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Genetic diversity, dietary preferences and natural enemies of *Halyomorpha halys* in Belgium.

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Thesis submitted to Ghent University in fulfilment of the requirements for the degree of Doctor (PhD) in Bioscience Engineering

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“The only impossible journey is the one you never begin.”

Tony Robbins

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List of abbreviations

AK	attract and kill
ANOVA	analysis of variance
ASV	amplicon sequence variant
BLAST	basic local alignment search tool
BMSB	brown marmorated stink bug
BOLD	barcode of life data system
bp	base pairs
CAP	common agricultural policy
CBC	conservation biological control
COI	cytochrome c oxidase subunit I
COII	cytochrome c oxidase subunit II
CRISPR	clustered regularly interspaced short palindromic repeats
CTAB	cetyltrimethylammonium bromide
cv.	cultivar
DD	degree days
df	degrees of freedom
DNA	deoxyribonucleic acid
E	east
EU	European Union
F1	first filial generation
GLM	generalized linear model
Hd	haplotype diversity
Hn	number of haplotypes
IPM	integrated pest management

ITS1	internal transcribed spacer 1
ITS2	internal transcribed spacer 2
L:D	light-dark cycle expressed in hours
LLN	long-lasting insecticidal net
matK	megakaryocyte-associated tyrosine kinase
MD	mating disruption
MDT	methyl (2E, 4E, 6Z)- decatrieonate
MGCA	molecular gut content analysis
mtDNA	mitochondrial deoxyribonucleic acid
N	north
NGAS	next generation amplicon sequencing
NGS	next-generation sequencing
nMDS	non-metric multidimensional scaling
OLS	ordinary least squares
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PERMANOVA	permutational multivariate analysis of variance
rbcL	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
RH	relative humidity expressed in percentage
RNA-seq	ribonucleic acid sequencing
RQ	research question
RRA	relative read abundance
s.l.	sensu lato
SD	standard deviation
SE	standard error

SIT	sterile insect technique
spp.	several species
T2	second metasomal tergite
trnF	transfer RNA-Phenalanine
trnH-psbA	histidine transfer gene – photosystem II protein D 1 gene
trnL	transfer RNA-Leucine
USA	United States of America
USDA-APHIS	United States Department of Agriculture Animal and Plant Health Inspection Service
UV	ultraviolet
VOC	volatile organic compound

Chapter 1

General introduction, objectives, and thesis outline

1.1. General introduction

Historically, pest control in fruit orchards was largely based on broad-spectrum pesticides. This practice was associated with a diversity of drawbacks, including human health and environmental effects, as well as pesticide resistance. The increasing concern about the potential adverse effects of chemical pesticides has forced growers to innovate and adapt their production methods (Vannoppen et al. 2002). More than a decade ago, the application of integrated pest management (IPM) evolved from a voluntary approach implemented by the most advanced and environmentally friendly fruit production systems to a compulsory framework for growers in the EU after the approval of the EU Directive 128/2009 (European Union, 2009).

Consumer demands for certified products have a significant impact on fruit production. Before IPM was mandatory in the EU, studies showed that European consumers were willing to pay a premium price when crops were produced under an organic-certified process (Govindasamy et al. 2001). In apple and pear production, particularly, there are high cosmetic standards for fresh fruit and its marketability. This represents formidable obstacles to the adoption of effective alternative means of pest control within IPM (Damos et al. 2015).

In Belgium, around 20,000 ha of land is used for fruit cultivation. With ~10,450 ha of pear orchards, ~5,348 ha of apple orchards and ~1,603 ha of land use for strawberries. In the 2023 season, Belgium produced 381,310 tonnes of pears, 203,005 tonnes of apples and 51,453 tonnes of strawberries. The total production value of fruit cultivated in Belgium was ~€661 million in 2023, or ~20% of the total agricultural production value. With pears, strawberries and apples holding the largest fraction of the fruit production value (European Commission, 2024; Vlam.be; Statbel.fgov.be). The largest area of fruit cultivation is in the Haspengouw region, in the south of Limburg, extending to the Hageland region in the province of Vlaams Brabant. Other notable regions are the Hoogstraten region near Antwerp, regions around Roeselare (West-Vlaanderen), the region around Liège, and the Waasland region, of which the latter is particularly important for pear production (Vlaanderen.be).

In addition to persistent challenges in IPM, such as regulatory pressures, and economic viability, the spread of invasive species into new regions presents a major concern, with implications for fruit production. The increase in global trade and international travel over the past decades has facilitated the spread of pests to new continents at unprecedented rates (Hulme 2009). Just within the last two decades, we have seen intercontinental movement of key economic pests (Isman 2019). For example, the South American tomato leafminer, *Tuta absoluta* (Lepidoptera:

Gelichiidae), has spread across Africa, Asia, and Europe, threatening tomato production in many invaded regions (Marchioro and Krechemer 2024). Similarly, the spotted wing drosophila, *Drosophila suzukii* (Diptera: Drosophilidae), native to Asia, is now established worldwide and is a significant pest in soft-skinned fruit agriculture (Clymans et al. 2022).

Amongst pip fruit growers in Belgium, there is a growing concern regarding the recent invasion of the brown marmorated stink bug (BMSB), *Halyomorpha halys* (Pentatomidae). The brown marmorated stink bug, native to East Asia is highly invasive due to its polyphagous nature and is known as one of the most important pests in fruit production in the USA, Switzerland, and Italy (Haye et al. 2015; Bulgarini et al. 2021). Due to their feeding habits, these stink bugs cause significant economic losses by damaging the fruits and causing them to develop malformation, pitting, and scarring, rendering the fruit unmarketable or even inedible (Rice et al. 2014; Powell 2020). Pip fruit, mainly apples, and pears, are particularly vulnerable as they are amongst the most produced fruits in Belgium and represent a revenue of more than €148 million (Verbond van Belgische Tuinbouwcoöperaties, 2022). In the fruit-growing areas of Northern Italy, *H. halys* has caused approximately €590 million in losses in 2019 (Bulgarini et al. 2021; Francati et al. 2021). A limited understanding of the invasion routes, dispersion behaviour, and distribution of *H. halys* hampers the development of efficient and successful strategies for the management of this stink bug in Belgian pip fruit orchards and underscores the need for monitoring of this invasive pest.

An additional concern in Belgian pip fruit orchards, especially pear, is the damage caused by indigenous stink bugs, mostly by the forest bug or *Pentatoma rufipes* (Pentatomidae) (Beliën et al. 2015). In certain organic pear plots in Belgium, up to 50% of the harvest was lost to damage from these shield bugs (Peusens and Beliën 2012). Pesticide-free management strategies for the control of stink bugs have often remained specific to well-studied species like *H. halys* or failed to contain populations below the economic threshold level. With the recent ban on almost all synthetic pyrethroid and neonicotinoid pesticides and the remaining knowledge gaps for both endemic and invasive stink bugs, there is a need for profound insights into their local natural enemies, landscape-dependent host plant preferences, and factors regulating densities of stink bugs within and around organic and IPM orchards. These insights could benefit existing IPM practices and underpin new management strategies for Belgian growers (Beliën et al. 2015).

1.2. Objective and thesis outline

The general objective of this thesis research is to provide insights into the invasion of *Halyomorpha halys* in Belgium, and to enhance our understanding of the natural enemies and host plant preferences of both *H. halys* and native shield bugs. This work aims to inform and improve local stink bug control strategies in Belgian pip fruit orchards within the framework of IPM.

This objective was translated into the following research questions (RQ):

1. Can we infer invasion routes and distribution patterns from the genetic diversity of *Halyomorpha halys* in Belgium?
2. What are the host plant preferences of *Halyomorpha halys* in its invasive area in Belgium?
3. What are the host plant preferences of the native *Pentatoma rufipes* in and around orchards?
4. Can a generalist predator like *Chrysoperla carnea* (Neuroptera: Chrysopidae) contribute to the biological control of stink bugs?
5. Which egg parasites of native stink bugs are present in and around pip fruit orchards?

In **Chapter 2**, the relevant literature that provides a background for Chapters 3-6 was reviewed. The concept of IPM and the role of natural enemies as biological control agents are introduced in the context of economically important shield bugs (Pentatomidae). A brief background was given on the concept of metabarcoding using next-generation sequencing, an instrumental technique for RQ 2 and 3.

