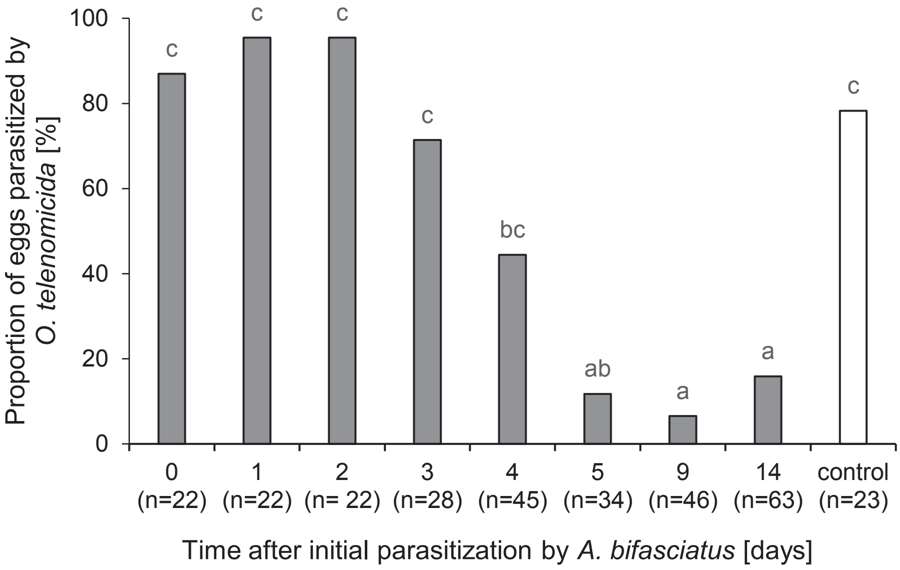

Figure 1 Proportion of *H. halys* eggs parasitized by *O. telenomicida* when eggs were exposed to *A. bifasciatus* 0-14 days before. Unparasitized, fresh (< 24 h) *H. halys* eggs were used as controls. Sample sizes are given in brackets; bars sharing the same letter are not significantly different by Tukey-adjusted mean separations (binomial GLM, df = 8, 294, χ^2 = 164.1, p < 0.01).

Superparasitism by *O. telenomicida* (more than one egg per *H. halys* egg) was common, with a mean of 3.26 ± 0.133 (SE) (n = 253) *O. telenomicida* eggs per host egg but had no significant influence on *A. bifasciatus* probing (binomial GLM, df = 1, 251, χ^2 = 0.184, p = 0.668). However, in terms of oviposition, *A. bifasciatus* females discriminated among *H. halys* of containing different developmental stages of *O. telenomicida* (binomial GLM, df = 8, 171, χ^2 = 49.9, p < 0.001) (Figure 3). No significant differences were found between oviposition in eggs parasitized by *O. telenomicida* 0 h to 9 days before

Superparasitism by *O. telenomicida* (more than one egg per *H. halys* egg) was common, with a mean of 3.26 ± 0.133 (SE) (n = 253) *O. telenomicida* eggs per host egg but had no significant influence on *A. bifasciatus* probing (binomial GLM, df = 1, 251, χ^2 = 0.184, p = 0.668). However, in terms of oviposition, *A. bifasciatus* females discriminated among *H. halys* of containing different developmental stages of *O. telenomicida* (binomial GLM, df = 8, 171, χ^2 = 49.9, p < 0.001) (Figure 3). No significant differences were found between oviposition in eggs parasitized by *O. telenomicida* 0 h to 9 days before
and controls (unparasitized eggs) (Tukey's all-pair tests, all p > 0.05) (Figure 3). However, oviposition in parasitized eggs that were 14 days old was significantly lower (20%) than in controls (90%) (p < 0.01) (Figure 3). The number of *O. telenomicida* eggs present inside the dissected *H. halys* eggs was 3.59 ± 0.188 (SE) (n = 142) and did not influence *A. bifasciatus* oviposition (binomial GLM, df = 1, 140, χ^2 = 1.91, p = 0.167).

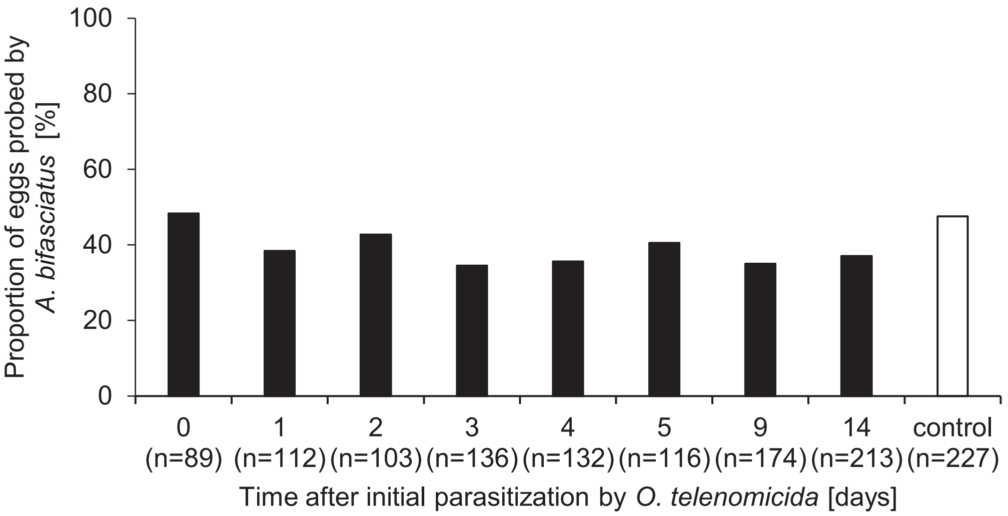

Figure 2 Proportion of *H. halys* eggs 'probed' (= drilling observed) by *A. bifasciatus* when eggs were parasitized by *O. telenomicida* 0-14 days before. Unparasitized, fresh (< 24 h) *H. halys* eggs were used as controls. Sample sizes are given in brackets; there were no significant differences between treatments (binomial GLM, df = 8, 1292, χ^2 = 13.68, p = 0.09051).

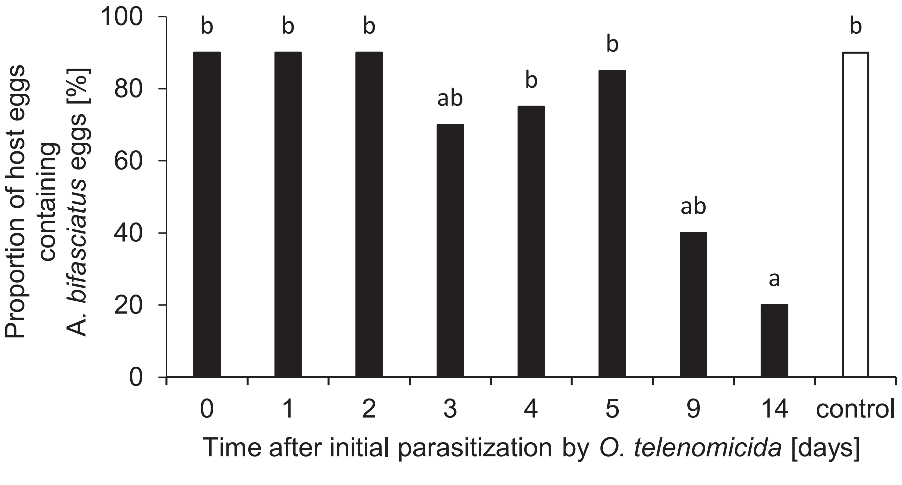

Figure 3 Proportion of *H. halys* eggs previously parasitized by *O. telenomicida* and subsequently probed by *A. bifasciatus* assessed in dissections. Unparasitized, fresh (< 24 h*) H. halys* eggs probed by A. bifasciatus were used as controls. Sample sizes equal 20 in each treatment and the control; bars sharing the same letter are not significantly different by Tukey-adjusted mean separations (binomial GLM, df = 8, 171, χ^2 = 49.9, p < 0.001).

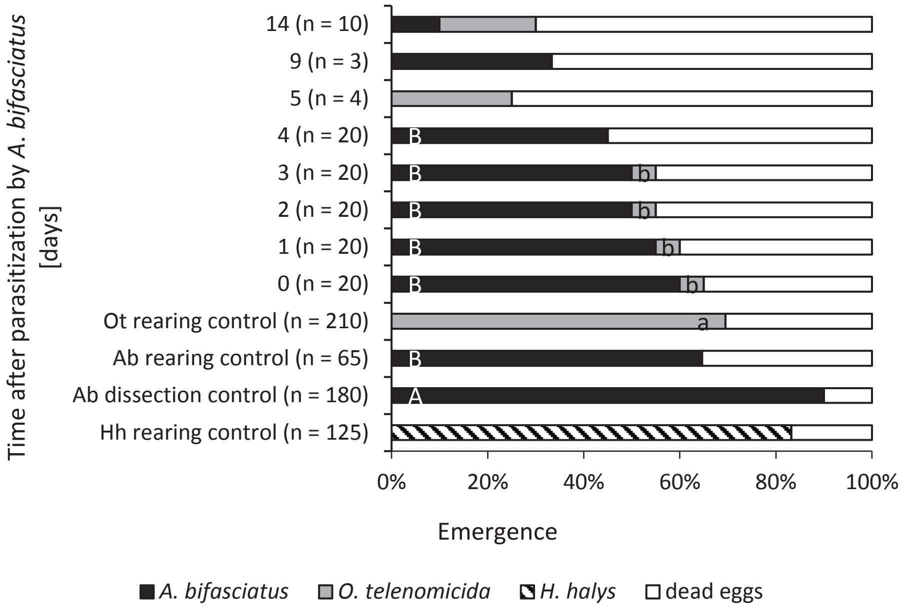

Figure 4 Proportion of *H. halys* eggs giving rise to *A. bifasciatus*, *O. telenomicida*, *H. halys* nymphs or nothing (dead eggs), following probing by *A. bifasciatus* (1st) and subsequent parasitism by *O. telenomicida* (2nd) at different time intervals between attacks. Sample sizes are given in brackets. Bars sharing the same letter are not significantly different by Tukey-adjusted mean separations (binomial GLM); capital letters show result for *A. bifasciatus*, and non-capitalized letters for *O. telenomicida*.

4.3.1.2 Intrinsic competition

In controls (n = 210) 70% of all parasitized eggs gave rise to *O. telenomicida* adults ('Ot rearing control', Figure 4 and 5), but developmental outcome depended on the number of *O. telenomicida* eggs deposited into each host egg (binomial GLM, df = 1, 197, $\chi^2 = 35.4$, $p < 0.001$), which amounted to 4.42 ± 0.191 (SE) (n = 199). For *H. halys* eggs with *O. telenomicida* emergence, the number of *O. telenomicida* supernumeraries was 3.65 ± 0.156 (SE) (n = 136) in contrast to 6.08 ± 0.434 (SE) (n = 63) in *H. halys* eggs without emergence. However, *O. telenomicida* emergence could be recorded from eggs containing up to eleven parasitoids. In the dissection controls for *A. bifasciatus*, 90% of all observed probing events ('probed eggs'; n = 180) resulted in oviposition ('Ab dissection control' Figure 4), while only 65% of 'probed' eggs (n = 65) gave rise to *A. bifasciatus* in rearing controls ('Ab rearing control' Figure 4 and 5), suggesting that some mortality occurs during development.

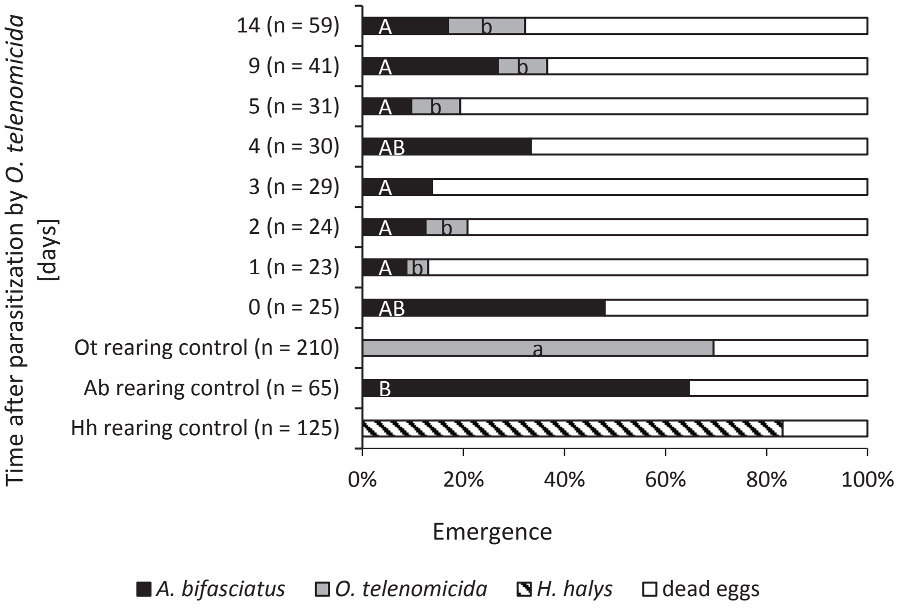

Figure 5 Proportion of *H. halys* eggs giving rise to *A. bifasciatus*, *O. telenomicida*, *H. halys* nymphs or nothing (dead eggs), following parasitism by *O. telenomicida* (1st) and subsequent probing by *A. bifasciatus* (2nd) at different time intervals between attacks. Sample sizes are given in brackets. Bars sharing the same letter are not significantly different by Tukey-adjusted mean separations (binomial GLM); capital letters show result for *A. bifasciatus*, and non-capitalized letters for *O. telenomicida*.

When multiparasitism of the same egg occurred, both *O. telenomicida* and *A. bifasciatus* could sometimes complete development on *H. halys* eggs previously parasitized by the other species, but overall far more *A. bifasciatus* than *O. telenomicida* adults emerged (Mann-Whitney U test, $W = 216.5$, $p < 0.001$) (Figure 4 and 5). The developmental outcome depended on the order of parasitization, and the time interval between the parasitization by the two competing species. In all cases, a proportion of multiparasitized eggs produced no parasitoids at all (Figure 4, 5). When *A. bifasciatus* was the *first* species to parasitize *H. halys* eggs, offspring production (45–60%) at the time intervals 0h to 4 days was not significantly different from rearing controls (65%) regardless of subsequent attack by *O. telenomicida* (binomial GLM, df = 5, 108, $\chi^2 = 2.96$, $p = 0.7068$) or superparasitism by the later species (binomial GLM, df = 1, 96, $\chi^2 = 2.03$, $p = 0.155$) (Figure 4). In contrast, in the same treatments, the offspring emergence of *O. telenomicida* was significantly lower (0–5%) than in rearing controls (70%) (binomial GLM, df = 5, 188, $\chi^2 = 103.1$, $p < 0.001$) (Figure 4). There was no impact of superparasitism by *O. telenomicida* (binomial GLM, df = 1, 85, $\chi^2 = 0.070$, $p = 0.792$). Little emergence of either species was recorded from the few multiparasitized eggs (due to low acceptance by both species) with five to fourteen days between initial parasitization events (Figure 4). These treatments (5, 9, 14 days) were excluded from the statistical analysis.

When *O. telenomicida* was the *first* species to parasitize *H. halys* eggs, multiparasitized eggs yielded no or very few *O. telenomicida* offspring (max. 4%) compared to controls (70%) (binomial GLM, df = 8, 464, χ^2 = 235.3, p < 0.001) (Figure 5). In contrast to the reverse order of parasitization (*A. bifasciatus* attacking first), multiparasitism also had a negative impact on *A. bifasciatus* offspring production (binomial GLM, df = 8, 322, χ^2 = 64.7, p < 0.001). When probing by *A. bifasciatus* occurred shortly after oviposition by *O. telenomicida* (0 d), *A. bifasciatus* offspring production (48%) was not significantly different from controls (65%), but reduced in all other treatments, varying from 8 to 33%. Due to the negative impact on both parasitoid species, the proportion of dead eggs (no emergence) was up to 87% (1 d) (Figure 5). Superparasitism by *O. telenomicida* did not influence the emergence of both parasitoid species (Ab: binomial GLM, df = 1, 258, χ^2 = 0.18, p = 0.673 and Ot: binomial GLM, df = 1, 259, $\chi2$ = 0.025, p = 0.875, respectively).

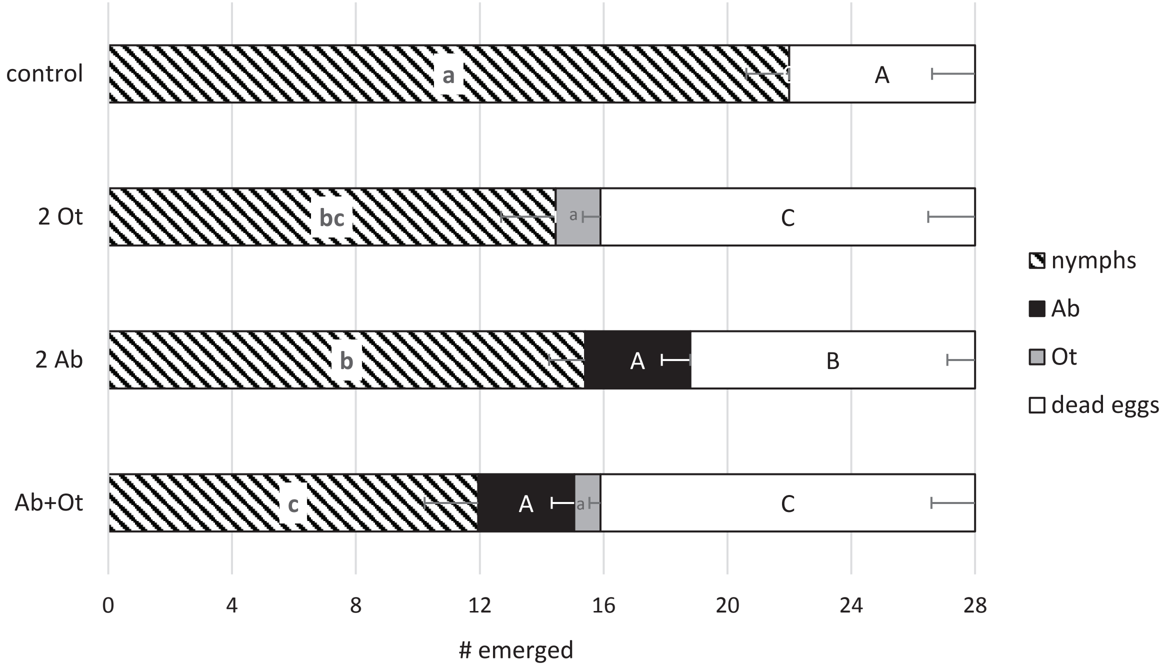

Figure 6 Mean emergence (-SE) from H. halys eggs (n = 20 for each treatment; 28 eggs per egg mass) exposed to: a) no parasitoids (control), b) two *O. telenomicida* females (2 Ot), c) two *A. bifasciatus* females (2 Ab), or d) one *A. bifasciatus* and one *O. telenomicida* female (Ab+Ot). Emergence between treatments (Ab+Ot, 2 Ab, 2 Ot, control) was compared within groups (nymphs, Ab, Ot, dead eggs); bars sharing the same letter are not significantly different by Tukey-adjusted mean separations (Poisson GLM).

4.3.2 Black box competition experiment

The number of dead *H. halys* eggs was significantly higher in all parasitoid treatments compared to the unparasitized control (21%) (Figure 6). The proportion of hatching nymphs was 55%

for the egg masses exposed to two *A. bifasciatus* females. It was lowest (52%) for the egg masses exposed to two *O. telenomicida* females and in the combined treatment with one *O. telenomicida* and one *A. bifasciatus* female, where it was 43% (Poisson GLM, df = 3, 76, $\chi^2 = 66.28$, $p < 0.001$) (Figure 6). *Anastatus bifasciatus* produced more offspring than *O. telenomicida* in all treatments, but parasitism never exceeded 12% (Mann-Whitney U test, $W = 591.5$, $p = 0.00526$) (Figure 6).

4.4 Discussion

Interspecies competition of parasitoids can cause changes in natural communities and influence the outcome of biological control programs (Meiners and Peri 2013). Many experiments with similar experimental setup measuring the intrinsic competition of egg parasitoids have been described in the literature, but this study is the first assessing the outcome of intrinsic competition between two European egg parasitoids, *A. bifasciatus* and *O. telenomicida*, considered for inundative releases against invasive brown marmorated stink bug in Europe. Apart from competition for *H. halys* eggs after inundative releases, competition may also naturally occur since their ecological host ranges overlap, including host such as *Iphiclides polalirius feisthamelii* (Duponchel) (Lepidoptera: Papilionidae) and *N. viridula* (Cusumano et al. 2015, Stefanescu et al. 2001, Stahl et al. 2018b).

Both *A. bifasciatus* and *O. telenomicida* females parasitized eggs that were previously attacked by the other species, but multiparasitism was dependent on the developmental stages of the competitors inside the host eggs. Separating the effect of the developmental stage of the competitor and the effect of the aging host egg is difficult, so these results should be interpreted with caution. Fresh host eggs containing eggs or early larval stages of their competitors were considered highly suitable for oviposition by both species, but oviposition noticeably declined when parasitoids were offered older host eggs containing late instar larvae or pupae. Since *O. telenomicida* females frequently rejected to oviposit in eggs containing mid and late instar larvae (5, 9 d) or pupae (14 d) of *A. bifasciatus*, females might be responding to internal markers by the means of sensory receptors (chemical or mechanical) on the ovipositor (Herman and Douglas 1976). Internal markers preventing super- or multiparasitism have been attributed to an injected secretion, physical changes, or changes in the hemolymph (reviewed in Vinson 1976). Parker (1933) conducted similar competition experiments with *O. kuvanae* and *A. japonicus*, egg parasitoids of the gypsy moth, and explained the decrease of oviposition by *O. kuvanae* with the movement of the larger *A. japonicus* larvae, touching its ovipositor and hindering its oviposition, which caused the *Ooencyrtus* females to leave the host eggs eventually. In *A. bifasciatus,* reduced oviposition was only observed for eggs containing late instar larvae (9 d) or pupae (14 d) of *O. telenomicida*, which might relate to the morphology of their larvae.

The respiratory stalk of the parasitoid egg remains attached to the *O. telenomicida* larva as it grows and only becomes disconnected shortly before pupation, which might restrict the movement of the larvae and thus hinder active defense against oviposition of *A. bifasciatus*. On the other hand, the frequent oviposition into host eggs containing eggs or young larvae of competitors suggests that females of both species were not deterred from oviposition, although the developmental outcome from multiparasitized eggs showed that for both species this would have been advantageous.

Since parasitoids often compete for the same host, many species have developed mechanisms to exclude competitors. In endoparasitoids, supernumerary larvae and eggs of conspecifics or competing species may be eliminated in two primary ways: physical attack, where a first instar larva uses its mandibles to attack a competitor ("killer larvae"), and physiological suppression caused by a toxin, anoxia, or nutritional deprivation (Vinson and Iwantsch 1980). These physiological factors originate with the ovipositing female wasp or her progeny and regulate the host's internal environment to optimize development, survival, and fitness of their eggs and larvae (Vinson and Iwantsch 1980, Harvey et al. 2013). In the case of multiparasitism, the first parasitoid female to oviposit injects biochemical factors (venoms, teratocytes, polydnaviruses or virus-like particles) into the host to optimize the physiological environment for her own immatures, whereas a second parasitoid female may inject factors into the same host that have requirements different from factors injected by the first parasitoid (Harvey et al. 2013).

In an earlier study, it was demonstrated that *O. telenomicida* females mediated physiological suppression by injecting factor(s) prior to the egg deposition which may alter the ooplasm inside the eggs of its host, *N. viridula,* making it unsuitable for larval development of its competitor *Trissolcus basalis* (Cusumano et al. 2012b). Remarkably, in the same study the emergence of *T. basalis* from multiparasitized eggs was always strongly reduced, regardless of whether host eggs were first parasitized by *T. basalis* and then punctured by *O. telenomicida*, or vice versa. Similarly, *O. pityocampae* (Mercet) was the superior intrinsic competitor over *Trissolcus agriope* Kozlov & Lê inside eggs of the green stink bug, *Brachynema signatum* Jakovlev (Hemiptera: Pentatomidae) (Cusumano et al. 2011). However, the results of the present study suggest that the venom injected by *O. telenomicida* did not have the same advantageous effects when competing with *A. bifasciatus.* The offspring emergence of *O. telenomicida* was drastically reduced (< 10%) in each treatment, regardless of the oviposition order. The mechanism preventing the development of *O. telenomicida* remains unknown. It is not known if *Anastatus* species have venom glands, but it seems probable since they were found in other Eupelmidae like *Eupelmus orientalis* (Crawford) and *E. urozonus* Dalman (Doury 1997, Moretti and Calvitti 2014). It is also conceivable that mechanical damage inflicted by the probing itself, or substances other than venom injected together with the egg or substances produced by the larvae could arrest development of competing larvae (Thompson 1980, Dahlmann 1991, Cusumano et al.

2018). On the other hand, first instar *Anastatus* larvae are equipped with simple sharp mandibles and a bifurcate tail (Parker and Thompson 1925), which makes them highly mobile (Harvey et al. 2013) and thus, they would be able to quickly reach competitors inside their hosts.

Interestingly, the outcome of intrinsic competition was less uniform for *A. bifasciatus*. If *A. bifasciatus* was the *first* species to oviposit and the same eggs were subsequently parasitized by *O. telenomicida* no later than 4 days after, no negative effects on *A. bifasciatus* emergence was observed. In other words, the piercing of the egg and the injection of venom by *O. telenomicida* females did not affect *A. bifasciatus*. Thus, it was confirmed that *A. bifasciatus* is a superior larval competitor, as shown previously when the intrinsic competition between *A. bifasciatus* and *Trissolcus japonicus* was investigated (Konopka et al. 2017). However, when the order of species was changed and *O. telenomicida* parasitized eggs prior to *A. bifasciatus*, the outcome of competition drastically changed. If *A. bifasciatus* parasitized the eggs immediately after *O. telenomicida* (0 to 24 h), *A. bifasciatus* remained the superior competitor and only *A. bifasciatus* offspring developed. In contrast, if parasitized eggs were attacked by *A. bifasciatus* at least 24 to 48 h later (1 d), only few *A. bifasciatus* emerged and nearly all multiparasitized eggs were dead (no parasitoid emergence). Although it is not entirely clear what caused the sudden change, one plausible scenario might be that *O. telenomicida* larvae that had hatched within the first 24 to 48 h had changed the physiology of the host in a way that *A. bifasciatus* larvae were not able to develop, before being killed by *A. bifasciatus*. This change could be due to substances released by secretions during egg hatching (e.g. Mackauer 1959), by the larvae (e.g. Moretti and Calvitti 2014) or by consumption of host resources to an extent that might be detrimental to the development of *A. bifasciatus*.

To increase the probability of parasitism and reduce the hours of observations, in this study individual *H. halys* eggs were used instead of egg masses. However, limiting the number of host eggs to one also increased the level of self-superparasitism of *O. telenomicida.* Even though in one case up to six *O. telenomicida* adults emerged from a single *H. halys* egg in the presented experiment, superparasitism negatively influenced *O. telenomicida* offspring emergence in rearing controls. This additional factor of intraspecies competition may have confounded the results for the interspecies competition when *O. telenomicida* was the first species ovipositing. However, despite self-superparasitism 70% of the parasitized *H. halys* eggs yielded *O. telenomicida* in the rearing control. Since superparasitized eggs were randomly distributed over all treatments, the conclusion that *O. telenomicida* offspring emergence was negatively influenced by *A. bifasciatus* parasitization before and after remains the same. In reverse, we, however, cannot exclude that the presence of several *O. telenomicida* reduced the offspring emergence of *A. bifasciatus* simply due to food limitation.

Additional experiments, using host eggs containing only single *O. telenomicida* larvae will be necessary for clarification.

It has been demonstrated in multiple systems that *Oooencrytus* species can act as facultative hyperparasitoids, mainly of *Trissolcus* species (Cusumano et al. 2011, Mohammedpour et al. 2014), and the same was assumed for *O. kuvanae* hyperparasitizing hibernating *A. japonicus* larvae in gypsy moth eggs (Howard and Fiske 1911). However, Parker (1933) was not able to naturally recreate this case and concluded that the few emerging *O. kuvanae* developed on *Anastatus* larva that were dead or dying at the time. Since parasitism in *A. bifasciatus* rearing controls was lower than in dissection controls, suggesting some baseline mortality occurs in *A. bifasciatus* larvae, the few *O. telenomicida* emerging in our experiments may have not only have resulted from eggs without *A. bifasciatus* oviposition, but also from eggs containing dead *A. bifasciatus* larvae. Accordingly, our study supports the assumption by Parker (1933) that *Oooencrytus* species cannot act as facultative hyperparasitoids of *Anastatus* species.

Exploiting a shared resource is possible by using different strategies (Amodeo 2015), but the two polyphagous species *A. bifasciatus* and *O. telenomicida* can easily avoid competition for *H. halys* eggs by exploiting alternative hosts (Cusumano et al. 2015, Stefanescu et al. 2001, Stahl et al. 2018b).

The black box experiment presented in this study was a simplified way to simulate combined releases. In contrast to other studies (Amodeo 2015), emergence of neither species was negatively impacted by the presence of a female of the competing species, which, if hosts are unlimited, is not contradicting the results of the larval competition. Overall parasitism was comparably low in contrast to the effect of host feeding inducing host egg abortion (Abram et al. 2016). The combination *O. telenomicida* and one *A. bifasciatus* and two *O. telenomicida* females caused the overall highest host mortality. Similarly, releasing *O. telenomicida* and *T. basalis* simultaneously in a simplified laboratory experiment resulted in highest mortality of *N. viridula* when compared to individual releases of the species (Cusumano et al. 2011). Results of the presented experiment might have been different if exposure times had been longer than 24 h: Mohammedpour et al. (2014) showed that offspring emergence of *O. pityocampae* doubled when exposure times were increased from 24 to 48 h.

In terms of biological control of *H. halys*, both species are considered candidates for augmentative biological control. A better overlap with the phenology of the pest, a wider geographical distribution across the areas invaded by *H. halys* and being a superior intrinsic competitor are factors that favor *A. bifasciatus*, but the main point to consider for biological control is the parasitism level that each of the two species can achieve on the target. For inundative biological control with *A.*

bifasciatus, no negative consequences are to be expected, but since it is difficult to draw conclusions from laboratory experiments alone, field experiments will be necessary.

4.5 Acknowledgements

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 641456. The authors are grateful to Serge Fischer (Agroscope Changins-Wädenswil, Switzerland) for providing the original *A. bifasciatus* colony. We would like to thank Darren Blackburn, Taylor Kaye, Lindsay Craig and Mariah Ediger for technical assistance in the laboratory. We appreciate the taxonomic guidance offered by Lucian Fusu (University of Iasi, Romania). We gratefully acknowledge the funding provided for this research by the following organizations and agencies: CABI is an international intergovernmental organisation, and we gratefully acknowledge the core financial support from our member countries (and lead agencies) including the United Kingdom (Department for International Development), China (Chinese Ministry of Agriculture), Australia (Australian Centre for International Agricultural Research), Canada (Agriculture and Agri-Food Canada), Netherlands (Directorate-General for International Cooperation), and Switzerland (Swiss Agency for Development and Cooperation). See http://www.cabi.org/about-cabi/who-we-work-with/key-donors/ for full details.

4.6 References

Abram PK, Brodeur J, Burte V, Boivin G (2016) Parasitoid-induced host egg abortion: an underappreciated component of biological control services provided by egg parasitoids. Biological Control 98, 52–60.

Amodeo V (2015) Intraguild interactions between egg parasitoids: from laboratory to field investigations. PhD thesis. University of Palermo, Italy.

Bográn CE, Heinz KM, Ciomperlik MA (2002) Interspecific competition among insect parasitoids: field experiments with whiteflies as hosts in cotton. Ecology 83, 653–668.

Bosco L, Moraglio ST, Tavella L (2017) *Halyomorpha halys*, a serious threat for hazelnut in newly invaded areas. Journal of Pest Science 91, 661-670.

Casula P, Wilby A, Thomas MB (2006) Understanding biodiversity effects on prey in multi-enemy systems. Ecology Letters, 9, 995-1004.

Costi E, Haye T, Maistrello L (2018) Surveying native egg parasitoids and predators of the invasive *Halyomorpha halys* in Northern Italy. Submitted to Journal of Applied Entomology.

Cusumano A, Peri E, Vinson SB, Colazza S (2011) Intraguild interactions between two egg parasitoids exploring host patches. BioControl 56, 173–184.

Cusumano A, Peri E, Vinson SB, Colazza S (2012a) Interspecific extrinsic and intrinsic competitive interactions in egg parasitoids. BioControl 57, 719–734.

Cusumano A, Peri E, Vinson SB, Colazza S (2012b) The ovipositing female of *Ooencyrtus telenomicida* relies on physiological mechanisms to mediate intrinsic competition with *Trissolcus basalis*. Entomologia Experimentalis et Applicata 143, 155–163.

Cusumano A, Peri E, Boivin G, Colazza S (2015) Fitness costs of intrinsic competition in two egg parasitoids of a true bug. Journal of Insect Physiology 81, 52–59.

Cusumano A, Peri E, Colazza S (2016) Interspecific competition/facilitation among insect parasitoids. Current Opinion in Insect Science 14, 12–16.

Cusumano A, Duvic B, Jouan V, Ravallec M, Legeai F, Peri E, Colazza S, Volkoff AN (2018) First extensive characterization of the venom gland from an egg parasitoid: structure, transcriptome and functional role. Journal of Insect Physiology 107, 68–80.

Dahlman DL (1991) Teratocytes and host/parasitoid interactions. Biological Control 1, 118–126.

Doury G, Bigot Y, Periquet G (1997) Physiological and biochemical analysis of factors in the female venom gland and larval salivary secretions of the ectoparasitoid wasp *Eupelmus orientalis*. Journal of Insect Physiology 43, 69–81.

Fisher RC (1961) A study in insect multiparasitism: I. Host selection and oviposition. Journal of Experimental Biology 38, 267-275.

Godfray HCJ (1994) Parasitoids: behavioral and evolutionary ecology. Princeton University Press, Princeton.

Halperin J (1990) Natural enemies of *Thaumetopoea* spp. (Lep., Thaumetopoeidae) in Israel Journal of Applied Entomology 109, 425-435.

Harvey JA, Poelman EH, Tanaka T (2013) Intrinsic inter-and intraspecific competition in parasitoid wasps. Annual Review of Entomology 58, 333–351.

Haye T, Wyniger D, Gariepy T (2014) Recent range expansion of brown marmorated stink bug in Europe. In Proceedings of the 8th International Conference on Urban Pests, 20–23 July 2014, Zurich, Switzerland (pp. 309–314). Executive Committee of the International Conference on Urban Pests.

Haye T, Fischer S, Zhang J, Gariepy T (2015) Can native egg parasitoids adopt the invasive brown marmorated stink bug, *Halyomorpha halys* (Heteroptera: Pentatomidae), in Europe? Journal of Pest Science 88, 693–705.

Heinz KM, Nelson JM (1996) Interspecific interactions among natural enemies of *Bemisia* in an inundative biological control program. Biological Control 6, 384-393.

Hermann HR, Douglas ME (1976) Comparative survey of the sensory structures of the sting and ovipositor of hymenopterous insects. Journal Georgia Entomological Society.

Hirose Y (1964) The activity of the egg parasites of the pinemoth, *Dendrolimus spectablis* Butler. Japanese black pine forest on the seacoast. Scientific Bulletin of the Faculty of Agriculture of Kyushi University 21, 13–24.

Hothorn T, Bretz F, Westfall P, Heiberger RM, Schuetzenmeister A, Scheibe S, Hothorn MT (2017) Package 'multcomp'. Obtenido de http://cran. statsfu. ca/web/packages/multcomp/multcomp.

Howard LO, Fiske WF (1911) The importation into the United States of the parasites of the gipsy moth and the brown-tail moth: a report of progress, with some consideration of previous and concurrent efforts of this kind (No. 91). US Government Printing Office.

Konopka JK, Haye T, Gariepy TD, McNeil JN (2017) Possible coexistence of native and exotic parasitoids and their impact on control of *Halyomorpha halys*. Journal of Pest Science 90, 1119–1125.

Liu Y-F, Zhang G-R, Gu D-X, Zhang W-Q (2000) The natural control effect of egg parasitoids on litchi stink bug *Tessaratoma papillosa* (Heteroptera: Pentatomidae) in litchi orchards. Insect Science 7, 322–328.

Mackauer M (1959) Histologische Untersuchungen an parasitierten Blattläusen. Zeitschrift für Parasitenkunde 19, 322–352.

Maistrello L, Vaccari G, Caruso S, Costi E, Bortolini S, Macavei L, Foca G, Ulrici A, Bortolotti PP, Nannini R, Casoli L (2017) Monitoring of the invasive *Halyomorpha halys*, a new key pest of fruit orchards in northern Italy. Journal of Pest Science 90, 1231-1244.

Meiners T, Peri E (2013) Chemical ecology of insect parasitoids: essential elements for developing effective biological control programmes. In: Wajnberg, E. and Colazza, S. (eds.): Chemical Ecology of Insect Parasitoids, John Wiley & Sons Ltd., Hoboke, USA, pp.191–224.

Mohammadpour M, Jalali MA, Michaud JP, Ziaaddini M, Hashemirad H (2014) Multiparasitism of stink bug eggs: competitive interactions between *Ooencyrtus pityocampae* and *Trissolcus agriope*. BioControl 59, 279–286.

Moretti R, Calvitti M (2014) Natural and artificial envenomation of *Ceratitis capitata* by *Eupelmus urozonus* and the search for new bioinsecticides. Entomologia Experimentalis et Applicata 150, 250–258.

Nanta P (1988) Biological control of longan stink bug, *Tessaratoma papillosa* Drury in Thailand. Biological control of longan stink bug, *Tessaratoma papillosa* Drury in Thailand. 43, 525–526.

Parker DL (1933) The interrelations of two hymenopterous egg parasites of the gypsy moth, with notes on the larval instars of each. Journal of Agricultural Research, 46, 23–34.

Parker DL, Thompson WR (1925) Notes on the larvae of the Chalcidoidea. Annals of the Entomological Society of America 18, 384–398.

Pemberton CE, Willard HF (1918) Interrelations of fruit-fly parasites in Hawaii. Journal of Agricultural Research, 12, 285–295.

Rondoni G, Bertoldi V, Malek R, Foti MC, Peri E, Maistrello L, Haye T, Conti E (2017) Native egg parasitoids recorded from the invasive *Halyomorpha halys* successfully exploit volatiles emitted by the plant–herbivore complex. Journal of Pest Science 90, 1087–1095.

Roversi P, Binazzi F, Marianelli L, Costi E, Maistrello L, Sabbatini Perevieri G (2016) Searching for native egg-parasitoids of the invasive alien species *Halyomorpha halys* Stal (Heteroptera Pentatomidae) in Southern Europe. Redia 99, 63–70.

Samra S, Ghanim M, Protasov A, Mendel Z (2016) Comparative study of development parameters of four *Ooencyrtus* spp. (Hymenoptera: Encyrtidae) on natural and factitious hosts. Journal of Applied Entomology 140, 334–345.

Stahl JM, Babendreier D, Haye T (2018a) Life history of *Anastatus bifasciatus*, a potential biological control agent of the brown marmorated stink bug in Europe. Biological Control. https://doi.org/10.1016/j.biocontrol.2018.10.016.

Stahl JM, Babendreier D, Haye T (2018b) Using the egg parasitoid *Anastatus bifasciatus* against the invasive brown marmorated stink bug in Europe: can non-target effects be ruled out? Journal of Pest Science 91, 1005–1017.

Stefanescu C, Pintureau B, Tschorsnig HP, Pujade-Villar J (2003) The parasitoid complex of the butterfly *Iphiclides podalirius feisthamelii* (Lepidoptera: Papilionidae) in north-east Spain. Journal of Natural History 37, 379–396.

Team RC (2014) R Foundation for Statistical Computing; 2015 R: A language and environment for statistical computing

Team RS (2017) RStudio: integrated development environment for R. RStudio, Inc. 2015

Thompson SN (1980) Preliminary characterization of phenyloxidase activity in the salivary secretion of the parasite, Exeristes roborator. Journal of Insect Physiology 26, 505–509.

Vinson SB (1976) Host selection by insect parasitoids. Annual Review of Entomology 21, 109–133.

Vinson SB, Iwantsch GF (1980) Host suitability for insect parasitoids. Annual Review of Entomology 25, 397–419.

Wermelinger B, Wyniger D, Forster B (2008) First records of an invasive bug in Europe: *Halyomorpha halys* Stal (Heteroptera: Pentatomidae), a new pest on woody ornamentals and fruit trees? Mitteilungen der Schweizerischen Entomologischen Gesellschaft 81, 1-8.

Zwölfer H (1971) The structure and effect of parasite complexes attacking phytophagous host insects. Proceedings of the Advanced Study Institute on Dynamics of Numbers in Population, pp.405–418.

Zwölfer H (1979) Strategies and counterstrategies in insect population systems competing for space and food in flower heads and plant galls. Fortschritte der Zoologie 25, 331–353.

5 Can *Anastatus bifasciatus* be used for augmentative biological control?

Abstract

Augmentative biological control is a widely used strategy to suppress insect pests in agricultural crops and forest plantations, when natural enemies are either absent or too scarce to provide efficient control. Apart from pest suppression, mass releases of generalist parasitoids can negatively influence populations of non-target species due to overflow of the agents into adjacent habitats. *Anastatus* species (Hymenoptera: Eupelmidae) are part of biological control programs worldwide and are, in China, also being used against the Asian *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) that is invasive in Europe. The generalist *Anastatus bifasciatus* is the most prevalent egg parasitoid of *H. halys* in Europe. To assess its potential to parasitize *H. halys* and non-target eggs in the field, it was released inundatively over three consecutive years in four Swiss and Italian fruit orchards. In every event 540 adult females were released in a plot of 60 trees in four rows. Parasitism of freeze-killed sentinel *H. halys* eggs, measured by parasitoid offspring emergence, was recorded after each release event, but only reached an average of 7% of all recovered eggs. Parasitism of exemplary non-target species reached an average of 8%. Dispersal of released *A. bifasciatus* females was recorded until up to 8 m from the closest release point. There were no indications that the parasitoids left the orchards. Molecular analyses showed that parasitism was likely underestimated by the preimaginal parasitoid mortality of 3.3%.

5.1 Introduction

Augmentative biological control is a widely used strategy to suppress insect pests in agricultural crops and forest plantations, when natural enemies are either absent (in greenhouses) or too scarce to provide efficient control (in large monocultures) (van Driesche et al. 2008). It is generally defined as the release of commercially mass-produced natural enemies against native or invasive pests, which can be divided into inundation and inoculation to distinguish between the two extremes of natural enemy release strategies. Inoculative biological control uses small numbers of the biological control agent as an initial population to ensure successful colonization at a critical period during the field season or during outbreaks of the pest species (Heimpel and Mills 2017). Inundative control differs from inoculation in that it is based on periodically releases of large numbers of the control agent to provide immediate suppression of the pest at any time of the season (Stinner 1977, Hoy 2008, Heimpel and Mills 2017).