To develop our understanding of the invasion and distribution of the brown marmorated stink bug, *H. halys*, in Belgium, a population genetic study using COI and COII barcode regions was conducted to address RQ1 in **Chapter 3**.

Chapter 4 is the result of a two-year field sampling campaign conducted between 2020 and 2022. A molecular gut content analysis was performed on *P. rufipes* and *H. halys* using next-generation amplicon sequencing (NGAS) to barcode and analyse the host plant relationships and preferences of these stink bugs in and around pear orchards (RQ 2 and 3).

In **Chapter 5**, the predatory efficacy of *C. carnea* larvae on the nymphs of *H. halys* and *N. viridula* was evaluated in laboratory experiments at different temperatures in both a simple and more complex arena (RQ4).

Chapter 6 provides an overview of some of the native egg parasitoids of economically important stink bugs in Belgium sampled during this research to address RQ 5.

Finally, **Chapter 7** provides a general discussion of the main findings of the research chapters and future research perspective.

Chapter 2

Stink bugs as pests in fruit production: a literature review

2.1. Stink bugs (Pentatomidae)

The Pentatomidae (common names: stink bugs, shield bugs, or pentatomids) is the third-largest family of the true bugs (Insecta: Heteroptera). More than 4000 of the estimated over 36,000 described species of Heteroptera belong to the Pentatomidae. Traditionally, the Pentatomidae are divided into as many as nine to ten subfamilies (Grazia et al. 2015; Rider et al. 2018). However, traditional classifications were recently challenged by (Roca-Cusachs et al. 2022). The Pentatominae, the largest, albeit nonmonophyletic subfamily of the Pentatomidae, contains the phytophagous stink bugs of economic importance. In contrast, the subfamily Asopinae, with around 300 taxa, contains predatory shield bug species, some of which are considered relevant for biological control (Roca-Cusachs et al. 2022).

Stink bugs derive their name from the distinct volatiles they emit, primarily composed of acrid compounds, including straight-chain unsaturated aldehydes. These compounds serve as allomones, defensive substances against predators and parasitoids from other species. Male pentatomids can produce pheromones, which are either specific to attracting females (sex pheromones) or appealing to both adult sexes (aggregation pheromones), depending on the species (Weber et al. 2018).

Generally, adult Pentatomidae possess several distinctive features; including five-segmented antenna. The name “Pentatomidae“ is derived from the Greek words “pente”, meaning five and “tomos” meaning segment, referring to this specific feature. Furthermore, adult stink bugs are characterized by a round, ovoid, or broadly elliptical body shape, three-segmented tarsi; a large triangular scutellum, usually narrowed posteriorly, and scent glands that open in the region of the metacoxae; these glands produce the abovementioned allomones that function as alarm and defence signals against natural enemies upon disturbance. The piercing-sucking mouthparts (rostrum) of stink bugs consist of a four-segmented labium forming a sheath that encloses two mandibular and two maxillary stylets (Fig. 2.1) (Schuh and Slater 1995; Schaefer and Panizzi 2000; Genevcius et al. 2021; Lima et al. 2021; Roca-Cusachs et al. 2022).

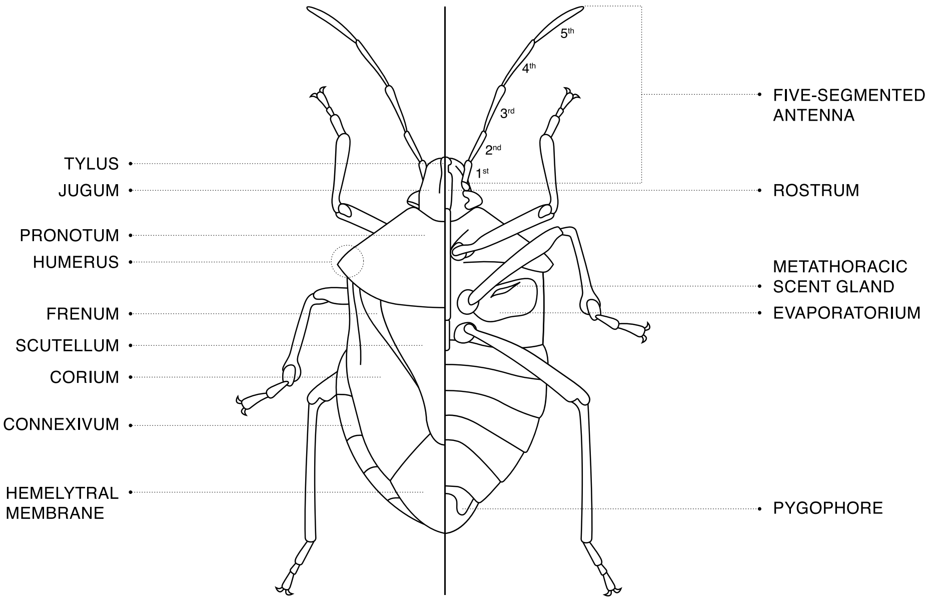

Figure 2.1. Schematic line drawing of an adult stink bug with indications of some major characteristics. (**left:** Dorsal view, **right:** Ventral view) (Credit: Author)

All stink bugs are hemimetabolous insects, and thus have three developmental stages: eggs, five nymphal instars, and a final winged adult stage (Fig. 2.2). Eggs of pentatomid stink bugs are usually barrel-shaped with a detachable cap (pseudoperculum), often the top of the chorion is ornamented with various structures that play a role in fertilization and gas exchange, commonly referred to as micropyles (Esselbaugh 1946; Javahery 1994; Matesco et al. 2014). Nymphs are wingless and lack one antennomere and one tarsomere compared to adults (Schuh and Slater 1995; Brugnera et al. 2022). The dorsal abdominal scent glands are important features at this stage, composed of three exocrine glands that produce semiochemicals used, among other functions, for intraspecific communication and protection against enemies (Aldrich 1988; Aldrich et al. 1997). Adults and nymphs display a piercing-sucking mode of feeding, during which they primarily feed on the cell content of fruits or seeds but also on stems and leaves of their host plants. They insert their stylets into the food source, inject saliva with digestive enzymes to pre-digest the plant matter, and then suck up the liquefied food (Schaefer and Panizzi 2000).

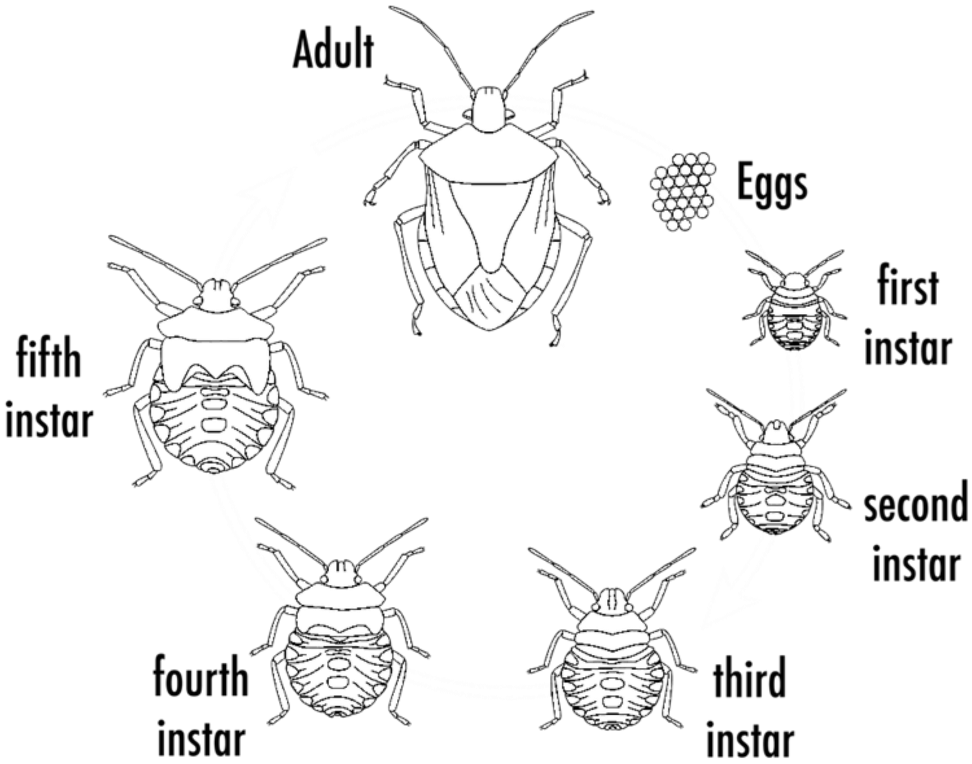

Figure 2.2. Overview of the life cycle of Pentatomidae. (Credit: Adapted from odaguides.us)

2.1.1. Economic Importance

Stink bugs of economic importance, both native and invasive, pose several significant challenges in Integrated Pest Management (IPM) (elaborated in section 2.3). While some pentatomids are carnivorous (mainly belonging to the subfamily Asopinae), most have a herbivorous diet, encompassing both wild and cultivated plants. Highly polyphagous stink bug species can therefore thrive in a variety of natural and managed habitats and can consequently become of economic concern.