For inundative releases in particular, factors such as timing (e.g. Butler and Hungerford 1971, Lopes et al. 2009), location and distribution of release spots (e.g. Hassan et al. 1986), densities of released natural enemies (e.g. Weseloh and Anderson 1975, Memmott et al. 1996) and developmental stage of the agent (Rabb et al. 1976) have to be considered prior releases to increase the efficacy of the agent and thus, the chances of successful biological control. Apart from pest suppression, mass releases of generalist parasitoids can negatively influence populations of non-target species due to the overflow of control agents into adjacent habitats (van Lenteren and Loomans 2006) and therefore, endangered and beneficial species within those habitats could be at risk. Consequently, potential risks associated with inundative releases should be assessed beforehand.

Egg parasitoids are advantageous for augmentative biological control because they reduce host populations before the damaging stages of the pest (Bigler et al. 1994). *Trichogramma* spp. are well-known biological control agents used against lepidopteran pests around the world (Smith 1996). A less well-known group of parasitoids is the genus *Anastatus* (Eupelmidae), which comprises primary endoparasitoids of a wide variety of hosts in the insect orders Hemiptera, Lepidoptera, Blattodea, Orthoptera and Mantodae (Narasimham and Sankaran 1982, Jones 1988, Askew 2005). *Anastatus* species are part of biological control programs worldwide and are used against a number of hemipteran pests such as fruitspotting bugs, *Amblypelta nitida* Stål and *A. lutescens lutescens* Distant (Hemiptera: Coreidae), in Australian macadamia orchards (Fay and De Faveri 1997, Huwer et al. 2011, Govender 2015), the citrus green stink bug, *Rhynchocoris humeralis* Thunberg (Hemiptera: Pentatomidae), in Nepal (Shrestha 2011) or the litchi stink bug, *Tessaratoma papillosa* Drury (Hemiptera: Pentatomidae), in China (Chen et al. 1990, He et al. 2001, Huang et al. 1974). In the Beijing Province of China, *Anastatus* sp. has been successfully mass released against the brown marmorated stink bug *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) with parasitism levels of more than 60% (Hou et al. 2009).

Halyomorpha halys is native to China, Japan as well as Korea, has become invasive in the Americas and Europe in the mid-1990s and early 2000s, respectively (Hoebeke et al. 2003, Wermelinger et al. 2008, Faúndez and Rider 2017). Since its arrival in Switzerland it has spread throughout Europe and has now been recorded from more than ten countries (reviewed in Haye et al. 2015a, Leskey and Nielsen 2018). It is a pest of a wide variety of economically important vegetable, fruit, and leguminous crops as well as ornamentals in both its native and invaded range (Kobayashi 1967, Lee et al. 2013). Severe damage has been caused particularly in fruit crops and hazelnuts in the USA, Georgia, and Italy (Leskey et al. 2012, Bosco et al. 2017, Maistrello et al. 2017).

In response, the number of insecticide treatments in certain regions has increased four-fold since the introduction of *H. halys,* thereby disrupting existing integrated pest management (IPM) programs, which led to outbreaks of secondary insect pests (Leskey et al. 2012). Due to the negative environmental effects of pesticide applications, environmentally friendly solutions such as biological control are urgently needed. Augmentative biological control using native natural enemies against invasive species is a fairly new approach and. To date, only a few examples exist, such as the use of *Trichopria drosophila*e Perkins (Hymenoptera: Diapriidae) against the spotted wing drosophila *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) (Chabert et al. 2013, Stacconi et al. 2013). In Switzerland and Italy, *Anastatus bifasciatus* (Geoffroy) is the most prevalent native parasitoid successfully parasitizing *H. halys* eggs in the field (Haye et al. 2015b, Roversi et al. 2016, Costi et al. 2018). It is one of the three European egg parasitoids capable of developing in viable *H. halys* eggs (Abram et al. 2017, Roversi et al. 2016, Moraglio et al. unpublished data) and thus, it was selected as potential candidate for inundative biological control of *H. halys* in Europe. Its host range, however, comprises more than 50 heteropteran and lepidopteran species (Noyes 2014, Stahl et al. 2018a) and there are concerns that mass releases of *A. bifasciatus* might lead to undesired non-target effects.

The present experimental field study aimed to assess the efficacy of *A. bifasciatus* against the pest *H. halys* and validate the potential risks for non-target species in a realistic field setting. Inundative releases were conducted over three consecutive years in four fruit orchards in Switzerland and Italy to develop a release strategy and answer the following questions: (1) What level of egg parasitism can be achieved by releasing *A. bifasciatus* against *H. halys* in fruit orchards?; (2) Are non-target species parasitized by *A. bifasciatus* in the field?; (3) Does *A. bifasciatus* disperse and persist in fruit orchards after releases?

5.2 Material and methods

5.2.1 Parasitoid rearing

Table 1 Field sites for experimental *A. bifasciatus* releases between 2016-2018

Site	Location (municipality, Canton/Region, country)	GPS coordiantes	Manage-ment	Host plants in plot (species, variety)	Pest presence	Release plot size [m²]
1	Lindau, Zurich, Switzerland	47°26'52.0"N 8°40'47.6"E	IPM	*Malus pumila*, Golden Delicious/Diwa/Braeburn	no	210
2	Bellinzona, Ticino, Switzerland	46°09'42.1"N 8°58'12.2"E	IPM	*Malus pumila*, Golden Delicious/Braeburn	yes	288
3	Manno, Ticino, Switzerland	46°01'52.8"N 8°55'20.4"E	Organic	*Malus pumila*, old varieties	yes	212
4	Carpi, Emilia-Romagna, Italy	44°43'46.8"N 10°52'30.0"E	Organic	*Pyrus communis*, Abate Fetel	yes	480

The laboratory rearing of *A. bifasciatus* originated from two parasitized *H. halys* egg masses collected by S. Fischer (Agroscope Changins-Wädenswil, Switzerland) in the Canton of Valais, Switzerland, in 2014 (Haye et al. 2015). Individuals of the founder population were identified by L. Fusu (University of Iasi, Romania). Adults were kept in 100 x 115 mm mesh-top cylindrical plastic containers, placed above a 90 x 20 mm Petri dish filled with honey water solution, which was provided to the parasitoids by two cotton wicks connecting the petri dish to the plastic container. The rearing containers were stored in an incubator at a light / temperature cycle of L 16h / 20°C and D 8h / 15°C. Twice a week the wasps were provided with new host eggs glued to cardboard pieces. The removed egg cards were stored at 26 °C until emergence of the new generation. Newly emerged wasps were collected daily and transferred to the rearing containers. In the first year of the study (2016), *A. bifasciatus* was exclusively reared on eggs of *H. halys*. When females were seven days old, they were provided constantly with new eggs until the day of release. In the second and third year (2017/2018), females were reared on a mix of *H. halys* and of *D. pini* eggs to increase the rearing and produce larger females (Stahl et al. 2018a). *Halyomorpha halys* eggs were produced following the methods described in Stahl et al. (2018a).

5.2.2 Release sites

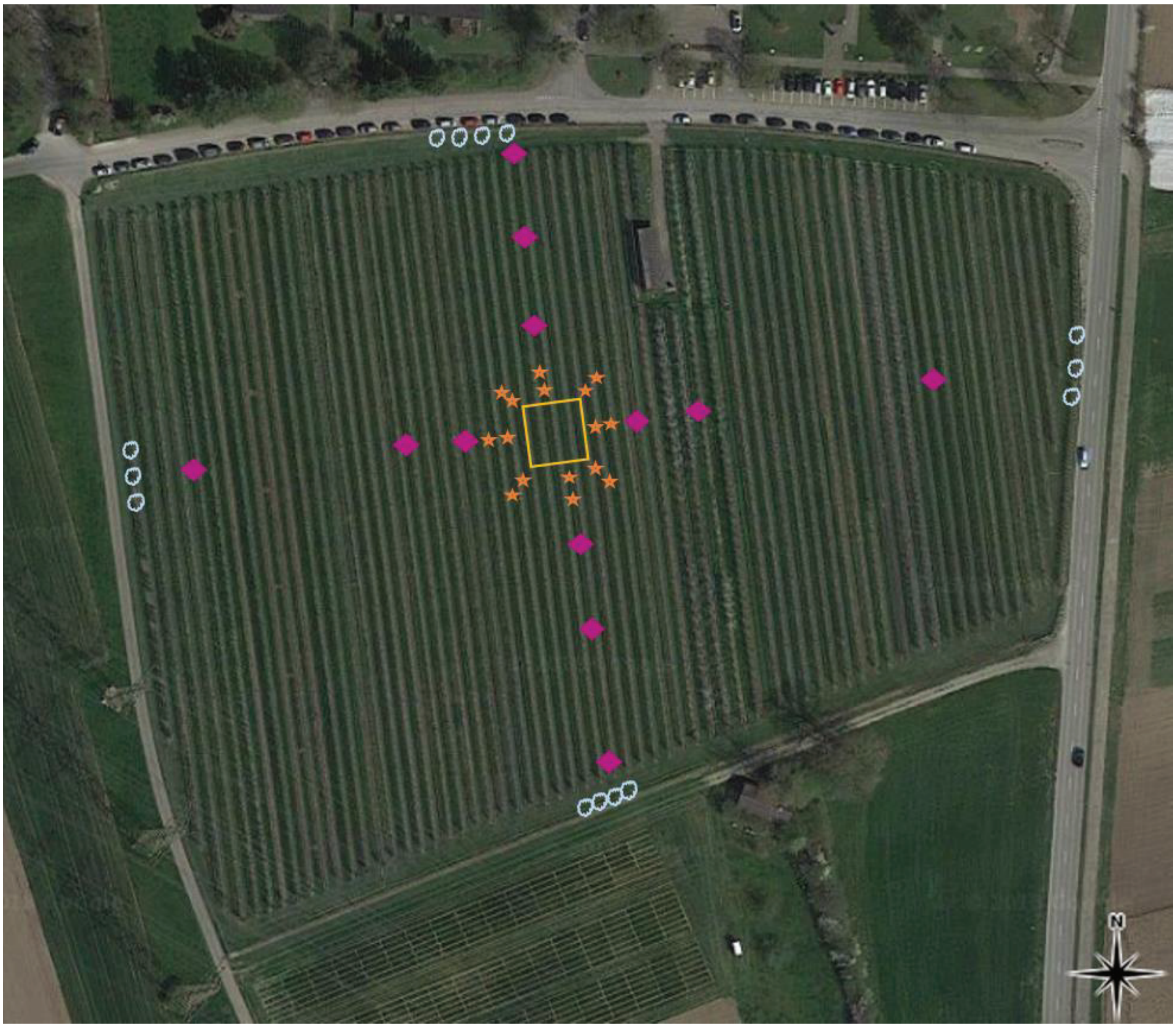

Figure 1 Site 1 with release plot (golden square) for releases 2016-2018, position of dispersal egg masses in 2016 (orange stars), 2017 (orange stars and purple diamonds) and 2018 (blue clouds). Changed after Google Maps (2018).

To assess the efficacy of A. bifasciatus under field conditions inundative releases were conducted in three apple orchards in Switzerland and a single pear orchard in Italy (Table 1). In each orchard, an area of 60 trees in 4 rows (15 trees per row) located in the center of the orchard was selected as release plot. Depending on the distance between rows and between trees within rows the size of the plots varied between 170 and 377 m2 (Table 1). All sites were equipped with a data logger ("HOBO Pendant Temperature/Light 64K", Onset Computer Corporation, Bourne, USA), to record the ambient temperature for the length of the experiment.

Table 2 Methods applied for attaching non-target eggs to apple leaves during experimental *A. bifasciatus* field releases 2016-2018

Species	Site	Year	Egg batch size	Total # eggs exposed/site	Exposure method
Samia cynthia (Drury) (Lepidoptera: Saturniidae)	1	2016	6	42	Glued individually
Euthrix potatoria (L.) (Lepidoptera: Lasiocampidae)	1	2016	6	252	Glued individually
Odonestis pruni (L.) (Lepidoptera: Lasiocampidae)	1	2016	4	168	Pre-glued to small, rectangular pieces of black mesh
Dendrolimus pini (L.) (Lepidoptera: Lasiocampidae)	1, 2	2017	4	192	Pre-glued to small, rectangular pieces of black mesh
Lasiocampa quercus (L.) (Lepidoptera: Lasiocampidae)	1, 2, 3, 4	2018	6 (3 in site 4)	288 (144)	Pre-glued to small, rectangular pieces of black mesh
Macrothylacia rubi (L.) (Lepidoptera: Lasiocampidae)	4	2018	3	144	Pre-glued to small, rectangular pieces of black mesh

5.2.3 Egg exposure

5.2.3.1 Parasitoid efficacy and persistence

Halyomorpha halys eggs were collected daily from the laboratory rearing, frozen for no longer than one month at -80°C and thawed earliest two days before the release date. For exposure, egg masses with at least 20 eggs were used, resulting in egg masses with 26.0 ± 0.09 (mean ± SE) eggs. In each step of the experiment, sentinel *H. halys* egg masses were glued directly on the underside of leaves of apple trees at a height of 50 to 180 cm. Branches of various host plants with feeding traces of *H. halys* were taken from the laboratory rearing cages and fixed next to the egg masses (one branch per egg mass) with a twist tie to increase the chance of parasitism by adding chemical cues of the host. Exposure times varied between four and seven days, depending on local weather conditions and pesticide application schedules.

Figure 2 Site 2 with release plot (golden square) for releases 2017-2018, position of dispersal egg masses in 2017 (purple diamonds) and 2018 (blue clouds). In 2018, the field to the north east of the apple orchard was planted with corn. Changed after Google Maps (2018).

In the first step, 25 *H. halys* egg masses were randomly distributed in each orchard to measure natural parasitism by *A. bifasciatus* at each site prior releases. On the day of the release, these egg masses were recollected, and 90 new egg masses were exposed inside the experimental plot. Within the plot, trees were randomly equipped with 0, 1, 2, or 3 egg masses. For each treatment there were 15 replicates (trees).

Figure 3 Site 3 with release plot (golden square) for release 2018, position of dispersal egg masses 2018 (blue clouds). Changed after Google Maps (2018).

Four to seven days after the parasitoid release, exposed egg masses were recollected. Fourteen days after the release, an additional 55 egg masses were placed out to monitor the persistence of the parasitoids. Twenty-five of those egg masses were randomly distributed over 25 trees within the entire orchard, while the remaining egg masses were placed on every second tree within the release plots.

5.2.3.2 Parasitoid dispersal capacity

To gain more information about the dispersal behavior of the parasitoids, additional *H. halys* egg masses were placed on trees outside the release plots on the day of the release. In 2016, a total of thirty-two egg masses were exposed in eight radial lines starting from the edges of the plot. Each line consisted of two trees which were approximately 3.5 m apart from each other and equipped with two egg masses each (Figure 1). In 2017, the same design was used at site 1 and site 2, but the distance from the release plot was extended in four of the eight directions up to approximately 90 m and 80 m from the closest release point, respectively, and the total amount of egg masses exposed increased to 56 (Figure 1 and 2).

Since natural *A. bifasciatus* populations were detected at site 2 in 2017, we focused on whether released *A. bifasciatus* females would leave the orchards. Since it could not be excluded that natural A. *bifasciatus* populations also occurred outside the orchards, egg masses used for the dispersal study at site 2 and site 3 were exposed as the same time as the egg masses used for monitoring natural parasitism before and after the releases. The number of egg masses exposed and the distance towards the release plots varied with sites: at site 1, 14 egg masses were exposed on *M. pumila* trees at the north and south side of the plot (blue clouds, Figure1), and on *M. pumila* trees in the furthest row towards the east and the west (Figure 1). The maximum distance to the release plot was approximately 120 m. At site 2, 50 sentinel egg masses were exposed three times at a maximum of 80 m away from the release plot in a bordering corn field and on young *M. pumila* trees (Figure 2). At site 3, 8 egg masses were exposed three times on eight trees, which were a maximum of 50 m away from the release plot. (Figure 3). At site 4 no egg masses were exposed.

5.2.3.3 Non-target parasitism

Depending on the availability of non-target species in each year of the study, eggs of six different species were exposed in the four release events: *Samia cynthia* (Drury) (Lepidoptera: Saturniidae), *Euthrix potatoria* (L.), *Odonestis pruni* (L.), *D. pini*, *Lasiocampa quercus* (L.) and *Macrothylacia rubi* (L.) (Lepidoptera: Lasiocampidae) (Table 2). Apart from *E. potatoria*, all non-target species were obtained from commercial insect breeders, the majority as pupae, and *M. rubi* as eggs. *Euthrix potatoria* caterpillars were collected in Bärschwil, Switzerland, and reared until the adult stage in 50 x 50 x 50 cm gauze cages (“BugDorn-4090 Insect Rearing Cage”, MegaView Science Co. Ltd., Taichung, Taiwan) on *Dactylis glomerata* L. (Poaceae), which was replaced daily. Once the adult stage was reached, all non-target species were kept in a 50 x 50 x 50 cm gauze cage for oviposition and provided with honey water and if necessary, their associated host plants as oviposition stimulus.

Newly laid eggs (< 24 h) were frozen for no longer than one month at -80°C and thawed no more than two days before the release date. Freeze-killed non-target egg batches of three to six eggs (see Table 2) were added to half of the trees inside the release plot that were previously equipped with *H. halys* egg masses (28 and 24 out of 45 trees in 2016 and 2017/18, respectively). The density of non-target egg masses was matched with the densities of *H. halys* egg masses (0, 1, 2, or 3 egg masses), but not with the number of eggs within the egg masses (Table 2). Non-target egg masses were randomly assigned to trees within the plot and egg batches were glued on the underside of leaves in close vicinity of the *H. halys* egg masses.

In the first release event in 2016, mainly *E. potatoria* eggs were exposed, but to better understand the potential influence of different non-target hosts, seven additional trees within the plot were equipped with one *H. halys* and one *S. cynthia* egg mass each. In all following release events, only eggs of single non-target species were used, apart from the release at site 4, where each tree equipped with non-target eggs contained three eggs of *M. rubi* and three eggs of *L. quercus* (Table 2).

5.2.4 Parasitoid releases

All females used for releases were considered experienced with the target host, because they were provided with *H. halys* eggs prior to releases. Females were deprived of hosts the week before release, so they would store their eggs and have a higher egg load when released. The day before release, females were transferred into release devices and stored overnight under the rearing conditions described above. Release devices were made of clear plastic cups (8 cm high, 6 cm wide at the top). At the bottom, a small opening used for transferring the parasitoids was covered with a piece of foam dipped in honey to provide parasitoids with food. The top of the cup was covered with mesh wide enough (1.9 x 1.8 mm) to allow *A. bifasciatus* females to pass. For transport the cups were closed with screw lids. In addition, cups were wrapped with black paper to ensure parasitoids would move upwards towards the natural light when cups were opened for release. Twelve cups containing 45 females each were equally distributed along the four rows of the plot (3/row) and placed at least five meters apart from each other. Cups were hung into the canopy at a height of 80 cm and fixed with twist ties. For release, screw lids were removed, and within the first hour all parasitoids had left the containers.

5.2.5 Treatment of recollected eggs

Recollected eggs were stored individually in small 54 x 14 mm Petri dishes at 26°C, 70% RH, and a 16L:8D photoperiod. Eggs were counted and assigned to one of the following categories: collapsed, chewed, sucked (see Morrison et al. 2016) or healthy eggs. Collapsed eggs, defined as eggs that looked undamaged but had lost more than half of their volume, were not considered for calculating parasitism levels.

Emerging wasps were counted, sexed and collected daily until no emergence was observed for four weeks. Parasitism was measured by the total number of eggs producing offspring divided by the sum of all healthy eggs in appearance. Eggs that had been attacked by chewing or sucking predators or had collapsed in the field were excluded from the analysis, since it was not possible to detect whether these eggs had been parasitized. Predation was calculated by the number of eggs showing signs of chewing or sucking divided by the sum of eggs with signs of predation and healthy eggs. 'Host location' was defined as proportion of egg masses with at least one parasitoid emerging divided by the total number of egg masses recovered and 'host exploitation' as the proportion of eggs parasitized within each parasitized egg mass (Bin and Vinson, 1991). *Halyomorpha halys* and non-target egg masses recovered in 2017 were overwintered in 54 x 14 mm Petri dishes under outdoor conditions in an open wooden shelter [Canton Jura, Switzerland (N47°22'23; E 7°19'32)] four weeks after emergence had stopped. The following year, egg masses were checked daily for emergence from May onwards. Since, in 2017 only 22% of exposed *H. halys* egg masses were recovered at site 1 (Figure 4), unemerged eggs within parasitized egg masses were analyzed with an *Anastatus* specific molecular marker six month after recollection (see chapter 6).

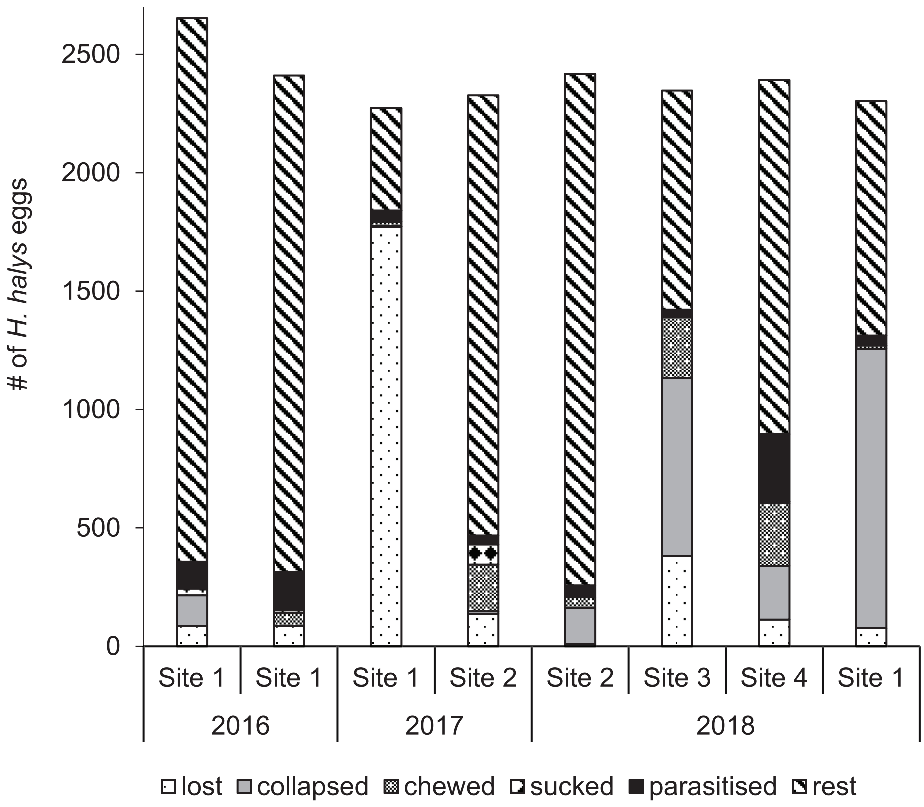

Figure 4 Fate of sentinel *H. halys* eggs exposed inside the release plots in Switzerland and Italy for 4-7 days after *A. bifasciatus* releases. Site numbers correspond with Table 1.

5.2.6 Statistical analyses

To estimate parasitoid dispersal, the influence of the distance to the closest release point on egg parasitism levels (= host impact) was analyzed for the first two years of the experiment, using a linear regression with the Theil-Sen estimator modified by Siegel repeated medians. The relationship between host density (measured as recovered *H. halys* eggs from a tree) and host impact was investigated with a generalized linear model (GLM) of the Poisson distribution using the log link function. Statistical analyses were conducted with R version 3.2.3 (Team RC 2014) using the development environment RStudio Version 1.0.136 (Team RS 2017). The package applied for the Theil-Sen estimator was 'mblm' (Komsta 2005).

5.3 Results

5.3.1 Parasitoid efficacy and persistence

Pre-release parasitism: Natural parasitism levels of sentinel egg masses by *A. bifasciatus* prior to releases were overall low (site 2) or completely absent (Table 3), with the exception of site 4 (Carpi), where it reached 28% (7 out of 25 egg masses). At site 1 (Lindau), parasitoids had been released in 2016, but no parasitism was detected in eggs exposed prior to releases in 2017.

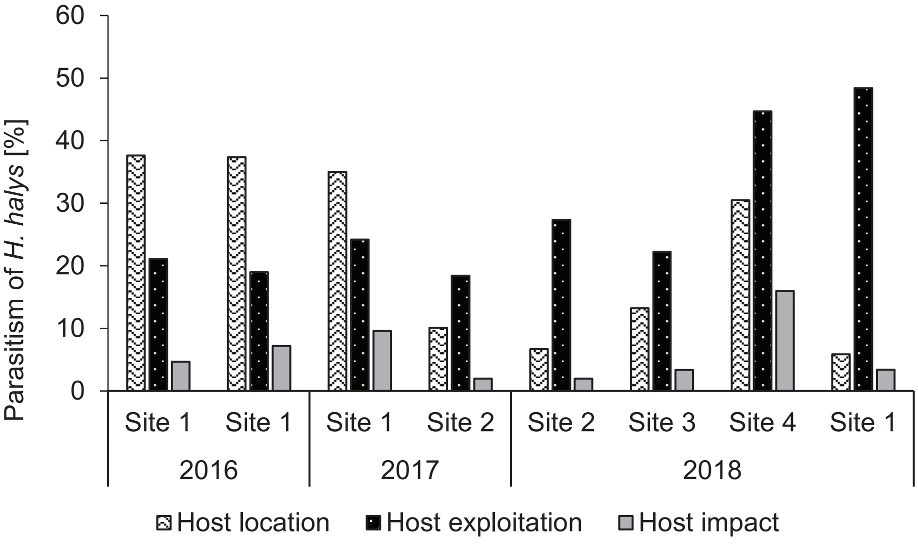

Figure 5 Parasitism measured by parasitoid offspring emergence of sentinel *H. halys* egg masses exposed after *A. bifasciatus* releases for 4–7 days between 2016 and 2018 in Switzerland and Italy. Site numbers correspond with Table 1. Host location is defined as proportion of recovered egg masses that yielded *A. bifasciatus* offspring; host exploitation as the proportion of eggs parasitized within in an egg mass; host impact as the proportion of parasitized eggs of the total recovered eggs.

Post-release parasitism and predation: After releasing *A. bifasciatus*, parasitism of sentinel *H. halys* eggs was detected in each orchard. On average, 20% ± 5.41 (SE) (range: 8–31%) of the recovered egg masses were parasitized (= host location). Since only an average of 27% ± 1.73 (SE) (range: 4–90%) of the eggs in parasitized egg masses yielded parasitoid offspring (= host exploitation), the actual egg parasitism (= host impact) was much lower, averaging 7% ± 3.20 (SE) (range: 2–16%) when data from all years and release events were combined. Eggs that were exposed at sites 1 and 2 in July/August 2017 did not produce any *A. bifasciatus* offspring after overwintering in 2018. There was no significant relationship between host density, measured as number of recovered eggs per tree, and number of parasitized eggs (Poisson GLM, df = 1, 80, z = -0.167, p = 0.867). The molecular analysis of unemerged eggs from *H. halys* egg masses exposed at site 1 in 2017, which yielded *A. bifasciatus* offspring, contained an additional 14% eggs (16 out of 113 eggs) with *A. bifasciatus* DNA. This amounts to 3.3% preimaginal mortality when egg masses without parasitoid emergence from the release event were included. Both chewing and sucking predation was recorded at all sites (Table 3), averaging 8% ± 3.27 (SE) (range: 0.04–27%) and 0.93% ± 0.52 (SE) (range: 0–5%), respectively (Figure 5).

Dispersal: *Halyomorpha halys* egg masses placed outside the release plots to measure *A. bifasciatus* dispersal only yielded parasitoid offspring at sites 1 and 2 in 2016 and 2017, respectively, but not at any other site (Table 4). At site 1, egg masses up to eight meters from the closest release point were successfully parasitized, but the level of parasitism was low (Table 4). At site 2, parasitized egg masses were found 37 m away from the closest release point but the presence of naturally occurring *A. bifasciatus* (Table 3) meant that it was inconclusive if the observed parasitism was caused by the released parasitoids. The results of the first year, however, showed that the number of parasitized *H. halys* eggs increased with decreasing distance to the nearest release point (linear regression, df = 119, v = 254.5, p < 0.001; Figure 6).

Persistence: Sentinel *H. halys* egg masses exposed two weeks after *A. bifasciatus* releases yielded parasitoid offspring in three out of the eight release events. However, in the one case where natural *A. bifasciatus* population were found in the orchard, it remains unclear if detected parasitism was caused by released parasitoids or naturally occurring ones (site 4) (Table 3). On average, *A. bifasciatus* offspring emerged from 3.24% ± 1.73 (SE) (range: 0–16%) of the recovered egg masses (= host location) and from 2.30% ± 1.75 (SE) (range: 0–16%) of the recovered eggs (= host impact).

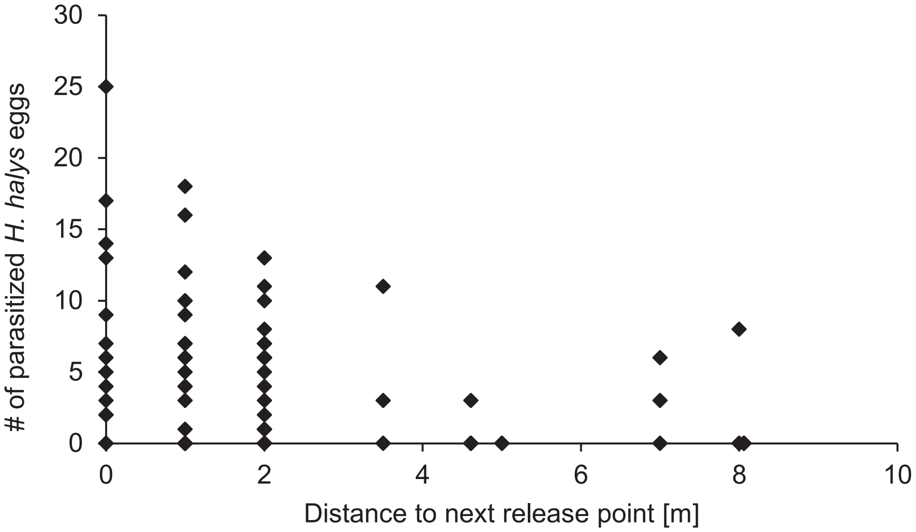

Figure 6 Number of *H. halys* eggs parasitized by *A. bifascatus* depending on distance to the closest release point during the first two releases in 2016 (site 1).

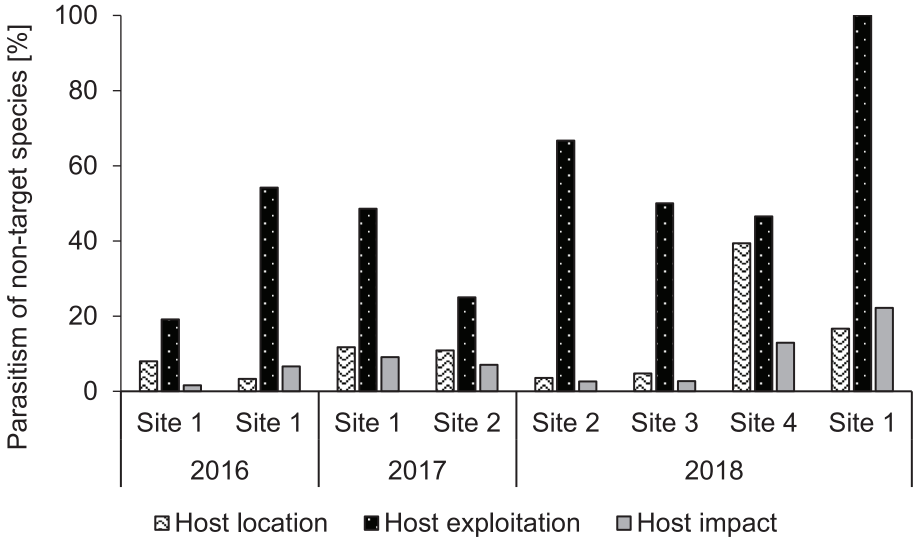

Figure 7 Parasitism measured by parasitoid offspring emergence of sentinel non-target egg masses exposed after *A. bifasciatus* releases for 4–7 days between 2016 and 2018 in Switzerland and Italy. Site numbers correspond with Table 1. Host location is defined as proportion of recovered egg masses that yielded *A. bifasciatus* offspring; host exploitation as the proportion of eggs parasitized within in an egg mass; host impact as the proportion of parasitized eggs of the total recovered eggs.

5.3.2 Non-target parasitism

Non-target parasitism occurred at all release sites, and five out of six non-target species yielded *A. bifasciatus* offspring. There was no indication of parasitism of *S. cynthia* eggs (recovered n = 29). The average parasitism of non-target eggs was 8.1% ± 2.42 (SE) (= host impact), when eggs of the five parasitized species were combined (*E. potatoria:* 2.3%, n =175; *O. pruni:* 6.7%, n =120; *D. pini:* 7.6% n = 145; *L. quercus:* 11.7%, n = 103; *M. rubi:* 16.9%, n =124) and ranged from 2% to 22% between release events (Figure 7). On average, 12% ± 4.20 (SE) (range: 3–40%) of the recovered non-target egg masses were parasitized (= host location), and an average of 25% ± 8.04 (SE) (range: 19–100%) of the eggs in parasitized egg masses yielded parasitoid offspring (= host exploitation) (Figure 7).

Table 3 Overview of *H. halys* egg masses exposed before parasitoid releases ("pre-release monitoring"), directly after releases within the release plot ("release") and two weeks after releases ("post-release monitoring")

Exposure date	Location	Treatment	Mean temperature (min – max) [°C]	Egg masses (eggs) exposed	Egg masses (eggs) recovered[1]
2016					
23.-28. Jul	1 - Lindau	Pre-release monitoring	22.4 (12.9 - 36.0)	25 (657)	22 (575)
28. Jul – 2. Aug		Release	21.7 (12.4 - 38.3)	97 (2653)	93 (2409)
11.-16. Aug		Post-release monitoring	21.8 (9.6 - 36.6)	55 (1480)	54 (1459)
16.-21. Aug		Release	20.4 (12.1 - 36.6)	90 (2410)	83 (2258)
2017					
17.-21. Jul	1 – Lindau	Pre-release monitoring	23.0 (13.8 - 34.5)	25 (623)	15 (298)
29. Jul – 3. Aug		Release	23.2 (15.3 - 35.0)	90 (2273)	20 (479)
10.-17. Aug		Post-release monitoring	18.8 (10.2 - 32.7)	55 (1410)	23 (562)
2.-10. Aug	2 – Bellinzona	Pre-release monitoring	23.4 (15.3 - 38.5)	25 (637)	20 (416)
14.-21. Aug		Release	23.1 (10.9 - 35.2)	90 (2327)	89 (1897)
28. Aug – 4. Sep		Post-release monitoring	21.2 (12.3 - 33.5)	55 (1387)	53 (774)
2018					
19.-25. Jul	2 – Bellinzona	Pre-release monitoring	25.0 (13.9 - 38.3)	25 (685)	22 (1371)
25.-30. Jul		Release	26.1 (15.5 - 37.9)	90 (2418)	90 (2205)
8.-13. Aug		Post-release monitoring	23.7 (16.8 - 36.4)	55 (2697)	51 (1940)
19.-25. Jul	4 – Carpi	Pre-release monitoring	25.0 (16.0 - 35.0)	25 (694)	25 (363)
25.-30. Jul		Release	26.5 (17.0 - 36.5)	90 (2392)	82 (1780)
9.-14. Aug		Post-release monitoring	25.4 (17.0 - 35.5)	55 (1469)	55 (958)
25.-30. Jul	3 – Manno	Pre-release monitoring	26.6 (15.3 - 44.5)	25 (752)	20 (545)

Exposure date	**Location**	**Treatment**	**Mean temperature (min – max) [°C]**	**Egg masses (eggs) exposed**	**Egg masses (eggs) recovered[1]**
30. Jul – 3. Aug		Release	28.0 (17.5 - 39.4)	90 (2347)	68 (957)
13.-17. Aug		Post-release monitoring	23.1 (14.6 - 34.7)	55 (1677)	51 (1345)
6.-10. Aug	1 – Lindau	Pre-release monitoring	na	25 (611)	10 (58)
11.-15. Aug		Release	na	90 (2303)	85 (1026)
24.-28. Aug		Post-release monitoring	na	55 (1760)	51 (1536)
Total				**1287 (35,662)**	**1082 (25,340)**

[1] healthy looking eggs, neither collapsed nor predated on

5.4 Discussion

After experimental releases of *A. bifasciatus* females, parasitism of sentinel *H. halys* eggs was detected in all eight release events at the four experimental sites. Since in two release events natural A. bifasciatus populations were present before releases, it remains unclear if detected parasitism was caused by released parasitoids or naturally occurring ones. In comparison, field releases of another European egg parasitoid, *Ooencyrtus telenomicida* (Vassiliev) (Hymenoptera: Encyrtidae) only elicited parasitism at 3 out of 6 release sites (Roversi et al. 2016). When estimating parasitism as the proportion of trees carrying at least on parasitized sentinel egg mass, *A. bifasciatus* was recovered from 30% of the trees. This is comparable with the results of experimental releases of *Anastatus* sp. in Australian macadamia orchards against fruitspotting bugs *Amblypelta nitida* Stål and *A. lutescens lutescens* Distant (Hemiptera: Coreidae), where parasitized host eggs were recovered from 24% of the plot trees (Govender 2015).

Table 4 Overview of *H. halys* egg masses measuring dispersal exposed before parasitoid releases ("pre-release monitoring"), directly after releases ("release") and two weeks after releases ("post-release monitoring")

Exposure date	Location	Treatment	Egg masses (eggs) exposed	Egg masses (eggs) recovered[1]	% parasitism by *A. bifasciatus* of egg masses (eggs)
2016					
28. Jul – 2. Aug	1 – Lindau	Release	32 (874)	32 (839)	12.5 (1.7)
16.-21. Aug		Release	32 (858)	29 (753)	10.3 (2.3)
2017					
29. Jul – 3. Aug	1 – Lindau	Release	56 (1438)	24 (540)	0 (0)
14.-21. Aug	2 – Bellinzona	Release	56 (1440)	52 (1107)	11.5 (2.9)
2018					
19.-25. Jul	2 – Bellinzona	Pre-release monitoring	50 (1300)	40 (802)	0 (0)
25.-30. Jul		Release	50 (1418)	22 (371)	0 (0)
8.-13. Aug		Post-release monitoring	50 (1323)	42 (829)	0 (0)
25.-30. Jul	3 – Manno	Pre-release monitoring	8 (187)	7 (140)	0 (0)
30. Jul – 3. Aug		Release	7 (190)	4 (89)	0 (0)
13.-17. Aug		Post-release monitoring	8 (211)	6 (143)	0 (0)
6.-10. Aug	1 – Lindau	Pre-release monitoring	14 (360)	11 (102)	0 (0)
11.-15. Aug		Release	14 (369)	10 (80)	0 (0)
24.-28. Aug		Post-release monitoring	14 (365)	12 (260)	0 (0)
Total			**399 (9,787)**	**291 (6,055)**	**4.5 (1.0)**

Host exploitation by *A. bifasciatus* was rather low (28%), which may be explained by the low weekly fecundity of the parasitoids (Stahl et al. 2018b) if each parasitized egg mass in the field was found by only one female. However, these data were obtained from trials with unlimited numbers of host. To increase host exploitation *A. bifasciatus* females were deprived of host eggs for one week prior releases. This approach may increase the number of stored parasitoid eggs and increase the parasitoids' host searching motivation (Mills and Wajnberg 2008). Unfortunately, there is no information about the behavior of *A. bifasciatus* in the field that might help explain the low exploitation values and improve release efficacy. To increase host exploitation by decreasing patch-leaving stimuli, dispersal capacities can be reduced by arresting natural enemies within the patch with chemical cues (Mills and Wajnberg 2008).

When data from all releases were combined, total egg parasitism (host impact) was on average 7%. Based on the mean host exploitation value in the field, if each *A. bifasciatus* female released in the orchards either parasitized three to six eggs, only 1 to 10% of the 540 released females would have been responsible for the observed parasitism. This could also be connected to the relatively small experimental plots, where dispersal is more likely than from bigger areas such as a whole orchard. Calculations carried out by Andow and Prokrym (1991) after releases of *Trichogramma nubilale* Ertle and Davis (Hymenoptera: Trichogrammatidae) revealed that every day 40% of the parasitoid females disappeared, which was attributed to the hot and dry weather conditions. In the present study, weather conditions at release sites were extremely hot and dry over the three years of the study and may have caused some degree of adult mortality. Another factor that might have influenced observed parasitism levels is intraguild predation. Some sentinel eggs that showed signs of predation also had *A. bifasciatus* host feeding marks, indicating that at least some parasitized eggs had been eaten by predators. As interactions between parasitoids and predators of *H. halys* have not been studied so far (Abram et al. 2017) future studies on the subject could provide valuable input.

Host location of parasitoids is facilitated by a number of cues that can either be emitted by the host plant (Vinson 1981), the host itself (Weseloh 1981) or a combination of the two factors. The experimental design of the current study was aimed to provide natural conditions and include host cues provided by branches with feeding and walking traces of *H. halys* next to the sentinel egg masses, assuming that these traces guide parasitoids to their hosts, as demonstrated for *T. basalis* locating egg masses of *N. viridula* (Colazza et al. 1999). At the beginning of the release experiments, the factors influencing the host finding of *A. bifasciatus* were unknown, but in the meantime Rondoni et al. (2017) demonstrated that *A. bifasciatus* positively responded to adult *H. halys* male volatiles and to *H. halys*-induced plant volatiles, indicating ability to exploit cues associated with the new host for egg location. Consequently, sentinel *H. halys* egg masses exposed in this study may have lacked important chemical cues associated with *H. halys* egg masses in nature, resulting in much lower host finding and thus, lower parasitism by *A. bifasciatus*. Similarly, Morrison et al. (2018) found no indication that *H. halys* egg parasitoids in North America used the *H. halys* aggregation pheromone as a kairomone.

Apart from trying to increase host exploitation, releasing higher numbers of parasitoids is another alternative to increase pest suppression. In the present study, the release density was 540 female *A. bifasciatus* per 60 trees, which translates to 11,000 (site 4) to 26,000 (site 1) females per hectare, depending on the distances between rows and trees within rows. In comparison, many commercial and experimental releases involve larger quantities of parasitoids, such as several times 100,000 *Trichogramma* per hectare (Os and Varshney 2018). In China, commercial releases of the closely related *A. japonicus* against less severe infestations of the litchi stink bug, *T. papillosa,* required approximately 180 females per medium-sized tree, which is nearyl 20 times more than the present experiments, and yield an average of 52–94% parasitism in the first year of its releases (Li et al. 2014).