When they invade cropping systems, their specific feeding activity causes necrotic spotting, loss of turgidity, stunted growth, delayed maturation, dimpling of fruits, abortion of seeds, and fruiting bodies (Fig. 2.3), rendering the crop unmarketable or even inedible (Rice et al. 2014; McPherson 2018). They also cause yield loss by feeding on ornamental plants. Moreover, punctures left by their feeding activity can increase the vulnerability of their host plant to pathogens. For example, the southern green stink bug, *Nezara viridula* (L.), the green stink bug

Chinavia hilaris (Say), and *Euschistus* spp. can act as vectors for certain phytopathogenic organisms like the fungal pathogen *Nematospora coryli* Peglion (Daugherty 1967; Medrano et al. 2007, 2009; Ademokoya et al. 2022).

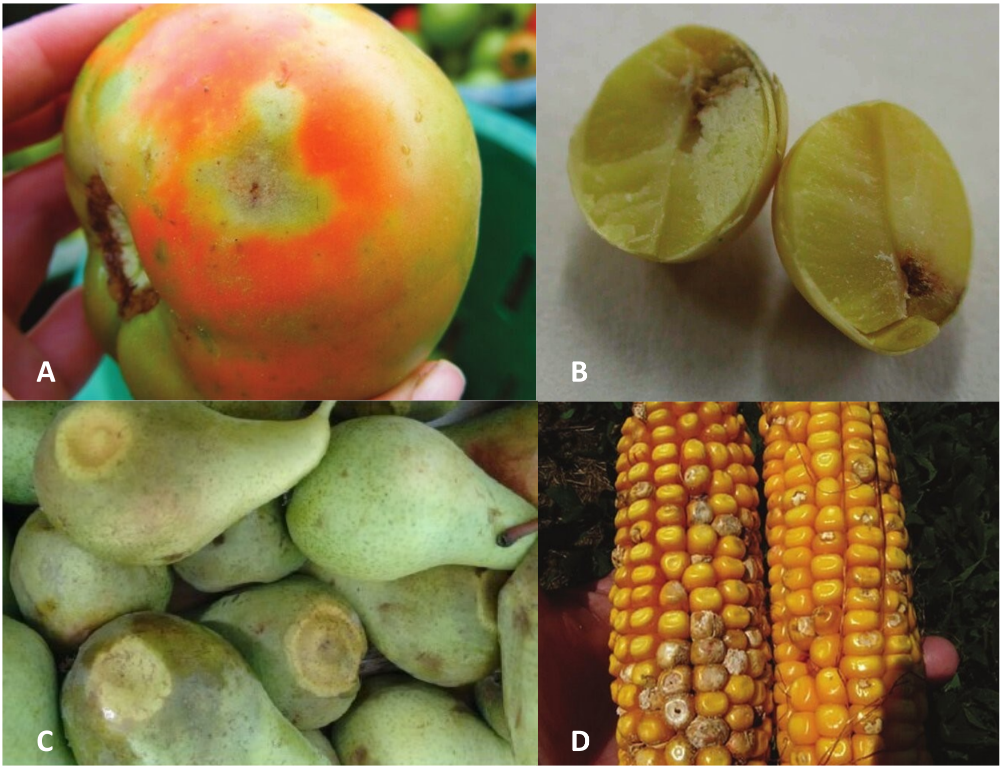

Figure 2.3. Examples of damage done to crops by stink bugs. **A.** Damage to tomato by *N. viridula*. **B.** Damage to soybean by *N. viridula.* **C.** Damage to pears by *Halyomorpha halys*. **D.** Damage to corn by *H. halys*. (Credit A & B: Kamminga et al. 2012, C: Bariselli et al. 2016, D: Rice et al. 2014)

Furthermore, adults often have strong flight abilities, aiding them in long-range dispersal. As such, they can move between cultivated hosts and wild hosts and from one crop to another, with wild hosts serving as sources or sinks for pest buildup before dispersal into cultivated crops (Panizzi 1992; Panizzi and Saraiva 1993; Ademokoya et al. 2022). Highly polyphagous species like *Halyomorpha halys* can thus switch from one host plant to another through the seasons, invading a perimeter of susceptible host plants (Martinson et al. 2015; Venugopal et al. 2015; Philips et al. 2017).

These characteristics have led to the adoption of area-wide management strategies to efficiently suppress stink bug populations, achieved by combining broader area-based management approaches with localized practices tailored to control resident stink bug populations within specific crop systems (Ademokoya et al. 2022).

Efforts to manage stink bugs, including behavioural techniques using semiochemicals and physical techniques using nets (see section 2.3), have both shown promise but are not always adopted by growers. Broad-spectrum insecticides are still commonly used due to the lack of more selective options targeting stink bugs. However, environmental concerns and insecticide resistance are growing issues. Biological control (see section 2.3.2) faces challenges as well, like the impact of broad-spectrum pesticides on natural enemies and the variability in the effectiveness of natural enemies across habitats and stink bug species.

2.1.2. The brown marmorated stink bug: *Halyomorpha halys*

2.1.2.1. Life history and biology

The brown marmorated stink bug (BMSB), *Halyomorpha halys* (Stål), is a successful invader in both agricultural and domestic settings in North America and Europe (Leskey and Nielsen 2018). It is native to East Asia and originates from China, Japan, Taiwan, and Korea (Lee et al. 2013).

Adults exit their overwintering sites in the spring, around April or May to mate and reproduce. Under ideal conditions, adults require between 14-15 days from imaginal ecdysis to reach sexual maturity. Females lay an average of 240 eggs in their lifetime. Eggs are usually laid in clusters of 20-30 eggs and are soft green in colour. They can develop between 15 and 33 °C and can take between 22 and 26 days to hatch at 15 °C and 2-4 days at 30 °C (Haye et al. 2014). The optimal temperature for the development of *H. halys* is approximately 27-28 °C, with individuals requiring around 520 degree days (DD) to progress from egg to adult; however, this requirement can vary among different populations (Mermer et al. 2023). The first instars remain aggregated near the egg cluster and have an orange-to-reddish abdomen. Nymphs become darker in colour, and white markings appear on their legs and antennae (Hoebeke and Carter 2003). Sexual dimorphism is observed in adults; females are often larger and heavier than their male counterparts. Adults are on average 12-17 mm in length and are often variable in colour and size. Generally brownish, greyish, or ochraceous (Fig. 2.4). The number of generations per year varies depending on the geographical location. Throughout most of its native range, only one generation is produced annually. However, some multivoltine populations have been reported in Southern China and invasive areas (Lee et al. 2013). In Italy, two generations per year have been documented (Maistrello et al. 2017). When day length diminishes, and temperatures start to drop in the autumn, adults undergo facultative diapause and start aggregating around their overwintering spots (Toyama et al. 2006).