Measuring parasitism by offspring emergence is less labor-intensive than dissections, but underestimates the actual levels of parasitism if a proportion of individuals cannot undergo complete development. Dissections of parasitized hosts often have the disadvantage that tiny eggs and early instar larvae of parasitoids can be hard to detect inside hosts, and remains of dead parasitoids may be difficult to recognize when hosts decay. An alternative method to detect parasitoids inside their hosts is the use of molecular markers (Gariepy et al. 2008, 2014). Since the molecular marker for *A. bifasciatus* just became available in the last year of the study (see chapter 6), a single sample from an individual release event was processed. The analysis of remaining unemerged eggs from parasitized egg masses showed that 14% contained dead developmental stages of *A. bifasciatus*, suggesting that host exploitation was indeed much higher than what was measured by offspring emergence. Another important behavioral trait of *A. bifasciatus* is host feeding (Stahl et al. 2018b), the consumption of haemolymph and host tissue by the adult female. Since many parasitoids kill hosts by host feeding as well as parasitism, this is factor that should not be neglected when estimating the efficacy of a parasitoid. To avoid additional damage by increasing *H. halys* densities in the apple orchards, freeze-killed *H. halys* egg masses had to be used for the experiments. As a consequence, it was not possible to assess the number of host eggs killed by *A. bifasciatus* host feeding. However, data from previous laboratory studies suggest that the number of eggs killed by host feeding is nearly as high as the number of eggs killed by parasitization, which may double the estimated host mortality (Konopka et al. 2017, Stahl et al. 2018b). In addition, parasitoid efficacy might be distinctly higher in a real infestation situation because using sentinel egg masses may underestimate parasitism (Jones et al. 2014). If preimaginal parasitoid mortality (3.3%) and host feeding (7%; chapter 2) are added to the observed parasitism (7% offspring emergence, chapter 5), the actual induced mortality of *H. halys* eggs may have been 17.3 %.

Higher parasitism of *H. halys* may be accompanied by higher non-target parasitism, since the host impact values for non-targets (8%) and the target (7%) are similar. These findings agree with studies on the physiological host range of *A. bifasciatus,* showing that most non-target species where as frequently parasitized and suitable for development as the target host (Stahl et al. 2018a). Even though arthropod biodiversity in apple orchards tends to be higher than in annual crops (Risch et al. 1983, Kozár 1992, reviewed by Simon et al. 2010), insect diversity, in other words the number of potential non-targets, was still comparably low in conventional apple orchards (JMS and TH personal observation). Consequently, potential dispersal of released *A. bifasciatus* into habitats outside the orchards is a more important factor in its risk assessment (Rand et al. 2006). In this study, parasitoid dispersal could only be confirmed up to eight meters from the closest release point. Other *Anastatus* species, however, can disperse up to 60 meters (Li et al. 2014, Govender 2015) and, with wind dispersal, up to 100 meters (Li et al. 2014). Since parasitism levels of sentinel eggs were low, the results of the dispersal study are not conclusive and further investigations are needed. As *A. bifasciatus* has the potential longevity of three months (97.5 days when provided with honey water) (Stahl et al. 2018b), released wasps were expected to persist in the fruit orchards, but their presence was only retained for two weeks in three out of eight release events which could also be attributed to to dispersal. Since releases were conducted in late summer, nectar sources were not available in the orchards, which may have caused a large proportion of parasitoids to leave the orchards and disperse into other habitats.

In conclusion, field releases of *A. bifasciatus* can increase parasitism of *H. halys* eggs in fruit orchards, but parasitism levels achieved with the current release strategy were not high enough to effectively suppress the pest. However, the overall impact may be higher when parasitoid mortality inside eggs and egg mortality by host feeding are taken into account. If cost effective, releasing higher densities of parasitoid or combined releases with other species, e.g. *O. telenomicida* should be considered (Roversi et al. 2016). To quantify the impact of the parasitoids, parasitism and host feeding, in the orchards more precisely, naturally laid egg masses should be used in future trials to include host finding cues of the host and host plants and avoid potential adverse effects of frozen sentinel eggs. Some degree of non-target parasitism after mass releases can be expected, but whether non-targets would be negatively affected at population level will require further investigations, including further dispersal studies.

5.5 Acknowledgements

This project has received funding from the European Union’s Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 641456. The authors are grateful to Serge Fischer (Agroscope Changins-Wädenswil, Switzerland) for providing the original *A. bifasciatus* colony. We would like to thank Warren Wong, Giorgia Frei, Patrick Mrazek, Taylor Kaye, Chelsey Blackman, Darren Blackburn, Jessica Fraser, Lindsay Craig, Christie Laing, Anna Grunsky, Mariah Ediger and Giorgia Mattei for technical assistance. We appreciate the taxonomic guidance offered by Lucian Fusu (University of Iasi, Romania). The authors would further like to thank the farmers who allowed the use their orchards. We gratefully acknowledge the funding provided for this research by the following organizations and agencies: CABI is an international intergovernmental organisation, and we gratefully acknowledge the core financial support from our member countries (and lead agencies) including the United Kingdom (Department for International Development), China (Chinese Ministry of Agriculture), Australia (Australian Centre for International Agricultural Research), Canada (Agriculture and Agri-Food Canada), Netherlands (Directorate-General for International Cooperation), and Switzerland (Swiss Agency for Development and Cooperation). See http://www.cabi.org/about-cabi/who-we-work-with/key-donors/ for full details.

5.6 References

Abram PK, Hoelmer KA, Acebes-Doria A et al. (2017) Indigenous arthropod natural enemies of the invasive brown marmorated stink bug in North America and Europe. Journal of Pest Science 90, 1009-1020.

Andow DA, Prokrym DR (1991) Release density, efficiency and disappearance of *Trichogramma nubilale* for control of European corn borer. Entomophaga 36, 105-113.

Askew A (2005) A new European species of Anastatus Motschulsky (Hym., Eupelmidae). Entomological Magazine 141, 1697-1699.

Bigler F, Wajnberg E, Hassan SA (1994) Biological control with egg parasitoids. Quality control in Trichogramma production. CAB International, Wallingford, UK.

Bosco L, Moraglio ST, Tavella L (2017) *Halyomorpha halys*, a serious threat for hazelnut in newly invaded areas. Journal of Pest Science 91, 661-670.

Butler GD, Hungerford CM (1971) Timing field releases of eggs and larvae of *Chrysopa carnea* to insure survival. Journal of Economic Entomology 64, 311-312.

Chabert S, Allemand R, Poyet M, Eslin P, Gibert P (2013) Ability of European parasitoids (Hymenoptera) to control a new invasive Asiatic pest, *Drosophila suzukii*. Biological Control 63, 40–47.

Chen Y, Lin L, Chen J (1990) Preliminary report of study on the release of Anastatus for the control of the stink bug (*Tessaratoma papillosa*) Fujian Agricultural Science and Technology 2, 15-16.

Colazza S, Salerno G, Wajnberg E (1999) Volatile and contact chemicals released by *Nezara viridula* (Heteroptera: Pentatomidae) have a kairomonal effect on the egg parasitoid *Trissolcus basalis* (Hymenoptera: Scelionidae). Biological Control 16, 310-317.

Costi E, Haye T, Maistrello L (2018) Surveying native egg parasitoids and predators of the invasive *Halyomorpha halys* in Northern Italy. Submitted to Journal of Applied Entomology.

Faúndez EI, Rider DA (2017) The brown marmorated stink bug *Halyomorpha halys* (Stål, 1855) (Heteroptera: Pentatomidae) in Chile. Arquivos Entomolóxicos, 17, 305-307.

Fay H, De Faveri S (1997) Egg parasitoids of fruitspotting bugs (Amblypelta spp.): potential for their mass-rearing and strategic release. Final report HG308:23.

Gariepy TD, Kuhlmann U, Gillott C, Erlandson M (2008) A large-scale comparison of conventional and molecular methods for the evaluation of host–parasitoid associations in non-target risk-assessment studies. Journal of Applied Ecology, 45, 708-715.

Gariepy TD, Haye T, Zhang J (2014) A molecular diagnostic tool for the preliminary assessment of host–parasitoid associations in biological control programmes for a new invasive pest. Molecular Ecology, 23, 3912-3924.

Govender AW (2015) Australian fruitspotting bugs, *Amblypelta nitida* Stål and *A. lutescens lutescens* Distant (Hemiptera: Coreidae), and the potential for their biologically based management in macadamia orchards. PhD thesis. University of Queensland.

Hassan SA, Stein E, Dannemann K, Reichel W (1986) Massenproduktion und Anwendung von Trichogramma: 8. Optimierung des Einsatzes zur Bekämpfung des Maiszünslers *Ostrinia nubilalis* Hbn. Journal of Applied Entomology 101, 508-515.

Haye T, Wyniger D, Gariepy TD (2014a) Recent range expansion of brown marmorated stink bug in Europe. In Proceedings of the 8th International Conference on Urban Pests, 20-23 July 2014, Zurich, Switzerland (pp. 309-314). Executive Committee of the International Conference on Urban Pests.

Haye T, Fischer S, Zhang J, Gariepy T (2015b) Can native egg parasitoids adopt the invasive brown marmorated stink bug, *Halyomorpha halys* (Heteroptera: Pentatomidae), in Europe? Journal of Pest Science 88, 693-705.

He J, Guo L, Tang F, Zhang F, Li F, Zhao Z (2001) The study of control Tessaratoma papillosa by releasing *Anastatus japonicus*. Guangxi Zhiwu 21, 163-165.

Heimpel GE, Mills NJ (2017) Biological control: ecology and applications. Cambridge University

Press.

Hoebeke ER, Carter ME (2003) *Halyomorpha halys* (Stål) (Heteroptera: Pentatomidae): a polyphagous plant pest from Asia newly detected in North America. Proceedings of the Entomological Society of Washington, 105, 225-237.

Hou Z, Liang H, Chen Q, Hu Y, Tian H (2009) Application of *Anastatus* sp. against *Halyomorpha halys*. Forest Pests and Diseases 4, 39.

Hoy MA (2008) Augmentative Biological Control. In: Capinera J.L. (eds) Encyclopedia of Entomology. Springer, Dordrecht.

Huang M, Mai S, Wu W, Poo C (1974) The bionomics of *Anastatus* sp. and its utilization for the control of lichee stink bug *Tessaratoma papillosa* Drury. Acta Entomol. Sin. 17, 362-375.

Huwer R, Maddox CDA, Fay H et al. (2011) A multi target approach to fruitspotting bug management. Research agreement MT10049:50.

Jones WA (1988) World review of the parasitoids of the southern green stink bug, *Nezara viridula* (L.)(Heteroptera: Pentatomidae). Annals of the Entomological Society of America 81, 262-273.

Kobayashi T (1967) The developmental stages of some species of the Japanese Pentatomoidea (Hemiptera): XVI. Homalogonia and an allied genus of Japan (Pentatomidae). Applied Entomology and Zoology 2, 1-8.

Konopka JK, Haye T, Gariepy TD, McNeil JN (2017) Possible coexistence of native and exotic parasitoids and their impact on control of *Halyomorpha halys*. Journal of Pest Science, 90, 1119-1125.

Kozár, F. (1992). Organization of arthropod communities in agroecosystems. Acta Phytopathologica et Entomologica Hungarica 27, 365-373.

Lee D-H, Short BD, Joseph SV, Bergh JC, Leskey TC (2013) Review of the biology, ecology, and management of *Halyomorpha halys* (Hemiptera: Pentatomidae) in China, Japan, and the Republic of Korea. Environmental Entomology 42, 627–641.

Leskey TC, Nielsen AL (2018) Impact of the Invasive Brown Marmorated Stink Bug in North America and Europe: History, Biology, Ecology, and Management. Annual Review of Entomology 63, 599–618.

Leskey TC, Hamilton GC, Nielsen AL et al. (2012) Pest status of the brown marmorated stink bug, *Halyomorpha halys* in the USA. Outlooks on Pest Management 23, 218–226.

Li DS, Liao C, Zhang BX, Song ZW (2014) Biological control of insect pests in litchi orchards in China. Biological Control 68, 23-36.

Lopes C, Spataro T, Lapchin L, Arditi R (2009) Optimal release strategies for the biological control of aphids in melon greenhouses. Biological Control 48, 12-21.

Maistrello L, Vaccari G, Caruso S, Costi E, Bortolini S, Macavei L, Foca G, Ulrici A, Bortolotti PP, Nannini R, Casoli L (2017) Monitoring of the invasive *Halyomorpha halys*, a new key pest of fruit orchards in northern Italy. Journal of Pest Science 90, 1231-1244.

Memmott J, Fowler SV, Harman HM, Hayes LM (1996) How best to release a biological control agent. In Proceedings of the 9th international symposium on biological control of weeds, Stellenbosch, South Africa, 19-26 January 1996. (pp. 291-296). University of Cape Town.

Mills NJ, Wajnberg E (2008) Optimal foraging behavior and efficient biological control methods. In: Behavioral ecology of insect parasitoids: From theoretical approaches to field applications, pp.3-30.

Morrison WR, III, Blaauw BR, Nielsen AL, Talamas E, Leskey TC (2018) Predation and parasitism by native and exotic natural enemies of *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) eggs augmented with semiochemicals and differing host stimuli. Biological Control 121, 140-150.

Narasimham AU, Sankaran T (1982) Biology of *Anastatus umae* (Hymenoptera: Eupelmidae), an oothecal parasite of *Neostylopyga rhombifolia.* Colemania 1, 135-140.

Noyes J (2014) Universal Chalcidoidea Database – World Wide Web electronic publication. Natural

History Museum Update August. http://www.nhm.ac.uk/our-science/data/chalcidoids/. Accessed 15 Oct 2018.

Os N, Varshney R (2018) Utilization of Trichogrammatid egg parasitoids in pest management. Acta Scientific Agriculture 2, 49–53.

Rabb RL, Stinner RE, van den Bosch R (1976) Conservation and augmentation of natural enemies (Vol. 233). Academic Press, New York.

Risch SJ, Andow D, Altieri MA (1983) Agroecosystem diversity and pest control: data, tentative conclusions, and new research directions. Environmental Entomology, 12, 625–629.

Rondoni G, Bertoldi V, Malek R, Foti MC, Peri E, Maistrello L, Haye T, Conti E (2017) Native egg parasitoids recorded from the invasive *Halyomorpha halys* successfully exploit volatiles emitted by the plant–herbivore complex. Journal of Pest Science 90, 1087–1095.

Roversi PF, Marianelli L, Costi E, Maistrello L, Sabbatini PG (2016) Searching for native egg-parasitoids of the invasive alien species *Halyomorpha halys* Stål (Heteroptera Pentatomidae) in Southern Europe. Redia 99, 63-70.

Shrestha R (2011) Productivity improvement of citrus fruits through effective fruit drop management technique in the mid and far western development region of Nepal. Technical report

Simon S, Bouvier JC, Debras JF, Sauphanor B (2011) Biodiversity and pest management in orchard systems. In Sustainable Agriculture Volume 2 (pp. 693-709). Springer, Dordrecht.

Smith SM (1996) Biological control with Trichogramma: advances, successes, and potential of their use. Annual Review of Entomology 41, 375–406.

Stacconi RM, Grassi A, Dalton D, Miller B, Ouantar M, Loni A, Ioriatti C, Walton V, Anfora G (2013) First field records of *Pachycrepoideus vindemiae* as a parasitoid of *Drosophila suzukii* in European and Oregon small fruit production areas. Entomologia 1, 11-16.

Stahl JM, Babendreier D, Haye T (2018a) Using the egg parasitoid *Anastatus bifasciatus* against the invasive brown marmorated stink bug in Europe: can non-target effects be ruled out? Journal of Pest Science 91, 1005-1017.

Stahl JM, Babendreier D, Haye T (2018b) Life history of *Anastatus bifasciatus*, a potential biological control agent of the brown marmorated stink bug in Europe. Biological Control. https://doi.org/10.1016/j.biocontrol.2018.10.016.

Stinner RE (1977) Efficacy of inundative releases. Annual Review of Entomology, 22, 515-531.

van Driesche RG, Lyon S, Sanderson JP, Bennett KC, Stanek III, EJ, Zhang R (2008) Greenhouse trials of *Aphidius colemani* (Hymenoptera: Braconidae) banker plants for control of aphids (Hemiptera: Aphididae) in greenhouse spring floral crops. Florida Entomologist, 583-591.

van Lenteren JC, Loomans AJ (2006) Environmental risk assessment: methods for comprehensive evaluation and quick scan In: Bigler F, Babendreier D, Kuhlmann U (eds.) Environmental impact of invertebrates for biological control of arthropods: methods and risk assessment. CABI Publishing, Wallingford, pp. 254-272.

Vinson SB (1981) Habitat location. Semiochemicals: their role in pest control.

Wermelinger B, Wyniger D, Forster B (2008) First records of an invasive bug in Europe: *Halyomorpha halys* Stal (Heteroptera: Pentatomidae), a new pest on woody ornamentals and fruit trees? Mitteilungen der Schweizerischen Entomologischen Gesellschaft 81, 1-8.

Weseloh RM (1981) Host location by parasitoids. In: Nordlund, D.A.: Semiochemicals: their role in pest control, pp.79–95.

Weseloh RM, Anderson JF (1975) Inundative release of *Apanteles melanoscelus* against the gypsy moth. Environmental Entomology 4, 33-36.

6 A molecular tool to identify *Anastatus* parasitoids of the brown marmorated stink bug

Submitted to: Entomologia Experimentalis et Applicata, Special Issue Next Generation Biocontrol

Judith M. Stahl[1,2]*, Tara D. Gariepy[3], Leo W. Beukeboom[4], Tim Haye[1]

Affiliations:
[1]CABI, Rue des Grillons 1, 2800 Delémont, Switzerland,

[2]Institute of Ecology and Evolutionary Biology, University of Bremen, Leobener Str. NW2, 28359 Bremen, Germany,

[3]London Research and Development Centre, Agriculture and Agri-Food Canada, 1391 Sandford Street, London, Ontario N5V 4T3, Canada,

[4]Groningen Institute for Evolutionary Life Sciences, University of Groningen, Post Office Box 11103, 9700 CC, Groningen, The Netherlands

Abstract

Globally, *Anastatus* Motschulsky (Hymenoptera: Eupelmidae) species are associated with the invasive agricultural pest *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae). In Europe, the polyphagous *A. bifasciatus* (Geoffroy) (Hymenoptera: Eupelmidae) is the most prevalent native egg parasitoid on *H. halys* eggs and is currently being tested as a candidate for augmentative biological control. *Anastatus bifasciatus* frequently displays oviposition behavior without oviposition, and induces additional host mortality through host stinging and host feeding that is not with offspring emergence. This exacerbates accurate assessment of parasitism and host impact, which is crucial for efficacy evaluation as well as for pre- and post-release risk assessment. To address this, a general *Anastatus* primer set amplifying a 318-bp fragment within the barcoding region of the cytochrome oxidase I (COI) gene was developed. When challenged with DNA of three *Anastatus* species (*A. bifasciatus*, *A. japonicus*, *Anastatus* sp.), five scelionid parasitoid species that might be encountered in the same host environments and eleven pentatomid host species, only *Anastatus* DNA was successfully amplified. When applied to eggs of the target host *H. halys* and an exemplary non-target host, *Dendrolimus pini* L. (Lepidoptera: Lasiocampiae), subjected to host feeding no *Anastatus* amplicons were produced. Eggs of the two host species containing different parasitoid stages from 1 h old *A. bifasciatus* eggs to pupae, and emerged eggs, yielded *Anastatus* fragments. Confirmation of parasitoid presence with dissections and subsequent PCRs resulted in 95% success for 1 h old parasitoid eggs. For both host species, field-exposed sentinel emerged eggs stored dry for 6 months, 100% of the specimens produced Anastatus amplicons. This DNA-based screening method can be used in combination with conventional methods to better interpret host-parasitoid and parasitoid-parasitoid interactions. It will help address ecological questions related to an environmentally-friendly approach for the control of *H. halys* in invaded areas.

6.1 Introduction

In the field of biological control, molecular diagnostic tools are useful for evaluating population genetics of natural enemies, assessing non-target effects, and accurately identifying biocontrol agents (Bigler et al. 2005, Gariepy et al. 2007). These tools may supplement traditional methods of species identification that, for parasitoids, mainly consist of rearing the natural enemies and dissecting their hosts.

Of recent interest globally, is the brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), and the potential to use parasitoids in a biological control strategy for this pest. *Halyomorpha halys* is a polyphagous species of Asian origin that has become highly invasive in North America and Europe following accidental introduction in the mid-1990s and around 2004, respectively (Hoebeke and Carter, 2003, Wermelinger et al. 2008, Haye et al. 2014a, b). A more recent introduction occurred in 2017 in South America (Faúndez and Rider 2017). In North America and Europe, *H. halys* has caused tremendous damage as an agricultural pest on a number of economically important host plants, including apple, pear, peach, hazelnut, and corn (Leskey et al. 2012, Bosco et al. 2017, Maistrello et al. 2017).

In its native range, *H. halys* is attacked by a complex of scelionid and eupelmid egg parasitoids, with *Trissolcus japonicus* Ashmead (Hymenoptera: Scelionidae) as the most prevalent species (Yang et al. 2009, Zhang et al. 2017). In Europe, native scelionid species typically associated with other pentatomids attempt to attack *H. halys* eggs, but are not able to complete development (Haye et al. 2015). As such, European *Trissolcus* and *Telenomus* species are not promising candidate agents for biological control of *H. halys*. In contrast, another European egg parasitoid, *Anastatus bifasciatus* (Geoffroy) (Hymenoptera: Eupelmidae), is the species most commonly found parasitizing *H. halys* eggs, and can complete its development on live *H. halys* eggs. This polyphagous, solitary species has been reared from sentinel and natural field-collected *H. halys* eggs in several European countries (Haye et al. 2015, Costi et al. 2018) and is currently being tested as a biological control agent in the field in Switzerland and Italy. Natural field parasitism assessed by rearing is generally quite low (e.g. Costi et al. 2018). However, *A. bifasciatus* induces additional host mortality through oviposition damage and host feeding, which in fact is equivalent to host mortality related to offspring production (Stahl et al. 2018a). In addition to *H. halys*, *A. bifasciatus* successfully parasitizes eggs of other European Heteroptera and Lepidoptera, including several species of conservational concern (Noyes 2014, Stahl et al. 2018b).