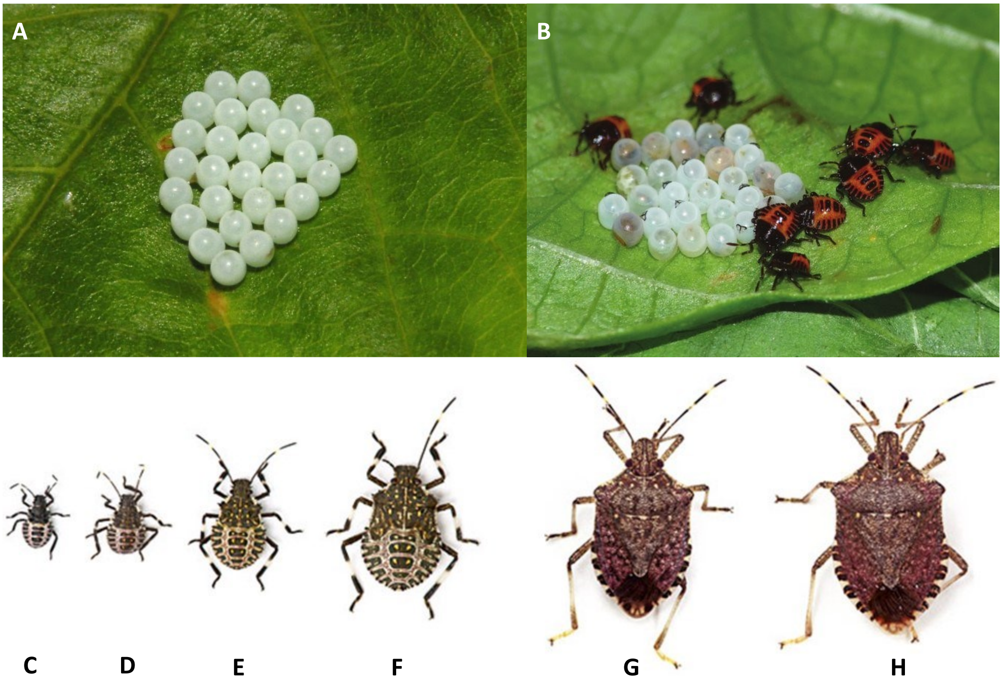

Figure 2.4. Life stages of *H. halys*. **A.** Egg mass. **B-F.** Nymphal stages. **G.** Male adult. **H.** Female adult. (Credit: Adapted from: A: Cabi.org, B: Rice et al. 2014, C-H: stopBMSB.org)

2.1.2.2. Invasion history and pest status

Adults are highly mobile and show strong flight capacity, actively dispersing (Lee and Leskey 2015). Passive, human-mediated pathways of movement have been well documented for *H. halys* and include individuals dispersing in or on cargo, packing crates, aircraft, machinery, ships, vehicles, and personal luggage (Hoebeke and Carter 2003; Nielsen and Hamilton 2009). Transportation events become even more probable knowing that *H. halys* has a typical aptitude to hide in dark microhabitats (e.g., in crevices of vehicles or objects, inside the packaging of different types of goods like empty boxes and fruit containers, clothes, and suitcases), in particular when they aggregate to overwinter (Toyama et al. 2006; Maistrello et al. 2016). These characteristics make *H. halys* an excellent invader.

The first record of *H. halys* outside of its native region was in Allentown (Pennsylvania, USA), in 1996. However, the original introduction is believed to have occurred several years earlier

(Hoebeke and Carter 2003; Leskey and Nielsen 2018). Ever since, *H. halys* has rapidly invaded the USA and Canada (Fogain and Graff 2011; Gariepy et al. 2014). Later, in 2017, *H. halys* was also recorded in South America (Chile) (Faúndez and Rider 2017).

In Europe, it is likely that *H. halys* began its invasion via Switzerland, with the first record in 200 (Wermelinger et al. 2008; Haye et al. 2015). Followed by the colonization of neighbouring countries, findings have been documented across many European countries: Belgium (Claerebout et al. 2019), Germany (Heckmann 2012), France (Callot and Brua 2013), Italy (Pansa et al. 2013), Greece (Milonas and Partsinevelos 2014), Hungary (Vétek et al. 2014), Liechtenstein (Arnold 2009; Haye et al. 2015), Romania (Macavei et al. 2015), Serbia (Šeat 2015), Austria (Rabitsch and Friebe 2015), Spain (Dioli et al. 2016), Georgia (Gapon 2016), Russia (Gapon 2016), Bulgaria (Simov 2016), Slovakia (Hemala and Kment 2017), Slovenia (Rot et al. 2018), the Czech Republic (Kment and Březíková 2019), Croatia (Šapina and Šerić Jelaska 2018), Portugal (Cianferoni et al. 2018), The Netherlands (Aukema 2019), Bosnia and Herzegovina (Zovko et al. 2019), Turkey (Güncan and Gümüş 2019) and Malta (Tassini and Mifsud 2019).

2.1.2.3. Damage

The brown marmorated stink bug has a broad polyphagous feeding range, primarily targeting the fruit structures of plants but also feeding on vegetative parts of its host plants, such as leaves and stems. It is known to feed on over 170 host plant species, many of which are economically significant (Rice et al. 2014; Bergmann et al. 2016). Known notable field crops are pear (*Pyrus* spp.), apple (*Malus* spp.), peach and cherry (*Prunus* spp.), corn (*Zea mays* L.), soybean (*Glycine max* L.), and hazelnut (*Corylus* spp.) (Nielsen and Hamilton 2009; Maistrello et al. 2014). It also feeds on small fruits like grapes (*Vitis* spp.), blueberry (*Vaccinium corymbosum* L.), raspberry and blackberry (*Rubus* spp.) (Bergmann et al. 2016) (Fig. 2.3c-d). They further have the potential to invade vegetable cropping systems including tomato (*Solanum lycopersicon* L.), eggplant (*Solanum melongena* L.), pepper (*Capsicum* spp.), and common bean (*Phaseolus vulgaris* L.). Lastly, it is also able to feed on a wide range of ornamental plants (Haye et al. 2015).

2.1.3. The red-legged shield bug: *Pentatoma rufipes*

2.1.3.1. Life history and biology

Another notable pentatomid pest species is *Pentatoma rufipes* (L.) (other common name: forest bug) native to the Palearctic region. This species is widely distributed across the Palearctic, with numerous observations reported in several European countries, such as Austria, Belgium, the Czech Republic, Denmark, France, Germany, Italy, the Netherlands, Switzerland, and the United Kingdom (Powell 2020).

It has a distinctive appearance with several features to distinguish it from other European pentatomids. The dorsal surface has a glossy dark brown colour, while the legs, scutellum tip, and sometimes the basal antennal segments have a vibrant orange/red shade. Notably, the hooked front corners of the pronotum feature tiny pointed barbs on each side, a hallmark of this species. Adults typically measure 12-15 mm long, with females often larger and heavier than males (Fig. 2.5).

First instar nymphs are darker and gradually become lighter as they mature. Later nymphal stages have a mottled appearance with patches of yellow/green and brown/black, which effectively camouflages them against tree bark, an evolutionary adaptation that enhances their survival in arboreal habitats (Powell 2020).

Populations of *P. rufipes* are univoltine (Hawkins 2003; König 2015). They undergo obligatory diapause during the winter as second-instar nymphs, sheltering on or under the bark of the main trunk or on branches (Kehrli et al. 2011), setting them apart from most other species in the Pentatomoidea superfamily (Saulich and Musolin 2012).

Second-instar nymphs, measuring approximately 3 mm in length, can wedge their small, flattened bodies into tree bark crevices to evade the cold, rendering them elusive during winter surveillance (Kehrli et al. 2011). Warmer spring days make them crawl out of their winter refuges, after which they start to sap-feed from buds, flowers, and developing fruits. In April-May, the overwintering second-instar nymphs undergo moulting, progressing to the third instar, succeeded by the fourth instar in May and June, and culminating in the fifth and final instar in June and July (Hawkins 2003). By July and August, adults emerge, initiating mating and egg-laying activities (Hawkins 2003). Adults have sustained flight capabilities, facilitating dispersal across considerable distances. It remains unclear whether they have a preference for the host tree species on which they developed for mating and egg-laying purposes.

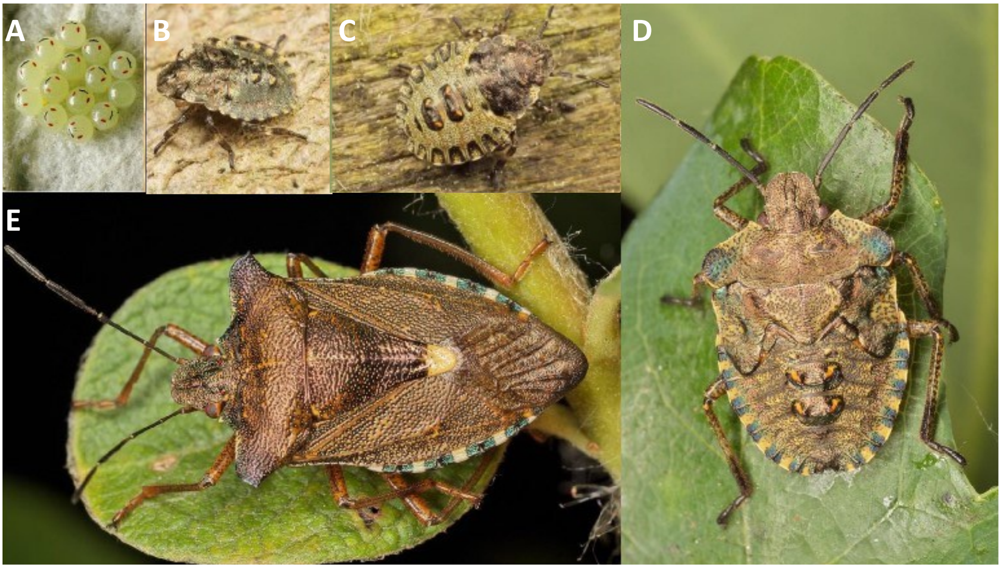

Figure 2.5. Life stages of *P. rufipes.* **A.** Egg mass. **B.** Second-instar nymph. **C.** Third-instar nymph. **D.** Fifth-instar nymph. **E.** Adult. (Credit: Adapted from Powell 2020)

2.1.3.2. Damage

This species is polyphagous but seems to mainly feed on deciduous tree species. Oak (*Quercus robur* L.) is notably preferred as a host plant, although other species such as alder (*Alnus glutinosa* L.), hazel (*Corylus avellana* L.), beech (*Fagus sylvatica* L.), apple (*Malus domestica* Borkh.), pear (*Pyrus communis* L.), cherry (*Prunus avium* L.), plum (*Prunus domestica* L.) and apricot (*Prunus armeniaca* L.) are also commonly used as host plants. It is a particularly abundant species in pear and apple orchards and causes damage to developing fruits shortly after flowering, causing fruit deformity (Fig. 2.6) (Kehrli and Pasquier 2012; Hradil et al. 2013; Šťastná and Psota 2013). Secretions of defensive allomones can also contaminate soft fruits like raspberries and cherries (Alford 2019).

Figure 2.6. Samples of UK apple and pear fruits reportedly damaged by *P. rufipes*. Mixed fruit showing surface pitting (top right) and cut to show internal lesions (middle). Pre-harvest damage to Comice pear (bottom right) and post-harvest damage to Gala apple (left). (Credit: Adapted from Powell 2020, Michelle Fountain, Ralph Hayter & Andy Bull)

2.1.4. The southern green stink bug: *Nezara viridula*

2.1.4.1. Life history and biology

The southern green stink bug, *Nezara viridula* (L.), is one of the most economically important pest species of agricultural crops worldwide. Its polyphagous feeding habits and extensive mobility, coupled with its passive dispersal through human-assisted allocation, have facilitated its global establishment (McPherson 2018). Although *N. viridula* is less important in fruit production, this shield bug species was utilized in experiments in Chapter 5 to compare with *H. halys* as efforts to rear *P. rufipes* and attempts to achieve sufficient reproductive output with shield bug species more relevant in fruit production were unsuccessful.

Adults of *N. viridula* are shield-shaped with an overall dull green colour, while diapausing adults have a more brownish colour (Fig. 2.7). The eyes are dark red or black. Small black dots can be found along the side of their abdomen. Males average 12 mm and females 13 mm in length. Copulation usually lasts a few minutes to a few days. Females can lay eggs two to three weeks after becoming adults. An adult female can lay up to 260 eggs over her lifespan (Esquivel et al. 2018). Eggs are pale yellow or cream-coloured and generally deposited in polygonal clusters of 60 to 90 eggs (Todd 1989; Esquivel et al. 2018).

Figure 2.7. Habitus of a *N. viridula* in dorsal view. Non-diapausing state (left). Diapausing state (right) (Credit: Wikimedia Commons)

The optimum temperature for the development of *N. viridula* is around 25 - 30 °C. Variations in optimal temperature for development and the number of eggs and egg masses laid are observed depending on their geographical occurrence. Egg incubation averages 5 days during optimal summer temperatures but may reach 2 to 3 weeks in early spring and late autumn. First-instar nymphs cluster on or near the egg mass. At the first moult, the nymphs begin to disperse slightly, still showing gregarious behaviour. At the fourth instar, the clustering behaviour ends as the nymphs disperse (Knight and Gurr 2007). Nymphal aggregation (Fig. 2.8) may be a measure of protection by pooling their defence against predators such as ants (Esquivel et al. 2018).

Figure 2.8. Nymphal aggregation of *N. viridula* on black nightshade. (Credit: Patrick De Clercq)

Developmental time from egg to adulthood is approximately 30 days, but will vary based on temperatures and food availability (Esquivel et al. 2018). At optimal temperatures, the developmental time can be as short as 23.2 days (Todd 1989). In general, *N. viridula* is multivoltine, producing multiple generations per year; however, at more northern latitudes, it has been observed to only produce one or two generations (Jones and Sullivan 1981). Like many other pentatomids in temperate zones, adults of *N. viridula* overwinter in a state of facultative diapause in sheltered spaces such as litter, under tree bark, in buildings, or other suitable shelters (Saulich and Musolin 2012; Esquivel et al. 2018).

2.1.4.2. Invasion history and pest status

Its geographic origin is uncertain, but it is assumed to have originated in the Ethiopian region of East Africa or the Mediterranean (Hokkanen 1986; Todd 1989). The distribution of *N. viridula* is often referred to as cosmopolitan since it is widely distributed throughout tropical and subtropical regions of Eurasia, Africa, Australia, and the Americas (Schaefer and Panizzi 2000; Gard et al. 2022). During the last decades, breeding populations have been established in Eastern and Western Europe (Schaefer and Panizzi 2000; Rédei and Torma 2003; Barclay 2004; Werner 2005; Musolin 2012; Dethier and Chérot 2014; Aukema 2019; Grozea et al. 2016; Rabitsch 2016; Hemala and Kment 2017) through natural dispersal and human-mediated transport (Esquivel et al. 2018).

2.1.4.3. Damage

It feeds on more than 150 species of plants, and over 40 plant families have been recorded as hosts for *N. viridula* (Esquivel et al. 2018). Despite its high polyphagy, this species shows a preference for leguminous and brassicaceous plants (Panizzi 2004). It inflicts damage to crops in the open field as well as in greenhouses (Fig. 2.3a-b). In Europe, it is a key pest in greenhouse vegetable crops such as tomatoes, sweet peppers, cucumbers, and eggplants (Vandekerkhove and De Clercq 2004; Gard et al. 2022). For example, in 2019 the economic impact of *N. viridula* was estimated to be between €25,000–35,000 per ha for eggplant production under plastic tunnels in France (Gard et al. 2022). It is also known to be a pest on soybean (Molina and Trumper 2012), peaches (Kamminga et al. 2012), wheat (Reay-Jones 2014), and can develop on fruit and nut trees (Esquivel et al. 2018).

2.2. Principles of IPM in fruit production

Integrated Pest Management (IPM) is a sustainable approach to manage pests by combining biological, cultural, physical, and chemical tools in a way that minimizes economic, health, and environmental risks (FAO, 2020). Originating in the late 1950s, IPM emphasizes healthy crop growth and natural pest control, that aims to maintain pest populations below economically harmful thresholds while minimizing chemical pesticide use, which can lead to ecosystem contamination and health issues. This approach strategically integrates various control methods, including cultural, genetic, and biological practices, to achieve effective pest management (Barzman et al. 2015; Deguine et al. 2021). When implementing IPM strategies, management practices happen at the ecosystem level, and potentially harmful species will continue to exist at tolerable levels of abundance. The adoption of IPM has become compulsory in the EU since the EU Directive 128/2009 (European Union, 2009) as well as in the USA (United States Department of Agriculture, 2010). Barzman et al. (2015) delineate eight principles that form the foundational framework of IPM, ensuring its effectiveness and sustainability in agricultural practices.