Accurate assessment of parasitism of *H. halys* in the field can be difficult. First, species-level identification of the host eggs is challenging, as there are few distinguishing morphological characters in the egg stage (Esselbaugh 1946, Bundy and McPherson 2000, Gariepy et al. 2013). Usually, recovered egg masses are found when nymphs have already hatched, which further exacerbates identification efforts. Identifying the associated egg parasitoids after their emergence is similarly challenging. There are species identification methods described for parasitoids of Heteroptera, based on exit holes and morphology of the frass the parasitoid larva has left behind in the egg (Viggiani and Mineo 1970), but the applicability and reliability of those methods in multispecies systems could be questioned. Even if the parasitoid was still present inside the host egg, preimaginal mortality can prevent identification via rearing and even dissections are of little use once the parasitoids have disintegrated. To overcome some of these difficulties, a modified DNA barcode approach was developed with general primers for Pentatomidae and Scelionidae in order to evaluate host-parasitoid associations in this system (Gariepy et al. 2013, 2018).

For risk assessment and to evaluate biocontrol efficacy of *A. bifasciatus* a molecular approach using PCR primers that can detect the presence of *A. bifasciatus* DNA within a host would greatly improve the ability to rapidly and accurately identify *A. bifasciatus* in *H. halys* and other potential hosts, and would provide a more accurate estimate of parasitism level. Once designed, newly-developed primers need to be tested in terms of their specificity and sensitivity: if the primers are not specific enough, they can amplify each other, or DNA from species other than the one they were designed for, both of which would lead to false positives (Admassu et al. 2006). Since hosts are normally associated with a limited number of parasitoids, primer specificity testing can be restricted to species closely related to the parasitoid the primers were designed for and species that parasitize closely related hosts (Gariepy et al. 2007). The sensitivity of a primer set refers to which stages of the parasitoid can be detected. It is important to ensure that all developmental stages are detectable, to prevent an underestimation of parasitism level due to false negatives. To gain more information regarding host-parasitoid associations in this system, a set of general PCR primers nested within the DNA barcode regions were developed for *Anastatus*, and evaluated in terms of specificity, sensitivity and applicability to field samples using the target host *H. halys* and an exemplary non-target host.

6.2 Material and Methods

6.2.1 Primer design

Publically-available DNA sequences for *Anastatus* species associated with pentatomid eggs (generated as part of another research project; Gariepy, unpublished) were retrieved from the DNA barcode of life datasystems (BOLD), project NASTA. These included 3 species, *A. japonicus* Ashmead (BIN ACE0140) collected from fresh *H. halys* egg masses in Asia (37 specimens), *A. bifasciatus* (BIN ACX9265) collected from sentinel *H. halys* egg masses in Europe (3 specimens), and *Anastatus* sp. (BIN ACP8173) collected from an unidentified pentatomid egg mass in Canada (10 specimens). The 652-bp COI sequences for *Anastatus* were aligned using Clustal W algorithm (Thompson et al. 1994) in Codon Code Aligner (v 4.0.4). Visual inspection of regions of DNA sequence similarity were investigated to design primers nested within the DNA barcode region that would amplify all three species of *Anastatus*. Further, *Anastatus* DNA sequences were aligned with representative members of the Scelionidae (BOLD project NSCEL) and Pentatomidae (BOLD project HCNC) to ensure adequate sequence variation existed within these regions to prevent non-specific amplification of the Pentatomidae and the Scelionidae, as one or more of these species could be present in mixed DNA samples extracted from host eggs.

6.2.2 Primer specificity

The specificity of the primer pair (Ana-361F and HCO-2198) was challenged in separate PCR reactions with DNA from *A. bifasciatus* (n = 11), *A. japonicus* (n = 17), and *Anastatus* sp. (n = 6), as well as with DNA of 11 scelionid parasitoids (*Telenomus chloropus* (Thomson), n = 10; *Trissolcus semistriatus* (Nees), n = 10; *Trissolcus japonicus*, n = 10; *Telenomus podisi* Ashmead, n = 11; *Trissolcus euschisti* (Ashmead), n = 4), and the pentatomid hosts (*Acrosternum hilare* Say, n = 3; *Banasa dimidiate* (Say), n = 2; *Brochymena quadripustulatus* (Fabricius), n = 3; *Cosmopepla bimaculate* (Thomas), n = 2, *Dolycoris baccarum* (L.), n = 2; *Euschistus variolarius* (Palisot), n = 2; *Halyomorpha halys*, n = 8, *Nezara viridula* (L.), n = 2; *Podisus maculiventris* (Say), n = 3; *Rhaphigaster nebulosi* (Poda), n = 3; and *Thyanta acerra* McAtee, n = 2). Amplification of DNA was performed in an Eppendorf Mastercycler Pro PCRs in a 25 µL volume containing 0.125 µL of Taq Platinum, 2.5 µL of 10x PCR buffer, 1.25 µL of 50 mM $MgCl_2$, 0.125

µL of 10 µM dNTPs (Invitrogen), 0.25 µL of 10 µM Ana-361F (5’-ATCACATAGGGGTCCTTCAGTA-3’), 0.25 µL of 10 µM HCO2198 (5’-TAAACTTCAGGGTGACCAAAAAATCA-3’) (Folmer et al. 1994), 19.5 µL ddH_20, and 1 µL of template DNA. Thermocycling conditions were: initial denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 40 sec, 58°C for 40 sec, and 72°C for 1 min, and a final extension period of 5 min at 72°C.

6.2.3 Primer sensitivity

The ability of the molecular markers to detect trace amounts of parasitoid DNA was tested in time trials to determine how soon parasitoid DNA could be detected following oviposition, which developmental stages of the egg parasitoid could be detected, and whether *A. bifasciatus* DNA can be detected in host eggs following emergence (based on trace levels of DNA present in parasitoid frass left in the egg). Since *A. bifasciatus* regularly displays host feeding and can display oviposition behavior without egg deposition, host mortality caused by these behaviors are of interest in the assessment of this species as a biological control agent. However, they cannot be measured by offspring emergence or with identification of punctured host eggs, particularly in field-collected samples. Therefore, the sensitivity of the primers was tested on *H. halys* eggs that had been probed and subjected to host feeding.

All tests apart from the host feeding treatment were conducted both with the hosts *H. halys* and *Dendrolimus pini* L. (Lepidoptera: Lasiocampidae), a non-target species recently shown to be a suitable host for *A. bifasciatus*. As a negative control, fresh (< 24 h), unparasitized *H. halys* and *D. pini* eggs were used.

To obtain different stages of the parasitoid, fresh host eggs (< 24 h) were exposed at 26°C to randomly selected *A. bifasciatus* females in 54 x 14 mm Petri dish arenas. Oviposition behavior was observed with a ‘Leica MS5’ microscope (Leica Biosystems Nussloch GmbH, Nussloch, Germany), drilled eggs were separated and stored for different periods at 26°C to allow the parasitoids to stay in the egg phase (1 h) or develop into an early instar larva (3 d), late instar larva (7 d) and the pupal stage (21 d). After the selected time periods the eggs were transferred to 2 ml Sarstedt tubes filled with 95% ethanol. For the probed and host-fed *H. halys* eggs, the parasitoid females were removed from the eggs after observed ovipositor insertion and the end of the first host feeding event and the eggs immediately transferred into ethanol. Each treatment was repeated 20 times.

To correct for parasitization behavior without oviposition, stored *H. halys* eggs were visually and non-invasively examined for *A. bifasciatus* presence after 7 and 21 days. Only those host eggs containing parasitoid larvae and pupae, respectively, were transferred into ethanol. The presence of earlier developmental stages (as well as all stages in *D. pini*) can barely be ascertained in a non-invasive way, so for these categories all host eggs were placed in ethanol. As a control for the presence of *A. bifasciatus*, additional potentially parasitized *H. halys* and *D. pini* eggs placed into ethanol 1 h (egg stage), 12 h (egg stage), 24 h (egg stage), 3 d, 7 d, and 21 d after observed oviposition behavior were dissected. When *A. bifasciatus* presence was confirmed by dissection, the remains of the dissected host and parasitoid were subjected to the same molecular procedures as described below.

In addition to the host eggs containing premature *A. bifasciatus* stages, empty eggs were transferred to ethanol < 24 h after parasitoid emergence to determine the ability to detect parasitoid DNA from empty eggs.

6.2.4 Application for field-collected samples

Since one of the most important applications of the developed molecular marker is the identification of *Anastatus* parasitoids from field-collected samples, field-exposed sentinel eggs of *H. halys* and *D. pini* were stored for approximately 6 months at 26°C and subjected to the same DNA extraction and amplification procedures as the time trial samples. This included empty eggs (from which *Anastatus* had already emerged), as well as unemerged eggs from the same egg masses that yielded *Anastatus* adults.

6.2.5 Molecular analysis

DNA was extracted with a high salt protocol with 6 M NaCl and pure chloroform. Samples were transferred to 1.5 ml Eppendorf vials individually with a clean, sterile paint brush after which 50 µl of digestion buffer (100 mM NaCl, 10 mM Tris:HCL pH 8.0 and 25 mM EDTA) was added. The material was shredded with a pellet mixer (VWR International, Leicestershire, UK) for approximately 1-2 min until homogenized. The paint brush and mixer tip were washed in diluted bleach solution, 70% ethanol and water between different samples to avoid contamination. 50 µl of a mixture of digestion buffer (48 µL) and proteinase K (0.2 mg/ml; QIAGEN GmbH, Hilden, Germany) (2 µL) was added to the homogenate, and vials were incubated for 2 – 5 h in a 56°C shaking water bath (1083, GFL Gesellschaft für Labortechnik mbH, Burgwedel, Germany) after which 40 µl 6M NaCl and 140 µl 100% chloroform was added and the samples mixed for 20 min with a horizontal shaker (HS 501 digital, IKA Labortechnik, Staufen, Germany). After 5 min of centrifuging at 15,000 rpm, the supernatant water phase with the isolated nucleic acids was transferred into a new 1.5 ml Eppendorf tube. 140 µl of 100% isopropanol was added to precipitate the DNA as well as 2 µl 15 mg/mL GlycoBlue (Thermo Scientific, Waltham, USA) to stain the forming pellet. The mixture was incubated for 5 min on the shaker and then centrifuged for 20 min at 15,000 rpm. Subsequently, the supernatant was discarded, and the remaining pellet washed three times with 150 µl of 70% ethanol (centrifuged for 5 min at 15,000 rpm and liquid discarded), after which the DNA pellets were vacuum dried (Concentrator 5301, Eppendorf, Hamburg, Germany) for 1–2 minutes at 45°C until all remaining liquid had evaporated. The DNA was resuspended in 20 µl Milli-Q water.

For the PCR, a 25 µl reaction volume was prepared with 2.5 µl 10x reaction buffer + Mg (Roche Diagnostics GmbH, Mannheim, Germany), 0.125 µl 10 mM dNTP mix (Fermentas, Waltham, USA), 0.25 µl 10 µM Ana-361F forward primer (5’-ATCACATAGGGGTCCTTCAGTA-3’) and 0.25 µl 10 µM HCO-2198 reverse primer (5’-TAAACTTCAGGGTGACCAAAAAATCA-3’), 20.75 µl ddH_2O, 0.125 µl Taq DNA polymerase (Roche Holding AG, Basel, Switzerland) and 1 µl template DNA. The PCR was run in an Applied Biosystems® Veriti® 96-Well Thermal Cycler (Thermo Scientific) with initial denaturation at 94°C for 1 min, 40 cycles of 40 s annealing at 58°C and 1 min extension at 72°C, followed by 5 min of final extension at 72°C.

PCR products were visualized with gel electrophoresis, 5 µl of template DNA was blended with 3 µl 6x DNA Gel Loading Dye (Thermo Scientific) and run on a 2% agarose gel with 1xTAE buffer and 0.05 µl/ml Ethidium bromide next to 1 µl 0.1 µg/µl flanking ladder GeneRuler 100 bp Plus (Thermo Scientific). The results were visualized with a GeneFlash Bio Imaging Gel Documentation System UV/VVIS Gene Flash (Syngene International Pvt Ltd., Bangalore, India).

6.2.6 Statistical analyses

The number of samples in which *Anastatus* DNA was detected was compared for time trials and field samples with a Generalized Linear Model (GLM) of the binomial distribution using the logit link function, with detection as the dichotomous response variable, and the hosts *H. halys* and *D. pini* as independent variable for each time interval/treatment, using R version 3.2.3 (Team 2014) and the development environment RStudio (Team 2017).

6.3 Results

6.3.1 Primer design

Alignment of the DNA barcode region of the COI gene from public sequences available on BOLD allowed the identification of short nucleotide sequences conserved within the genus *Anastatus*, but with sufficient variation to permit exclusion of members of the family Pentatomidae and Scelionidae. This resulted in the development of a unique forward primer, Ana-361F (5'-ATCACATAGGGGTCCTTCAGTA -3') that when used in combination with the universal reverse primer HCO-2198 (Folmer et al. 1994), yields a 318-bp PCR fragment for *Anastatus* spp.

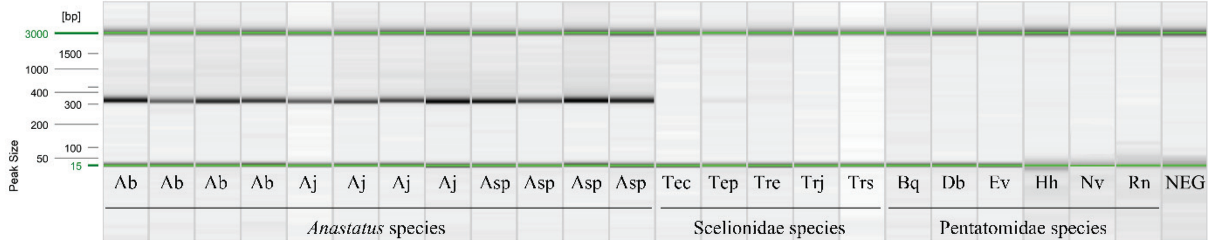

Figure 1 Specificity of Anastatus PCR primers (Ana-361F / HCO2198) when challenged with DNA from *Anastatus bifasciatus* (Ab), *Anastatus japonicus* (Aj), and *Anastatus* sp. (Asp), as well as several scelionid parasitoid species (*Telenomus chloropus*, Tec; *Telenomus podisi*, Tep; *Trissolcus euschisti*, Tre; *Trissolcus japonicus*, Trj; and *Trissolcus semistriatus*, Trs) and pentatomid species (*Brochymena quadripustulata*, Bq; *Dolycoris baccarum*, DB; *Euschistus variolarius*, Ev; *Halyomorpha halys*, Hh; *Nezara viridula*, Nv; and *Rhaphigaster nebulosa*, Rn)

6.3.2 Primer specificity

When challenged with DNA from different *Anastatus* species, the primer combination Ana-361F and HCO-2198 successfully amplified an approximately 320-bp fragment for all *Anastatus* specimens collected from Europe (*A. bifasciatus*, n = 11), China (*A. japonicus*, n = 17), and Canada (*Anastatus* sp., n = 6); whereas no DNA amplification was observed for specimens of Scelionidae and Pentatomidae (Figure 1). No PCR amplicons were observed for *D. pini*, the representative lepidopteran host used in subsequent experiments (data not shown).

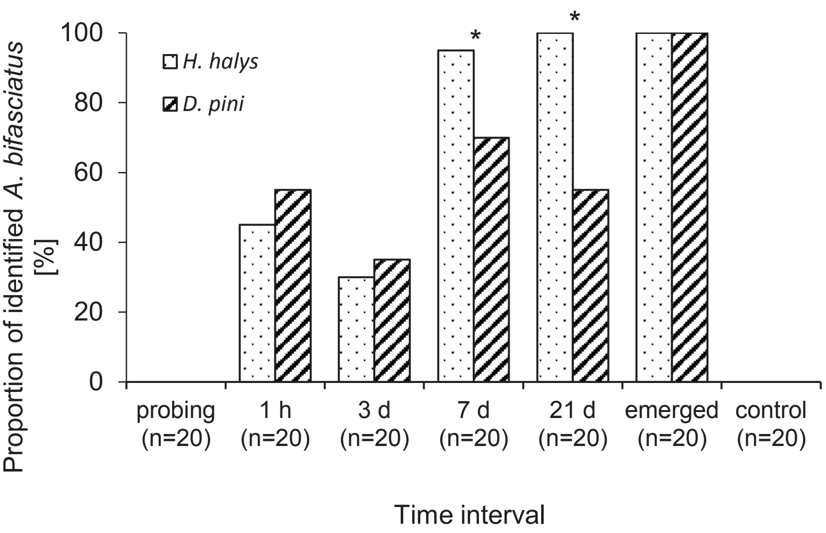

Figure *2* Proportion of detected parasitoid DNA in the different time intervals with the hosts *H. halys* and *D. pini*; specimens placed in alcohol directly after probing, but before oviposition (probing); samples with a development time of 1 h (egg), 3 d (early larva), 7 d (late instar larva) and 21 d (pupa) as well as those stored in ethanol < 24 h after adult parasitoid emergence (emerged), and unparasitized host eggs (control). For each treatment the number of host eggs tested is depicted as n in brackets. Asterisks show significant differences of pairwise comparisons between host species for each time interval after binomial GLM (link = logit; $\alpha < 0.05$).

6.3.3 Primer sensitivity

45% of *H. halys* and 55% of *D. pini* eggs placed in alcohol 1 h after observed parasitization behavior yielded *Anastatus*-specific products (Figure 2). In specimens transferred to ethanol after 3 d, 30% and 35% of *H. halys* and *D. pini*, respectively, were positive for *Anastatus* DNA (Figure 2). For both time intervals the values were not significantly different between host species (binomial GLM, df = 1, 38, $z = -0.631$, $p = 0.527$ for 1 h and df = 1, 38, $z = -0.337$, $p = 0.736$ for 3 d). After 7 d and 21 d intervals, *Anastatus* was more frequently detected in *H. halys* samples (95% and 100%) than in *D. pini* samples (70% and 55%) (binomial GLM, df = 1, 38, $z = 1.846$, $p = 0.0297$ for 7 d and df = 1, 38, $z = 0.008$, $p < 0.001$ for 21 d). For both host species, 100% of the tested samples transferred into alcohol < 24 h after adult *A. bifasciatus* emergence produced *Anastatus* amplicons (Figure 2).

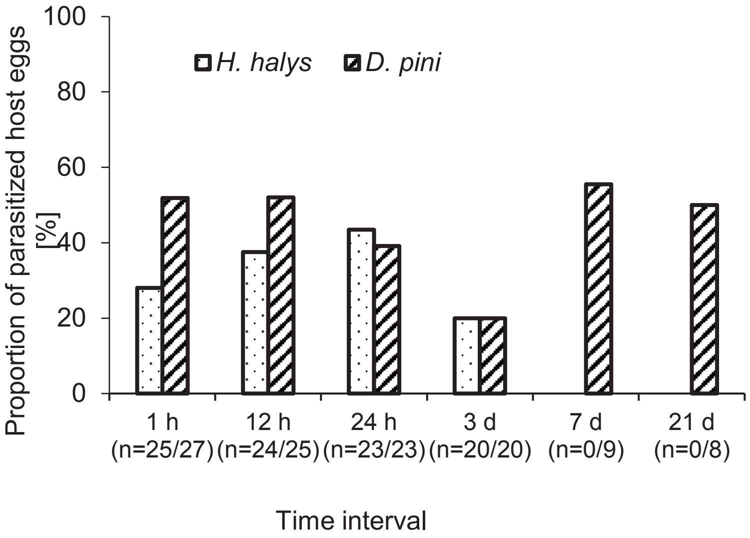

Figure *3* Proportion of dissected eggs containing *A. bifasciatus* in the different time intervals with the hosts *H. halys* and *D. pini*; samples with a development time of 1 h (egg), 12 h (egg) and 24 h (egg), 3 d (early instar larva), 7 d (late instar larva) and 21 d (pupa), and unparasitized host eggs (control). For each treatment the number of host eggs tested is depicted as n in brackets.

Dissection of eggs drilled by *A. bifasciatus* (from the same batch as those from which DNA was extracted and tested with the *Anastatus*-specific primers) revealed that 20–56% of the *H. halys* and *D. pini* eggs contained the expected *A. bifasciatus* stages (Figure 3). Upon testing only those eggs that were found to contain *A. bifasciatus*, DNA amplification resulted in 89% (24 h old eggs) to 100% (early instar larvae, late instar larvae, pupae) detection of *Anastatus* (Figure 4).

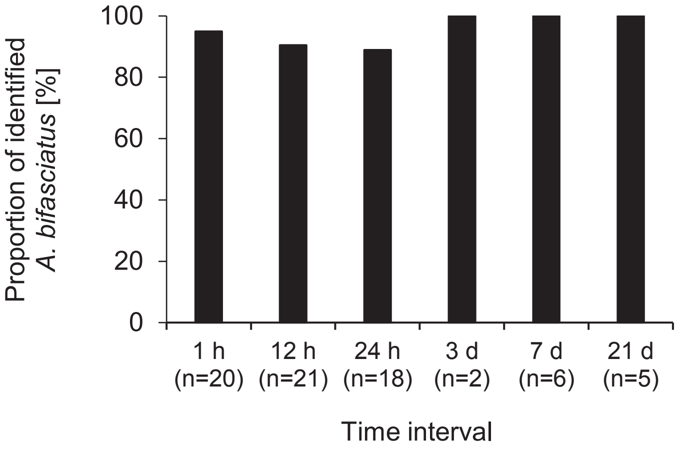

Figure *4* Proportion of detected parasitoid DNA of the dissected parasitoid stages; samples with a development time of 1 h (egg), 12 h (egg) and 24 h (egg), 3 d (early instar larva), 7 d (late instar larva) and 21 d (pupa). For each treatment the number of host eggs tested can be found as n in brackets.