2.2.1. Prevention and suppression

This principle mainly includes cultural strategies that reduce the vulnerability of crops to pests through management of soil properties, selection of resistant cultivars, crop rotation, canopy maintenance and enhancing beneficial insect populations by increasing interspecific plant diversity within and around the cultivated field. Many of these cultural strategies are applicable in both organic and IPM orchards (Barzman et al. 2015; Shaw et al. 2021).

Increased soil fertility and organic matter content have been associated with reduced pest pressure. However, excessive nutrient levels can have adverse effects, leading to new tree growth, which in turn can increase susceptibility to colonization by pests like aphids (Altieri and Nicholls 2003). In the case of pears, elevated nitrogen levels have been linked to greater numbers of pear suckers, specifically *Cacopsylla pyricola* (Förster) (Hemiptera: Psyllidae), due to increased foliar growth (Daugherty et al. 2007).

In pip fruit, the selection of apple tree cultivars (e.g. *Malus domestica* cv. Florina) resistant against its primary aphid pest, the rosy apple aphid, *Dysaphis plantaginea* (Passerini) (Homoptera: Aphididae) is an effective strategy (Qubbaj et al. 2005; Miñarro and Dapena 2007; Denoirjean et al. 2021). Although such resistant cultivars might not often be cultivated in professional fruit growing.

Increasing interspecific plant diversity is often achieved by cover crops, these can also affect soil fertility. For example, cv. Comice pears are often deficient in Magnesium, and thus cover plants like the legume hairy vetch, *Vicia villosa* Roth would be incompatible as they lower Mg levels in the soil (Oh et al. 2014).

The planting of hedgerows along orchard borders and cover cropping, often applied as sown-in wild flower mixes between tree rows in orchards, provides alternative food sources for many non-pest herbivorous invertebrates like predators and parasitoids and additionally provides habitats for other beneficial insects like pollinators as well (Bugg and Waddington 1994; Biddinger and Rajotte 2015; Beliën et al. 2021; Shaw et al. 2021; Fountain 2022).

2.2.2. Monitoring

Detection and biosurveillance of pests and beneficial insects on a field level is a cornerstone of IPM. Regular monitoring of pest populations allows for the timely implementation of control measures before pest outbreaks and helps to ensure that these measures are appropriate for the specific pests that are present in the agroecosystems. Monitoring data can for instance serve as input for insect phenology forecasting models (Beliën et al. 2019; Crimmins et al. 2020). The availability and nature of monitoring, warning, and forecasting systems vary according to the type of pest and the means locally available.

In many cases monitoring relies on the use of attractive traps for efficient sampling. The most common monitoring traps make use of long-range stimuli like chemical cues or more medium to short range visual stimuli.

Coloured sticky surfaces, usually yellow or water traps without chemical attractants are widely used to monitor insect pests in orchards. However, for instance, *C. pyricola* is attracted to shorter wavelength green, and this colour was subsequently used for the development of monitoring traps of pear sucker (De Jorge et al. 2023). Similarly, for the spotted wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) that expresses strong sensitivity to colour combinations pairing green as a background colour against other colours in the foreground (Little et al. 2019).

Chemical cues for trapping may involve the use of semiochemicals like pheromones, plant produced volatile organic compounds (VOCs), kairomones or fermented food baits. The latter is commonly used for the detection and monitoring of *D. suzukii* (Larson et al. 2021). Pheromones are often used for the monitoring of lepidopteran or hemipteran pests like leafrollers or shield bugs. Monitoring methods using chemical cues as mentioned above are closely related to behavioural strategies like mass trapping, mating disruption and attract-kill techniques (AK techniques) (El-Sayed et al. 2006; Clymans 2022) (see section 2.2.4).

2.2.3. Decision making based on monitoring and thresholds

The point at which the cost of pest damage exceeds the cost of control measures is a principle that helps farmers make informed decisions about when and how to intervene to manage pests, ensuring that control measures are economically justified. Present-day decision-support systems are usually based on real-time tactical decision-making rather than thresholds; they, however, often involve only one crop, one pest, and one control technique (Barzman et al. 2015).

In pome fruit IPM programs, action thresholds based on moth captures in traps are commonly employed to facilitate the control of codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) (Roelofs et al. 1971; Witzgall et al. 2008; Balaško et al. 2020). Growers utilize traps baited with sex pheromone for surveillance purposes, while orchards are treated with sex pheromone dispensers for the mating disruption of this pest at certain thresholds (see section 2.2.4).

2.2.4. Non-chemical control methods

Pesticide application-free methods can collectively generate synergies, resulting in satisfactory pest management, they include behavioural control techniques, genetic control techniques, physical control techniques and biological control methods.

The most implemented behavioural control technique is mating disruption (MD). It uses synthetic sex pheromones in the field to disrupt mate finding behaviour and is commonly used to control lepidopteran pests e.g., Codling moth, where mate finding relies on males flying towards a pheromone releasing female. Male codling moths are confused by the female sex pheromones that are dispensed in orchards (Howell et al. 1992; Witzgall et al. 2010; McGhee et al. 2016).

Attract-kill techniques are a form behavioural control that often involve mass trapping (i.e. immobilization or physical confinement) of the pest by attracting it towards a contraption sometimes containing a toxicant (or pathogen or sterilant) using semiochemicals like sex-pheromones, kairomones or visual cues (e.g., light) thereby killing the trapped pest insect.

AK techniques frequently constitute the *pull* component of push-pull strategies. While the *push* component includes alarm pheromones or other repellent chemicals produced by aromatic plants or synthetic alternatives to repel the pest away from the desired area (Xu 2017; Wallingford et al. 2018; Song et al. 2023). For example, French marigold, *Tagetes patula* (L.), *Ageratum* spp., and basil, *Ocimum basilicum* (L.) reduced spirea aphid, *Aphis spiraecola* Patch (Homoptera: Aphididae), infestation on apple in comparison to an untreated control (Souza et al. 2019).

AK techniques are widely applied to control tephritid fruit flies (Diptera: Tephriditae) (Lasa et al. 2015; Delgado et al. 2022; Cotoc-Roldan et al. 2023; Fezza et al. 2024) and other dipteran

(Clymans et al. 2022), lepidopteran (Erler and Tosun 2023; Rhainds 2024) and coleopteran (Piñero and Dudenhoeffer 2018) pests in fruit growing.

Genetic control can include sterile insect techniques (SIT), these rely on the release of an overwhelming number of sterile insects into the wild. Wild females mate with sterile males producing no offspring, which reduces pest populations (Dyck et al. 2021). SIT have been particularly successful in the control of tephritid fruit flies for example the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) (Suckling et al. 2016; Duarte et al. 2022), the Oriental fruit fly, *Bactrocera dorsalis* (Hendel) and the melon fly, *Bactrocera cucurbitae* (Coquillett) (Ito et al. 2003; Vargas et al. 2015; Ali et al. 2017). The development of sterile mutations was initially obtained by using ionizing irradiation (Hooper 1989). More recent techniques allow gene editing with CRISPR/Cas9 to create sterile insects by altering genes critical for reproduction (Ashok et al. 2023).

Incompatible insect techniques (IIT), similar to SIT, rely on releasing males infected with *Wolbachia*, a widespread endosymbiotic bacterium in invertebrates. This can prevent the production of viable offspring when infected males mate with wild-type females. *Wolbachia* and similar endosymbiotic bacteria induce a process called cytoplasmic incompatibility (CI), in which *Wolbachia*-infected male insects remain fertile in matings with *Wolbachia*-infected females but produce no viable offspring when they mate with wild females (or those with an incompatible strain of *Wolbachia*) (Laven et al. 1967; Pagendam et al. 2020). Unlike sterilization techniques such as irradiation or gene editing, which can significantly reduce insect fitness or face regulatory challenges, the *Wolbachia*-based IIT approach can provide an effective alternative framework for controlling insect populations without these drawbacks (Pagendam et al. 2020).

In physical control methods, the use of exclusion netting systems (see section 2.3) has become a more common practice for stone fruit growers over recent years (Shaw et al. 2021). Herein, nets or meshes are used as an environmentally-friendly alternative to pesticides, especially in organic farming (Castellano et al. 2008) and offer protection against *D. suzukii* and lepidopterans like codling moth and leopard moth, *Zeuzera pyrina* L. (Lepidoptera: Cossidae), additionally reducing damage from mirids and birds (Alaphilippe et al. 2016; Manja and Aoun 2019; Shaw et al. 2021; Bouvier et al. 2022).