6.3.4 Application to field-collected samples

In field-exposed sentinel eggs belonging to both host species, parasitoid DNA was detected in empty eggs following parasitoid emergence approximately 5 – 6 months prior to preservation in ethanol for subsequent molecular analysis. For both host species, 100% of the specimens (n = 20 for *H. halys* and n = 20 for *D. pini*) produced *Anastatus* amplicons (Figure 5). In contrast, *Anastatus* was detected in 15% of unemerged *H. halys* eggs and 58% of unemerged *D. pini* eggs collected from the same field-exposed sentinel egg masses that had also yielded *Anastatus* adults (Figure 4). There were no significant differences between the detection levels in the two hosts (binomial GLM, df = 1, 47, *z* = -1.92, *p* = 0.0519).

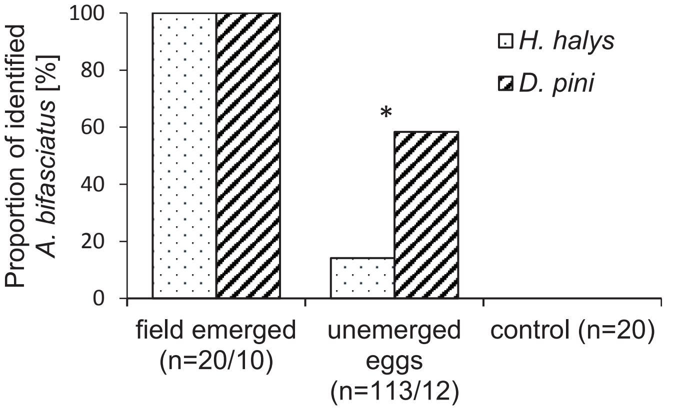

Figure *5* Proportion of detected parasitoid DNA in the different treatments of the field-collected samples with *H. halys* and *D. pini* as hosts: specimens stored in ethanol up to 6 months after adult parasitoid emergence (field emerged), eggs from the same egg masses that presumably contained dead *A. bifasciatus* (unemerged eggs) and unparasitized host eggs (control). Sample sizes are depicted as n in brackets.

6.4 Discussion

In the present study, a set of PCR primers capable of detecting *Anastatus* species DNA without non-target amplification of scelionid, pentatomid, and Lepidoptera DNA is reported. Although these primers are capable of amplifying the three *Anastatus* species from the present study, their broad applicability to other members with the same genus and/or in the Eupelmidae from other host-parasitoid systems is unknown and should be tested. However, in the current context, the number of species interactions that may occur within the present host-parasitoid system are limited and therefore the ability of the primers to separate *Anastatus* DNA from pentatomid and scelionid DNA is sufficient for the research questions addressed here.

A total of 57% of host eggs that potentially contained different stages of *A. bifasciatus* (egg, larva, pupa, post-emergence empty egg) produced *Anastatus* amplicons. In contrast, 95% of host eggs that were dissected 1 h post-parasitism and subjected to molecular analysis (following confirmation of the presence of a parasitoid egg) were positive for *Anastatus* DNA. This suggests that the molecular tool is sensitive enough to consistently detect parasitoid eggs, and the comparatively low detection level (57%) in the time trial series was largely the result of parasitoid probing behavior without oviposition. Successful oviposition by *A. bifasciatus* cannot be evaluated by visual inspection of the egg, and although probing of the egg can be observed, roughly only half of the eggs are used for oviposition, whereas the other half are exclusively used for host feeding (Konopka et al. 2017, Stahl et al. 2018a).

Recently, trophic interactions between Pentatomidae hosts, including *H. halys*, and their scelionid parasitoids were investigated in North America with a modified DNA barcode approach (Gariepy et al. 2018). Given that the *Anastatus* primers are also within the DNA barcoding region, they could potentially be used in combination with the primer pair for Scelionidae (SCEL: SCEL-F1/HCO-2198) (Gariepy et al. 2013) to assess multispecies interactions in the Pentatomidae. However, in order to determine species-level interactions, DNA sequencing of both scelionid and *Anastatus* amplicons would be required for species-level resolution (as was done for scelionids and pentatomids in Gariepy et al. 2018). This type of DNA sequencing approach allows broad applicability of the technique, regardless of geography, as the same primers amplify *Anastatus* from North America, Europe, and Asia, and the DNA barcode library for these species (BOLD Project NASTA) would allow species separation based on single nucleotide polymorphisms in the DNA barcode region. An improved understanding of species interactions is of particular interest, as *T. japonicus* has recently been discovered in Europe, and is established in Switzerland in locations where *A. bifasciatus* is frequently found (Stahl et al. 2018c). Laboratory studies on interactions between *T. japonicus* and *A. bifasciatus* suggest the potential for coexistence, but field data from Europe are needed to confirm this, and to determine whether the two species will compete for target and non-target host resources (Yang et al. 2009, Qiu et al. 2010, Konopka et al. 2017).

Traditionally, parasitism level is evaluated by rearing and dissection of host material; however, identification of immature stages of the host and parasitoid to the species level can be difficult, and in some cases impossible due to a lack of taxonomic expertise and/or lack of morphologically-distinguishing characteristics in the immature stages. This is particularly problematic in multispecies systems, where several closely-related taxa may be encountered. However, even in a simpler system that contains only one host and one parasitoid species, a molecular approach is valuable in quantifying parasitism level and monitoring movement and spread of a biocontrol agent following release. *Anastatus bifasciatus* is currently being investigated as a potential augmentative biocontrol agent for *H. halys* in Europe, and efficacy evaluations are a crucial part of the process (Haye et al. 2015, Stahl et al. 2018a). Rearing and dissections often yield different results due to preimaginal mortality of the parasitoids (Day 1994, Gariepy et al. 2008). Recent studies indicate that preimaginal mortality of *A. bifasciatus* in *H. halys* is indeed a variable that should not be neglected (Stahl et al. 2018a). However, accurate measurements with dissections are often exacerbated by the possibility that parasitoid development has not yet reached a detectable stage, or that dead parasitoids have decayed beyond the ability to detect or identify them (Ratcliffe et al. 2002). A large-scale comparison of parasitism estimates based on dissection, rearing, and molecular techniques in another host-parasitoid system has shown that molecular techniques provide more accurate estimates of parasitism and parasitoid species composition, whereas rearing and dissection tend to underestimate parasitoid-induced mortality (Gariepy et al. 2008). In the present system, parasitoid-induced mortality can be from oviposition and it can also it can be inflicted from host feeding, which occurs either with or without oviposition. Host mortality due exclusively to parasitoid feeding events cannot be detected by dissection (Cebolla et al. 2018, Day 1994), nor can it be detected with the *Anastatus* primers described here. Although we assume that minute traces of parasitoid DNA would be transferred to the host egg during probing (e.g., calyx fluid) or feeding (e.g., saliva), these are not detectable using the protocol developed here. Perhaps further refinement of the protocol to increase detection sensitivity will allow minute quantities of DNA to be detected.

Detection of parasitoid DNA within a host does not necessarily indicate parasitoid survival and associated host mortality; however, it does indicate that attack of a given host has occurred. This is extremely valuable as a non-target risk-assessment tool, as detection of parasitoid DNA clearly demonstrates the potential of a parasitoid to use a given host species as a resource (Gariepy et al. 2008). *Anastatus bifasciatus* is known to be an extremely polyphagous parasitoid that attacks and develops in a variety of Hemiptera and Lepidoptera, some of which are of conservational concern (Noyes 2014, Stahl et al. 2018b). With this molecular tool, recovered eggs from the field can be screened for the presence or absence of *Anastatus* species, and trace amounts of DNA can even be detected following parasitoid emergence. This forensic-style approach (screening of empty egg masses for traces of parasitoid DNA) can provide valuable information regarding the ecological host range and impact of a parasitoid on non-target species, which is often a decisive factor in pre- and post-release assessments of biocontrol agents (Gariepy et al. 2008, 2018). In the present study, field-exposed sentinel egg masses which had been stored for several months at 26°C nonetheless yielded *Anastatus* PCR products, despite the fact that storage conditions were less than ideal in terms of preserving DNA and preventing degradation. Although prolonged exposure under field conditions may result in quicker degradation of DNA, this clearly demonstrates that the *Anastatus* primers are capable of detecting DNA from dry, decaying eggs from emerged and unemerged eggs. As the PCR primers amplify a fairly short fragment of the COI gene (~320-bp), it can be considered a “mini-barcode”, which is more likely to be detectable for longer periods of time in comparison to longer fragments (Hajibabaei and McKenna 2012).

The availability of a molecular tool to detect the presence of *A. bifasciatus* is also very useful in laboratory studies. Oviposition behavior in *A. bifasciatus* may or may not result in the insertion of an egg within a host, and there is no way to determine whether oviposition was successful based on observation or visual inspection of the probed host egg. As such, results from laboratory experiments can be difficult to interpret, as they rely primarily on offspring emergence, which can take several weeks under regular conditions and several months if diapause has been induced, during which time the host and/or parasitoid may be subjected to increased mortality (Konopka et al. 2017, Stahl et al. 2018a). This delay between observed attack and confirmation of parasitism is time consuming and may not provide accurate results based on mortality experienced in rearing. In contrast, molecular tools can provide a rapid, accurate assessment of parasitism within < 24 h following observed oviposition. However, it is important to note that molecular detection of parasitism does not necessarily reflect successful parasitism, and molecular assessment requires destructive sampling, which therefore prevents the measurement of successful parasitoid development within a given host species. However, a combination of rearing and molecular analysis of separate samples would facilitate a more accurate representation of parasitism events, and an indication of whether successful development occurs.

The utility of the *Anastatus* primers as a molecular tool in both field and laboratory studies will permit the evaluation of a promising biological control agent for invasive *H. halys*, and will greatly facilitate pre- and post-release studies on *A. bifasciatus* in Europe. In addition, the fact that these primers amplify at least 3 species of *Anastatus* associated with the Pentatomidae provides additional flexibility in their application in other geographic areas where these species exist and where there may be interest in evaluating their biological control potential for *H. halys*. Although the current application of the primers is to detect presence/absence of *Anastatus*, their utility can be expanded by implementing them in a DNA barcoding approach. DNA sequencing of the resulting 320-bp PCR product, and comparison of sequences with publicly-available DNA barcodes (BOLD, Project NASTA) would allow species separation, which may be of interest in locations areas where multiple *Anastatus* species co-occur with overlapping host range. Overall, the development and application of such a tool will help address ecological questions related to a reduced-risk, environmentally-friendly approach for the control of *H. halys* in invaded areas, and can be used in combination with conventional methods to better interpret host-parasitoid and parasitoid-parasitoid interactions.

6.5 Acknowledgements

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 641456. The authors are grateful to Serge Fischer (Agroscope Changins-Wädenswil, Switzerland) for providing the original *A. bifasciatus* colony. We would like to thank Dalinda Baroui, Anna Rensink, Elzemiek Geuverink, Kelley Leung, and Marcela Buricova for advice and assistance in the laboratory. We appreciate the taxonomic guidance offered by Lucian Fusu (University of Iasi, Romania). We gratefully acknowledge the funding provided for this research by the following organizations and agencies: CABI is an international intergovernmental organisation, and we gratefully acknowledge the core financial support from our member countries (and lead agencies) including the United Kingdom (Department for International Development), China (Chinese Ministry of Agriculture), Australia (Australian Centre for International Agricultural Research), Canada (Agriculture and Agri-Food Canada), Netherlands (Directorate-General for International Cooperation), and Switzerland (Swiss Agency for Development and Cooperation). See http://www.cabi.org/about-cabi/who-we-work-with/key-donors/ for full details.

6.6 References

Admassu B, Juen A, Traugott M (2006) Earthworm primers for DNA-based gut content analysis and their cross-reactivity in a multi-species system. Soil Biology and Biochemistry, 38, 1308-1315.

Bigler F, Bale JS, Cock MJW, Dreyer H, Greatrex R, Kuhlmann U, Loomans AJM, van Lenteren JC (2005) Guidelines on information requirements for import and release of invertebrate biological control agents in European countries. Biocontrol News and Information, 26, 115N-123N.

Bosco L, Moraglio ST, Tavella L (2017) *Halyomorpha halys*, a serious threat for hazelnut in newly invaded areas. Journal of Pest Science 91, 661-670.

Bundy CS, McPherson RM (2014) Morphological examination of stink bug (Heteroptera: Pentatomidae) eggs on cotton and soybeans, with a key to genera. Annals of the Entomological Society of America, 93, 616-624.

Cebolla R, Vanaclocha P, Urbaneja A, Tena A. (2018) Overstinging by hymenopteran parasitoids causes mutilation and surplus killing of hosts. Journal of Pest Science, 91, 327-339.

Costi E, Haye T, Maistrello L (2018) Surveying native egg parasitoids and predators of the invasive *Halyomorpha halys* in Northern Italy. Submitted to Journal of Applied Entomology.

Day WH (1994) Estimating mortality caused by parasites and diseases of insects: comparisons of the dissection and rearing methods. Environmental Entomology, 23, 543-550.

Esselbaugh CO (1946) A study of the eggs of the Pentatomidae (Hemiptera). Annals of the Entomological Society of America, 39, 667-691.

Faúndez EI, Rider DA (2017) The brown marmorated stink bug *Halyomorpha halys* (Stål, 1855) (Heteroptera: Pentatomidae) in Chile. Arquivos Entomolóxicos, 17, 305-307.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology, 3, 294–299.

Gariepy TD, Kuhlmann U, Gillott C, Erlandson M (2007) Parasitoids, predators and PCR: the use of diagnostic molecular markers in biological control of Arthropods. Journal of Applied Entomology, 131, 225-240.

Gariepy TD, Kuhlmann U, Gillott C, Erlandson M (2008) A large-scale comparison of conventional and molecular methods for the evaluation of host–parasitoid associations in non-target risk-assessment studies. Journal of Applied Ecology, 45, 708-715.

Gariepy TD, Haye T, Zhang J (2014) A molecular diagnostic tool for the preliminary assessment of host–parasitoid associations in biological control programmes for a new invasive pest. Molecular Ecology, 23, 3912-3924.

Gariepy, TD, Bruin, A, Konopka, J, Scott-Dupree, C, Fraser, H, Bon, MC, Talamas, E (2018) A modified DNA barcode approach to define trophic interactions between native and exotic pentatomids and their parasitoids. Molecular Ecology. https://doi.org/10.1111/mec.14868.

Hajibabaei M, McKenna C (2012) DNA mini-barcodes. DNA barcodes. Humana Press, Totowa, NJ, pp. 339-353.

Haye T, Abdallah S, Gariepy TD, Wyniger D (2014a) Phenology, life table analysis and temperature requirements of the invasive brown marmorated stink bug, *Halyomorpha halys*, in Europe. Journal of Pest Science, 87, 407-418.

Haye T, Wyniger D, Gariepy TD (2014b) Recent range expansion of brown marmorated stink bug in Europe. In Proceedings of the 8th International Conference on Urban Pests, 20-23 July 2014, Zurich, Switzerland (pp. 309-314). Executive Committee of the International Conference on Urban Pests.

Haye T, Fischer S, Zhang J, Gariepy TD (2015) Can native egg parasitoids adopt the invasive brown marmorated stink bug, *Halyomorpha halys* (Heteroptera: Pentatomidae), in Europe? Journal of Pest Science, 88, 693-705.

Hoebeke ER, Carter ME (2003) *Halyomorpha halys* (Stål) (Heteroptera: Pentatomidae): a polyphagous plant pest from Asia newly detected in North America. Proceedings of the Entomological Society of Washington, 105, 225-237.

Konopka JK, Haye T, Gariepy TD, McNeil JN (2017) Possible coexistence of native and exotic parasitoids and their impact on control of *Halyomorpha halys*. Journal of Pest Science, 90, 1119-1125.

Leskey TC, Short BD, Butler BR, Wright SE (2012) Impact of the invasive brown marmorated stink bug, *Halyomorpha halys* (Stål), in mid-Atlantic tree fruit orchards in the United States: case studies of commercial management. Psyche: A Journal of Entomology.

Maistrello L, Vaccari G, Caruso S, Costi E, Bortolini S, Macavei L, Foca G, Ulrici A, Bortolotti PP, Nannini R, Casoli L (2017) Monitoring of the invasive *Halyomorpha halys*, a new key pest of fruit orchards in northern Italy. Journal of Pest Science 90, 1231-1244.

Noyes J (2014) Universal Chalcidoidea Database – World Wide Web electronic publication. Natural History Museum Update August. http://www.nhm.ac.uk/our-science/data/chalcidoids/. Accessed 15 Oct 2018.

Qiu L, Yang Z (2010) Population dynamics and interspecific competition between *Trissolcus halyomorphae* Yang and *Anastatus* sp. in parasitizing *Halyomorpha halys* (Stål) eggs. Chinese Agricultural Science Bulletin, 8, 049.

Ratcliffe ST, Robertson HM, Jones CJ, Bollero GA, Weinzierl RA (2002) Assessment of parasitism of house fly and stable fly (Diptera: Muscidae) pupae by pteromalid (Hymenoptera: Pteromalidae) parasitoids using a polymerase chain reaction assay. Journal of Medical Entomology, 39, 52-60.

Stahl JM, Babendreier D, Haye T (2018a) Life history of *Anastatus bifasciatus*, a potential biological control agent of the brown marmorated stink bug in Europe. Biological Control. https://doi.org/10.1016/j.biocontrol.2018.10.016.

Stahl JM, Babendreier D, Haye T (2018b) Using the egg parasitoid *Anastatus bifasciatus* against the invasive brown marmorated stink bug in Europe: can non-target effects be ruled out? Journal of Pest Science, 91, 1005-1017.

Stahl JM, Tortorici F, Pontini M, Bon MC, Hoelmer K, Marazzi C, Tavella L, Haye H (2018) First discovery of adventive populations of *Trissolcus japonicus* in Europe. Submitted to Journal of Pest Science.

Team RC (2014) R Foundation for Statistical Computing, 2015 R: A language and environment for statistical computing.

Team RS (2017) RStudio: integrated development environment for R. RStudio, Inc. 2015.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22, 4673-4680.

Viggiani G, Mineo G (1970) Identification of the parasitoids of *Gonocerus acuteangulatus* (Goeze). Bollettino dell'Istituto di Entomologia Agraria e dell'Osservatorio di Fitopatologia di Palermo, 8, 143-163.

Wermelinger B, Wyniger D, Forster B (2008) First records of an invasive bug in Europe: *Halyomorpha halys* Stal (Heteroptera: Pentatomidae), a new pest on woody ornamentals and fruit trees? Mitteilungen der Schweizerischen Entomologischen Gesellschaft 81, 1-8.

Yang ZQ, Yao YX, Qiu LF, Li ZX (2009) A new species of Trissolcus (Hymenoptera: Scelionidae) parasitizing eggs of *Halyomorpha halys* (Heteroptera: Pentatomidae) in China with comments on its biology. Annals of the Entomological Society of America, 102, 39-47.

Zhang J, Zhang F, Gariepy TD, Mason P, Gillespie D, Talamas E, Haye T (2017) Seasonal parasitism and host specificity of *Trissolcus japonicus* in northern China. Journal of Pest Science, 90, 1127-1141.

7 First discovery of adventive populations of *Trissolcus japonicus* in Europe

Published in: Journal of Pest Science
https://doi.org/10.1007/s10340-018-1061-2

Judith Stahl[1,2*], Francesco Tortorici[3], Marianna Pontini[3], Marie-Claude Bon[4], Kim Hoelmer[5], Cristina Marazzi[6], Luciana Tavella[3], Tim Haye[1]

[1] CABI, Rue des Grillons 1, 2800 Delémont, Switzerland

[2]Institute of Ecology and Evolutionary Biology, University of Bremen, Leobener Str. NW2, 28359 Bremen, Germany

[3]Dipartimento di Scienze Agrarie, Forestali e Alimentari (DISAFA), ULF Entomologia Generale e Applicata, University of Torino, Largo P. Braccini 2, 10095 Grugliasco (TO), Italy

[4]European Biological Control Laboratory, USDA Agricultural Research Service, Campus International de Baillarguet, Montferrier le Lez, France

[5]Beneficial Insects Introduction Research Unit, USDA Agricultural Research Service, Newark, DE, USA

[6]Servizio fitosanitario cantonale, Dipartimento delle finanze e dell'economia, Sezione dell'agricoltura viale S. Franscini 17, 6501 Bellinzona, Switzerland

Abstract

The brown marmorated stink bug, *Halyomorpha halys* (Stål), native to East Asia, emerged as an invasive pest in Europe in the 2000s. In its native range, *Trissolcus japonicus* (Ashmead) is the dominant egg parasitoid of *H. halys*, and thus it has been considered for classical biological control in countries invaded by the pest. A survey of native egg parasitoids conducted in 2017 and 2018 with frozen, sentinel egg masses of *H. halys* revealed that *T. japonicus* was already present in apple orchards in the Canton Ticino, Switzerland. *Trissolcus japonicus* was recovered in both years and from three different sites. In total, 17 egg masses were recovered from which 42 adult parasitoids emerged. A genetic analysis using the barcode mitochondrial DNA confirmed the morphological identification of *T. japonicus* and evidenced a best match of the "Ticino populations" to Japanese populations, but the pathways of entry remain unknown.