Biological control methods include the release, attraction and conservation of beneficials in the orchards (see section 2.3.2). Traditional hedgerows around orchards support anthocorid populations and foster other beneficial insects including earwigs, *Forficula auricularia* (L.) (Dermaptera: Forficulidae) that can help in the control of apple aphid, *Aphis pomi* (DeGeer) (Homoptera: Aphididae) (Carroll and Hoyt 1984; Shaw et al. 2021). Pirate bugs like *Orius* spp., *Anthocoris nemoralis* (Fabricius) and *Anthocoris nemorum* (L.) (Anthocoridae) are the most widely occurring predatory Heteropterans in pear and apple orchards, and help suppress pear sucker populations in orchards (Solomon et al. 1989; Lattin 1999; Sigsgaard 2010). Populations of predators can suppress a wide number of mite pests, including the two-spotted spider mite, *Tetranychus urticae* Koch (Amano 1999), fruit tree red spider mite, *Panonychus ulmi* (Koch) (Fitzgerald et al. 2007), apple rust mite, *Aculus schlechtendali* (Nalepa) (Strapazzon and Monta 1988), and pear rust mite, *Epitrimerus pyri* Nalepa (Easterbrook 1978). However, unlike egg parasitoids, the release of generalist predators in orchards is not common (further elaborated for stink bugs in section 2.3.1).

Perhaps at the interface of biological control agents and biopesticides are entomopathogenic fungi such as *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae), bacteria like *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) or granulovirusses, which are commonly available in the form of a wettable powder formulations. These formulations are suspended in water and then sprayed like traditional pesticides for the control of various pests, such as psyllids (Puterka 1999), leafrollers (Unruh et al. 2012), other lepidopteran pests (Sarker et al. 2020), sawflies (Świergiel et al. 2016), tephritids (Daniel and Wyss 2010), thrips (Wu et al. 2017), and mites (Wu et al. 2016; Canassa et al. 2020).

2.2.5. Pesticide selection, reduced pesticide use, and anti-resistance strategies

These three principles advocate for the judicious use of pesticides when necessary. This includes selecting the least harmful pesticides to minimize non-target effects and rotating modes of action to reduce the risk of developing pesticide resistance.

While many broad-acting pesticides including all organophosphates and all but one neonicotinoid (acetamiprid) for outdoor use are banned in the EU. Fruit growers have to resort to synthetic pyrethroids, botanical pyrethrins or compounds like spynosyns or avermectins derived from bacteria as a last resort to control pests in their IPM programs (fytoweb.be).

Many common pests in fruit production have developed resistances to various classes of pesticides in certain regions: leafrollers (Pree et al. 2001; Mota-Sanchez et al. 2008; Soleño et al. 2020), tephritids (Kakani et al. 2010; Jin et al. 2015; Demant et al. 2019), thrips (Gao et al. 2012; Guo et al. 2020b; Fu et al. 2022; Mocchetti et al. 2023), spider mites (Van Leeuwen et al. 2009, 2010; Wybouw et al. 2019; De Beer et al. 2022) and aphids (Bass et al. 2014; Guo et al. 2020a; Bass and Nauen 2023).

2.2.6. Evaluation

The evaluation of yield, yield stability, and profit over multiple years at the cropping system level helps to adapt strategies or develop new methods. Ryalls et al. (2024) demonstrated that with the increase of IPM measures adopted, the performance of natural enemies increased, which simultaneously reduced overall pest and disease pressure in apple cultivation. However, boring and mining herbivorous pests did not show a response to IPM measures.

2.3. Integrated management of stink bugs in fruit production

Grower initiatives for prevention and suppression often start with general cultural practices such as incorporating sown-in wildflower strips and establishing hedgerows in and around orchards (see section 2.2.1). While these practices are integral components in pip fruit IPM, they are not tailored to the suppression of stink bugs in orchards. However, they do play a dual role in attracting and sustaining predatory and parasitic insects for general pest management and drawing pollinators into orchards (Beliën et al. 2021; Fountain 2022).

In fruit production, the research to manage stink bugs primarily focuses on *H. halys.* Monitoring tools for *H. halys* use traps baited with semiochemical stimuli, relying on various chemicals to attract and capture individuals. The discovery of the aggregation pheromone of *H. halys* by Khrimian et al. (2014) led to a multitude of applications for the trapping of *H. halys*. The male-produced aggregation pheromone (3S,6S,7R,10S)-10,11-epoxy-1-bisabolen-3-ol and (3R,6S,7R,10S)-10,11-epoxy-1-bisabolen-3-ol was then combined with methyl (E,E,Z)-2,4,6-decatrienoate (MDT) lures, to attract more adult and nymphal *H. halys* than either the aggregation pheromone or MDT alone, offering new opportunities for the monitoring and control of *H. halys* (Weber et al. 2014; Leskey et al. 2015). This in turn led to the commercial availability of the aggregation pheromone mixture for *H. halys* eg. PHEROCON Trécé BMSB (‘Trécé’) and AgBio Inc. Stink Bug Xtra Combo (‘Xtra Combo’). In 2018, Akotsen-Mensah et al. demonstrated that *H. halys* adults and nymphs are attracted to black pyramid traps baited with these commercial aggregation pheromone lures in peach and apple orchards.

Besides pheromones, host plant stimuli, like VOCs were also reported to significantly influence *H. halys* behaviour in combination with the aggregation pheromone (Morrison III et al. 2016b). In a later study, Morrison III et al. (2017) concluded that the retention capacity of *H. halys* in the field using VOCs, was the most successful when combined with favourable host plants like peach and apple but not with unacceptable hosts like Japanese maple.

Along with the research and commercialization of the olfactory attractants for *H. halys*, several trap designs were developed and tested. These include: black pyramid traps, sticky traps, hanging pyramid traps, tunnel traps and multimodal traps that also use a visual stimulus (e.g., UV light) alongside the aggregation pheromone mixture (Rice et al. 2018; Suckling et al. 2019b, 2019a; Rondoni et al. 2022) (Fig. 2.9).

Figure 2.9. Different pheromone baited trap designs. **A.** Rocket trap. **B.** Sticky trap. **C.** Tunnel trap. **D.** Pyramid trap. **E.** Black pyramid trap. **F.** Multimodal trap with UV light. (Credit: Adapted from: A & D: Rik Clymans, B & C: PCFruit, E: Brett Blaauw, F: Rondoni et al. (2022))

The development of these different olfactory and visual stimuli-based trap designs has greatly improved monitoring strategies for *H. halys* that otherwise would have included sweep net sampling, limb jarring (striking limbs of a fruit tree) or visual surveys (inspecting the tree for a standardized duration) (Leskey and Nielsen 2018).

Based on these monitoring strategies, non-chemical control methods like AK techniques were developed as well. Pheromone-baited sticky traps (Fig. 2.9b) immobilize the shield bugs upon landing on the glue covered surface and eventually kill them (Acebes-Doria et al. 2018).

Long-lasting insecticidal nets (LLNs) which have insecticide incorporated within the fibres that are toxic to the stink bugs baited with commercial pheromone lures were also developed (Kuhar et al. 2017; Peverieri et al. 2018) or trap designs combined with LLNs permeated with alpha-cypermethrin like the “nazgûl trap” developed by Suckling et al. (2019b).

Lastly, these pheromone-based tools have also been used in combination with chemical control strategies like perimeter-based AK techniques, where only baited trees and those within a perimeter of 5 m are sprayed with insecticides (Morrison III et al. 2019) or techniques that involve monitoring-based decision-making using trap-based threshold-triggered spray applications of pesticides (Leskey et al. 2020, 2021).

Physical control methods like exclusion nets can also be a potential solution to reduce *H. halys* infestation on plants under netted plots and thus its damage, as reported in North America (Marshall and Beers 2021, 2024) and Italy (Candian et al. 2018, 2021; Fornasiero et al. 2023). Indeed, in nectarine orchards in Italy the damage was reduced by up to 78% for netted plants compared to those with insecticidal treatments alone (Candian et al. 2021). Even the use of a simple physical barrier like a tall barrier fence reduced movement into peach orchards by the pentatomid *Euschistus servus* (Say) (Cottrell and Tillman 2019).