8 General discussion

Figure 1 Released *A. bifasciatus* female on sentinel *H. halys* egg mass in site 1 of the release trial

In 2015, augmentative biological control was applied on more than 30 million ha worldwide (van Lenteren et al. 2018). With 170 species of invertebrate biological control agents, Europe is still the largest commercial market for augmentative biological control, which is partly due to political support of biological control within IPM programs, but also due to high demand by consumers, and a highly developed biological control industry (Cock et al 2010, van Lenteren et al. 2018). Augmentative biological control has been applied successfully for more than 100 years (Cock et al. 2010), but the use of native natural enemies against invasive species in the field is a relatively new strategy that might become more important in the future, if access to exotic biological control agents becomes more and more limited (Hassan 1994, Molla et al. 2013, Panzavolta et al. 2013, Stacconi et al. 2013). So far, only a few examples exist for this new approach, e.g. the augmentation of North American larval parasitoids against the invasive emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) (Gaudon and Smith personal communication) or the mass releases of *Trichopria drosophilae* Perkins (Hymenoptera: Diapriidae) against the spotted wing drosophila, *Drosophila suzukii* (Diptera: Drosophilidae), in Italy (Stacconi 2018). Following this new approach, in the present study the potential of the Palaearctic egg parasitoid *A. bifasciatus* for augmentative biological control against the invasive *H. halys* in Europe was investigated.

Due to the long oviposition period of *H. halys*, *A. bifasciatus* could be released inoculatively or inundatively during peak oviposition period(s) (Haye et al. 2014, Costi et al. 2017). Since the oviposition period of *A. bifasciatus* overlaps with that of *H. halys* (chapter 2), it seems well-suited for an inoculative release strategy. However, for inoculative releases multiplication of the parasitoid in the field is essential, and even if females can survive for three months (chapter 2) and progeny production on alternative hosts could be possible (chapter 3), the low reproductive capacity of *A. bifasciatus* may prevent a sufficient population growth throughout the season. On the other hand, the new findings on the biology of *A. bifasciatus* presented here, particularly the low fecundity and challenge arising therefrom to fully parasitize egg masses, are also providing evidence that an inundative approach might not necessarily result in high pest suppression either. To address this issue, over a three-year period, eight experimental augmentative field releases have been conducted in three different apple orchards and one pear orchard located in Switzerland and Northern Italy. In total, more than 4,300 *A. bifasciatus* females were released, equivalent to, depending on distances between trees, 11,000 (site 4) to 26,000 (site 1) females per hectare at each release event (chapter 5). With the observed average of 7% parasitism (range: 2–16%) pest control could not be achieved. However, parasitism was measured by offspring emergence and additional mortality induced by *A. bifasciatus* progeny that failed to develop was 3.3%, when tested with a new developed molecular marker (chapter 6). This value might not be representative for all sites, since only samples from a single release event were analysed. The low detection threshold of the molecular marker is advantageous in detecting *A.*

bifasciatus eggs directly after oviposition, but host feeding (without oviposition) – which accounts for nearly half of the mortality of *H. halys* eggs (chapter 2) – cannot be detected in field collected samples (chapter 6). Accordingly, the overall impact of *A. bifasciatus* on the mortality of *H. halys* eggs was likely underestimated. If preimaginal parasitoid mortality (3.3%; chapter 6) and host feeding (7%; chapter 2) are added to the observed parasitism (7% offspring emergence, chapter 5), the actual induced mortality of *H. halys* eggs might have been 17.3%.

This potentially more accurate estimate of induced *H. halys* egg mortality is more promising than the initial field data would suggest; however, it remains unrealistic that full pest control can be achieved. In comparison, augmentative releases of *Anastatus* sp. against *H. halys* in China were considered effective, resulting in parasitism levels of 53 to 65% (Hou et al. 2009). However, high parasitism of a particular developmental stage of the host does not necessarily translate to full pest suppression (Xu et al. 2001). In the end, the main goal is suppressing pest populations below the economic threshold, which has not yet been defined for *H. halys* (Nielsen et al. 2013).

Independently, optimisation of *H. halys* control with *A. bifasciatus* releases could be achieved by increasing the number of released females – if cost effective – or creating optimal conditions for the released parasitoids by providing suitable floral resources (e.g. flower strips) in or around the orchards (Bianchi and Wäckers 2008, Tillmann 2017). These should consist of plant species that improve the longevity and fecundity the parasitoid (Heimpel and Jervis 2005, Vattala et al. 2006, Génau et al. 2012).

The original *A. bifasciatus* colony, which was used for all experiments described within this thesis, was established from only two parasitized *H. halys* egg masses recovered in the Canton of Valais, Switzerland (Haye et al. 2015), representing a very small founder population. Since *A. bifasciatus* is haplodiploid, requiring lower levels of heterozygosity than diploid organisms, founder populations of less than 500 individuals can maintain healthy colonies (Smith 1996). The less than 50 *A. bifasciatus* individuals that founded our *A. bifasciatus* colony, however, represent a clear bottleneck. Since the same *A. bifasciatus* colony was maintained in the laboratory since 2014, selection pressure and conditioning during the process of mass rearing might have altered the behavior of *A. bifasciatus* produced under artificial conditions, which could have contributed to the low success of field-releases (Boller 1972).

Intraspecific variation within parasitoid species has been studied with increasing interest, because such variations may be used to optimise the selection of appropriate strains within a beneficial species (Roush 1990, Goldson et al. 1997). As mentioned above, for the present study a single population was used, but additional populations were collected during the experimental field releases in the Canton of Ticino, Switzerland, and the Region of Emilia-Romagna, Italy. Since DNA-based technologies are now available for strain differentiation, these new populations present an opportunity for future research.

At the beginning of the project in 2015, *A. bifasciatus* was the only known parasitoid in Europe that could complete its development on viable *H. halys* eggs, limiting the list of candidates for augmentative biological control to this species. In the meantime, two additional egg parasitoid species with the same capabilities but from different families, *Ooencyrtus telenomicida* (Vassiliev) (Encyrtidae) and *Trissolcus kozlovi* Ryakhovskii (Scelionidae), have been discovered, which are also considered for augmentative control (Roversi et al. 2016, Moraglio et al. unpublished). However, the efficacy of these species is still unknown and both species will need to undergo similar studies as presented in this thesis. Based on the results of the intrinsic competition experiments between *A. bifasciatus* and *O. telenomicida* (chapter 4), negative effects on the latter species may occur when co-existing in the same habitat or released simultaneously. Negative effects of intrinsic competition with *A. bifasciatus* might also be expected for *T. kozlovi*, being closely related to the Asian *T. japonicus*, which proved to be an inferior larval competitor to *A. bifasciatus* (Konopka et al. 2017).

Large scale inundative releases of native generalist biocontrol agents can also lead to non-target effects due to overflow into adjacent habitats (van Lenteren and Loomans 2006) putting endangered and beneficial species within those habitats at risk. Laboratory tests demonstrated that *A. bifasciatus* is able to develop on a wide variety of non-target species (chapter 3), and it was confirmed that some degree of non-target parasitism is also likely under field conditions (chapter 5). Accordingly, locally threatened species, such as *Saturnia pyri* (Denis and Schifermüller) (Lepidoptera: Saturniidae), which is part of the physiological host range of *A. bifasciatus* (chapter 3), could be affected due to mass-releases of the biocontrol agent. A more important aspect for the risk assessment for *A. bifasciatus* is the potential dispersal into non-crop habitats, where non-target species may be more abundant and diverse (Rand et al. 2006). In experimental field releases *A. bifasciatus* females dispersed at least eight meters (some egg masses were exposed more than 50 m) from the closest release point but these preliminary results suggest that that females did not leave into adjacent habitats (chapter 5). Other parasitoids such as *Trichogramma brassicae* Bezdenko and *Diadegma semiclausum* (Helen) (Hymenoptera: Ichneumonidae) can disperse more than 20 m and 80 m, respectively (Kuske et al. 2003, Lavandero et al. 2005), or even further with additional passive wind transport (Bigler et al. 1990). Since in the present study parasitism levels after field releases were generally low, and the presence of natural *A. bifascatus* populations in some of the orchards interfered with the dispersal experiments, further studies will be needed to draw final conclusions. Finally, the level of risk of different control methods needs to be put in perspective. Even though arthropod biodiversity, in apple orchards, the main habitat of the current studies, tends to be higher than in annual crops (reviewed in Simon et al. 2010), based on personal observation, insect diversity in conventional Swiss apple orchards is still comparably low and the chosen example *S. pyri* might be far more threatened by the frequent use of insecticides (Rappaz 1983).

With regard to biological control solutions against *H. halys*, the first discovery of adventive populations of *Trissolcus japonicus* (Ashmead) in Europe (chapter 7) is a major development, because it is considered the most promising classical biological agent, causing 50–90% egg parasitism in its native range (Qiu et al. 2007, Yang et al. 2009, Zhang et al. 2017). Recoveries were achieved over two consecutive years, showing that *T. japonicus* is already established in Switzerland. Prior to potential releases of the parasitoid, the efficacy and specificity of the discovered strain should be tested and compared with other Asian *T. japonicus* populations. In terms of natural biological pest control of *H. halys*, the arrival of its main antagonist will likely lead to a significant decrease of the pest in the near future if the parasitoid is able to spread and build up sufficient populations. To facilitate spread and population growth of *T. japonicus* populations, re-location and complementary mass releases could be considered, but current control methods against *H. halys*, mainly insecticide applications, may be counterproductive (Orr et al. 1989, Penca and Hodges 2017, Saber et al. 2005). In this respect, an integrated strategy to control *H. halys* in Europe might be most promising. This could combine attract-and-kill methods (Leskey et al 2015, Morrison et al. 2016), selective spraying of border rows (Aigner et al. 2015), and if cost effective, inundative *A. bifasciatus* releases during *H. halys* oviposition peaks at least until *T. japonicus* populations are high enough. Fortunately, the two latter parasitoid species are expected to coexist easily, as *T. japonicus* does with the Asian *Anastatus* species (Konopka et al. 2017, Zhang et al. 2017).

8.1 References

Aigner B, Kuhar TP, Hogue J, Malone SM, Herbert DA, Seymore E (2015) Brown marmorated stink bug, biology and management in mid-Atlantic soybeans.

Bianchi FJ, Wäckers FL (2008) Effects of flower attractiveness and nectar availability in field margins on biological control by parasitoids. Biological Control 46, 400-408.

Bigler F, Bosshart S, Waldburger M, Ingold M (1990) Dispersal of *Trichogramma evanescens* Westw. and its impact on parasitism of eggs of *Ostrinia nubilalis* Hbn. Mitteilungen der Schweizerischen Entomologischen Gesellschaft 63, 381-388.

Boller E (1972) Behavioral aspects of mass-rearing of insects. Entomophaga 17, 9-25.

Bourchier RS, Smith SM (1996) Influence of environmental conditions and parasitoid quality on field performance of *Trichogramma minutum*. Entomologia Experimentalis et Applicata 80, 461-468.

Cock MJ, van Lenteren JC, Brodeur J, Barratt BI, Bigler F, Bolckmans K, Cônsoli FL, Haas F, Mason PG, Parra JRP (2010) Do new access and benefit sharing procedures under the convention on biological diversity threaten the future of biological control? BioControl 55, 199-218.

Costi E, Haye T, Maistrello L (2017) Biological parameters of the invasive brown marmorated stink bug, *Halyomorpha halys*, in southern Europe. Journal of Pest Science 90, 1059-1067.

Géneau CE, Wäckers FL, Luka H, Daniel C, Balmer O (2012) Selective flowers to enhance biological control of cabbage pests by parasitoids. Basic and Applied Ecology, 13(1), pp.85-93.

Goldson SL, Phillips CB, McNeill MR, Barlow ND (1997) The potential of parasitoid strains in biological control: observations to date on Microctonus spp. intraspecific variation in New Zealand. Agriculture, Ecosystems & Environment 64, 115-124.

Haye T, Abdallah S, Gariepy, T, Wyniger D (2014a) Phenology, life table analysis and temperature requirements of the invasive brown marmorated stink bug, *Halyomorpha halys*, in Europe. Journal of Pest Science 87, 407–418.

Haye T, Fischer S, Zhang J, Gariepy T (2015) Can native egg parasitoids adopt the invasive brown marmorated stink bug, *Halyomorpha halys* (Heteroptera: Pentatomidae), in Europe? Journal of Pest Science 88, 693–705.

Hassan SA (1994) Strategies to select Trichogramma species for use in biological control. In: Wajnberg E, Hassan SA (eds.) Biological control with egg parasitoids, pp.55-71. CAB International, Berkshire, UK.

Heimpel GE, Jervis MA (2005) Does floral nectar improve biological control by parasitoids. Plant-provided food for carnivorous insects: a protective mutualism and its applications. Cambridge University Press, Cambridge, pp.267-304.

Hou Z, Liang H, Chen Q, Hu Y, Tian H (2009) Application of Anastatus sp. against *Halyomorpha halys.* Forest Pest and Disease 4, 39-43.

Konopka JK, Haye T, Gariepy TD, McNeil JN (2017) Possible coexistence of native and exotic parasitoids and their impact on control of *Halyomorpha halys*. Journal of Pest Science 90, 1119–1125.

Kuske S, Widmer F, Edwards PJ, Turlings TC, Babendreier D, Bigler F (2003) Dispersal and persistence of mass released *Trichogramma brassicae* (Hymenoptera: Trichogrammatidae) in non-target habitats. Biological Control 27, 181-193.

Lavandero B, Wratten S, Shishehbor P, Worner S (2005) Enhancing the effectiveness of the parasitoid *Diadegma semiclausum* (Helen): movement after use of nectar in the field. Biological Control 34, 152-158.

Leskey, T.C., Agnello, A., Bergh, J.C., Dively, G.P., Hamilton, G.C., Jentsch, P., Khrimian, A., Krawczyk, G., Kuhar, G.T.P., Lee, D.-H., et al. 2015. Attraction of the invasive *Halyomorpha halys* (Hemiptera: Pentatomidae) to traps baited with semiochemical stimuli across the United States. Environmental Entomology, 44, 746–756.

Mollá O, Biondi A, Alonso-Valiente M, Urbaneja A (2014) A comparative life history study of two mirid bugs preying on *Tuta absoluta* and *Ephestia kuehniella* eggs on tomato crops: implications for biological control. BioControl 59, 175–183.

Morrison WR, Lee DH, Short BD, Khrimian A, Leskey TC (2016) Establishing the behavioral basis for an attract-and-kill strategy to manage the invasive *Halyomorpha halys* in apple orchards. Journal of Pest Science 89, 81–96.

Nielsen AL, Holmstrom K, Hamilton GC, Cambridge J, Ingerson–Mahar J (2013) Use of black light traps to monitor the abundance, spread, and flight behavior of *Halyomorpha halys* (Hemiptera: Pentatomidae). Journal of Economic Entomology 106, 1495-1502.

Orr DB, Boethel DJ, Blake Layton M (1989) Effect of insecticide applications in soybeans on *Trissolcus basalis* (Hymenoptera: Scelionidae). Journal of Economic Entomology 82, 1078-1084.

Rappaz R (1983) Protection des papillons et des plantes nourricières des chenilles. Bulletin de la Murithienne 100, 185-194.

Panzavolta T, Bernardo U, Bracalini M, Cascone P, Croci F, Gebiola M, Iodice L, Tiberi R, Guerrieri E (2013) Native parasitoids associated with *Dryocosmus kuriphilus* in Tuscany, Italy. Bulletin of Insectology 66, 195–201.

Penca C, Hodges AC (2017) Pyriproxyfen treatment terminates *Halyomorpha halys* reproductive diapause, with an indirect mortality effect on its egg parasitoid *Trissolcus japonicus*. Journal of Pest Science 90, 1269-1276.

Qiu LF, Yang ZQ, Tao WQ (2007) Biology and population dynamics of *Trissolcus halyomorphae*. Scienca Silvae Sinicae 43, 62-65.

Rand TA, Tylianakis JM, Tscharntke T (2006) Spillover edge effects: the dispersal of agriculturally subsidized insect natural enemies into adjacent natural habitats. Ecology Letters 9, 603-614.

Roush RT (1990) Genetic variation in natural enemies: critical issues for colonization in biological control. In: Mackauer M, Ehler LE, Roland J (eds.), Critical Issues in Biological Control. Intercept, Andover, pp. 263-288.

Roversi PF, Marianelli L, Costi E, Maistrello L, Sabbatini PG (2016) Searching for native egg-parasitoids of the invasive alien species *Halyomorpha halys* Stål (Heteroptera Pentatomidae) in Southern Europe. Redia 99, 63-70.

Saber M, Hejazi MJ, Kamali K, Moharramipour S (2005) Lethal and sublethal effects of fenitrothion and deltamethrin residues on the egg parasitoid *Trissolcus grandis* (Hymenoptera: Scelionidae). Journal of Economic Entomology 98, 35-40.

Simon S, Bouvier JC, Debras JF, Sauphanor B (2011) Biodiversity and pest management in orchard systems. In: Sustainable Agriculture Volume 2 (pp. 693-709). Springer, Dordrecht.

Stacconi RM, Grassi A, Dalton D, Miller B, Ouantar M, Loni A, Ioriatti C, Walton V, Anfora G (2013) First field records of *Pachycrepoideus vindemiae* as a parasitoid of *Drosophila suzukii* in European and Oregon small fruit production areas. Entomologia 1, 11–16.

Tillman G (2017) Ecosystem-based incorporation of nectar-producing plants for stink bug parasitoids. Insects 8, 65.

van Lenteren JC, Loomans AJ (2006) Environmental risk assessment: methods for comprehensive evaluation and quick scan In: Bigler F, Babendreier D, Kuhlmann U (eds.) Environmental impact of invertebrates for biological control of arthropods: methods and risk assessment. CABI Publishing, Wallingford, pp. 254-272.

van Lenteren JC, Bolckmans K, Köhl J, Ravensberg WJ, Urbaneja A (2018) Biological control using invertebrates and microorganisms: plenty of new opportunities. BioControl 63, 39-59.

Vattala HD, Wratten SD, Phillips CB, Wäckers FL (2006) The influence of flower morphology and nectar quality on the longevity of a parasitoid biological control agent. Biological Control 39, 179-185.

Xu J, Shelton AM, Cheng X (2001) Comparison of *Diadegma insulare* (Hymenoptera: Ichneumonidae) and *Microplitis plutellae* (Hymenoptera: Braconidae) as biological control agents of *Plutella xylostella* (Lepidoptera: Plutellidae): field parasitism, insecticide susceptibility, and host-searching. Journal of Economic Entomology 94, 14-20.

Yang ZQ, Yao YX, Qiu LF, Li ZX (2009) A new species of Trissolcus (Hymenoptera: Scelionidae) parasitizing eggs of *Halyomorpha halys* (Heteroptera: Pentatomidae) in China with comments on its biology. Annals of the Entomological Society of America 102, 39–47.

Zhang J, Zhang F, Gariepy T, Mason P, Gillespie D, Talamas E, Haye T (2017) Seasonal parasitism and host specificity of *Trissolcus japonicus* in northern China. Journal of Pest Science 90, 1127–1141.

Acknowledgements

Without my supervisors this project would not have been possible. Thank you to Dr. Tim Haye and Dr. Dirk Babendreier who guided me through all PhD highs and lows. Tim, you have been supervisor, boss, mentor, colleague and friend. Thank you, Prof. Dr. Thomas Hoffmeister for being my supervisor from afar.

The wonderful Dr. Cristina Marazzi, who single-handedly facilitated our field work in the beautiful Ticino.

My co-authors, especially Dr. Tara Gariepy, who spent quite some time on my manuscripts.

Now Dr. Joanna Konopka, who introduced me to the world of stink bugs and their egg parasitoids and became a friend.

My students Giorgia Frei, Lara Gallmann, Dalinda Baroui and our summer students Patrick Mrazek, Warren Wong, Yamila Franco, Darren Blackburn, Chelsey Blackman, Jessica Fraser, Taylor Kaye, Christie Laing, Lindsay Craig, Anna Grunsky and Mariah Ediger, none of which are by order of importance. Thank you for your amazing help and for all the fun we had! I also want to apologize for all the long days, hyper-aggressive mosquitos, sunburns, ticks, stinging nettles, incessant rain and all other field day related nuisances. And obviously the many Anasthours most of you had to spend in the lab…

My colleagues and friends at CABI, especially my fellow PhD students now Dr. Pierre Girod, Iva Franic, Benno Augustinus, Theo Linders. Your company has been an integral part of my life at CABI. Other CABI colleagues: e.g. Céline Martinez for being the champion of all CABI students including myself and brightening the whole institute with your cheer and energy, and Patrick Haeflinger for making my time a real Swiss experience with all your fondues, raclettes and Bircher mueslis (a real treat and making the summer weekend work worth it!). Everyone at CABI Switzerland.

The lunch club for mostly great food and stimulating conversation, but mainly for ensuring my survival.

My fellow BINGO ESRs who quickly became my first PhD student family, I look forward to seeing each of you again. Everyone in the BINGO network.

Everyone in Bremen and Groningen that made my secondments successful and fun. Sophie Chattington for being a steady organizational (and emotional) support in the final stretches.

My parents for not questioning my choice to work with stinky bugs, but embracing it with lots of stink bug pictures from their garden (the first record of Halyomorpha in NS will be yours!), emotional and practical support and generally being great people.

Old friends who stuck by my side even though they had to listen to nothing but insect or otherwise work-related topics (and did so without complaining) for the last few years – if they heard from me at all. Special thanks to Agnes, Lisa, Anja and Tina.

Warren for being my partner, fighting your way through this thesis and helping me become a better person.

Everyone I forgot while writing this section (which does not mean I forgot your contribution).