Targeting hemipterans like stink bugs with the Sterile Insect Technique (SIT) poses a unique challenge, as all stages of their development and potential release can inflict damage on crops, this is reflected in the limited amount of research conducted. Despite this, research efforts have been done to successfully sterilize *N. viridula* with radiation (Horrocks et al. 2020). Efforts by Suckling et al. (2019c) to sterilize *H. halys* with radiation were unsuccessful, and did not alter their mating competency. Tools to affect development and reproduction of stink bugs using CRISPR/Cas9 gene editing or RNA interference-based strategies are still in early research stage but have been studied in *N. viridula* (Riga et al. 2020; Sharma et al. 2021; Souza et al. 2022), *H. halys* (Lu et al. 2017; Mogilicherla et al. 2018; Finetti et al. 2023) and *Euschistus heros* (Fabricius) (Cagliari et al. 2020).

More recent developments focus on host-symbiont association disruption by preventing stink bug nymphs to acquire obligate endosymbiont bacteria from their environment. For instance, treating *H. halys* egg surfaces with a biocomplex containing copper, zinc and citric acids resulted in high nymph mortality under open field conditions because nymphs were unable to acquire the *Candidatus Pantoea carbekii* endosymbiont (Gonella et al. 2020; Dho et al. 2025).

In the EU, for the management of stink bugs, growers still depend on broad-spectrum insecticides such as synthetic pyrethroids, insect growth regulators, botanical insecticides, spinosyns and to a lesser extent neonicotinoids. This is due to the lack of more selective insecticides that effectively target stink bugs. In Belgium, only spinosad (a spinosyn), a natural insecticide derived from the soil bacterium *Saccharopolyspora spinosa* Mertz & Yao (Pseudonocariales: Pseudonocardiaceae), is authorized for use against stink bugs by fruit growers (Dively et al. 2020; Fytoweb.be). However broad-spectrum insecticides applied for other pests can also have side effects on stink bugs.

Despite the judicious use and selection of insecticides, there’s a risk for development of resistance to these insecticides by stink bugs. For example, *E. heros*, has already developed a reduced susceptibility to organophosphates, pyrethroids, and neonicotinoids in Brazil (Sosa-Gomez et al. 2001; Sosa-Gómez et al. 2020). Moreover, *H. halys* may have a genetic predisposition to develop insecticide resistance, indicated by findings from Mittapelly et al. (2019) that reveal the presence of conserved P450 motifs and show the differential expression of CYP6 genes among tissues of *H. halys*. Cytochrome P450s are a superfamily of enzymes that help in the detoxification of xenobiotic substances and can play a role in pesticide resistance development.

Biological control methods leveraging natural enemies (see section 2.3.1) including invertebrate predators, egg parasites and to some extent mammals and birds also play a role in the management of stink bug populations (Ademokoya et al. 2022; Grabarczyk et al. 2023).

2.3.1. Natural enemies of stink bugs

2.3.1.1. General overview

Stink bugs are attacked by predators and parasitoids across all their developmental stages, including eggs, nymphs, and adults.

The presence of pathogens has been mainly documented in nymphs and adults. The entomopathogenic microsporidian, *Nosema maddoxi* Becnel, Solter, Hajek, Huang, Sanscrainte & Estep (Microsporidia: Nosematidae), infects *H. halys* populations in North America and Asia before, during and after overwintering, causing decreased fitness (Hajek et al. 2018). It was also discovered in *N. viridula* (Rivers et al. 2022). González et al. (2023) reported infections of the fungal pathogen *Colletotrichum fioriniae* Marcelino & Gouli (Glomerallales: Glomerellaceae) causing mortality during overwintering. Tozlu et al. (2019) tested bacterial strains against *H. halys* nymphs under laboratory conditions and found that all of the tested entomopathogens caused mortality rates of over 75% compared to untreated controls. Lastly the fungus *B. bassiana* has also shown promise as a biological control agent for *H. halys* (Gouli et al. 2012), *E. heros* (Silva-Santana et al. 2022) and *N. viridula* (Portilla et al. 2022).

Invertebrate predators of stink bug eggs, nymphs, and adults belong to insect families across various orders. These include Coccinellidae, Melyridae (Coleoptera), Forficulidae (Dermaptera), Asilidae (Diptera), Anthocoridae, Reduviidae, Geocoridae, Mantidae (Mantodea), Crabronidae (Hymenoptera) and Chrysopidae (Neuroptera) (see lacewings

2.3.1.2) (Rice et al. 2014; Abram et al. 2017; Ademokoya et al. 2022). In addition, spiders have also been observed feeding on all life stages, e.g., on *H. halys* (Rice et al. 2014).

Besides invertebrate predation, there is some evidence of vertebrates, mammals in particular, preying on stink bugs, including mice *Mus musculus* (L.) (Rodentia: Muridae) (Ehler 2000, 2002) as well as big brown bats *Eptesicus fuscus* (Beauvois) (Chiroptera: Vespertilionidae) (Whitaker 1995; Valentin et al. 2016; Maslo et al. 2017) and rather low incidences of avian predation (Grabarczyk et al. 2023).

Egg parasitoids of stink bugs (see section 3.2.1.1) are all hymenopterans belonging to the superfamily Platygastroidea (including the family Scelionidae), the families Encyrtidae, Eupelmidae, and occasionally some Braconidae and Mymaridae. Nymphal and adult parasitoids mostly include Encyrtidae (Hymenoptera) and Tachinidae (Diptera).

2.3.1.2. Egg parasitoids

Most of the parasitic wasps of pentatomid eggs belong to the superfamily Platygastroidea (Hymenoptera: Proctotrupomorpha) except for a few other species of hymenopterans belonging to the genera *Ooencyrtus* and *Anastatus*. The recently revised monophyletic superfamily Platygastroidea is a diverse group and contains multiple families of parasitic wasps that parasitize on nine orders of insects as well as spiders (Austin et al. 2005). The largest family, Scelionidae, comprises over 3000 species and contains the main genera of interest with egg parasites of stink bugs (Pentatomidae): *Trissolcus* and *Telenomus* and to a lesser extent *Gryon*. Within the Scelionidae, the second largest family, Platygastridae, arises as a paraphyletic family to the Scelionidae, containing over 1000 species (Chen et al. 2021).

All Scelionidae are endoparasitic wasps of insect or spider eggs and have a hypodermic-like ovipositor that they use to pierce the chorion of a host egg and lay their own single (or sometimes several) eggs in. The larva of the wasp that hatches consumes the content of the host egg, effectively killing the host embryo, and then pupates within the egg. Together with a high-efficiency rate in parasitizing the eggs of their host species, these scelionid wasps are specialized and efficient agents for the biological control of harmful pentatomid pests (Austin et al. 2005).

The genera *Trissolcus* and *Telenomus* are cosmopolitan and contain most of the known stink bug egg parasitoids. These parasitic wasps are typically small, only a few millimetres in length, and generally dark-coloured. The monophyly of these genera has never been demonstrated as of yet (Taekul et al. 2014; Chen et al. 2021). The morphological diversity among species of these genera has made it difficult to unambiguously diagnose genera and species morphologically. Species from these genera are classified in either genus by comparison of some specific morphological characters (Fig. 2.10). Firstly, the frons is smooth or with a microsculpture in *Telenomus*, however often with coarse sculpture, nevertheless variable in *Trissolcus* (not on figure). Secondly, in *Telenomus* eye-setation is present or absent, but usually absent in *Trissolcus*. The development of the notauli, which are external indications of the separation between the dorsolongitudinal and dorsoventral flight muscles, is almost always absent in *Telenomus* but usually present in *Trissolcus*. The shape of the second metasomal tergite (T2) is longer than wide in *Telenomus* and wider than long in *Trissolcus*. Most females of *Telenomus* species have four clavomeres but this can range from 3 to 6, whereas female *Trissolcus* have five clavomeres (except for 4 in two species). Clavomeres are defined by the presence of papillary sensilla on the antennae. Another useful character to distinguish *Trissolcus* is the subacropleural sulcus, and the prespecular sulcus that form a V shape in the antodorsal portion of the mesopleuron, in *Telenomus* this is not the case, although the subacropleural sulcus can be present. In general, *Trissolcus* are robust and stout-bodied, whereas many *Telenomus* species are more elongated and gracile, however *Telenomus* has highly variable body shapes (Fig. 2.10). Many of these Scelionidae are egg parasites of important stink bug pests, e.g., *N. viridula*, *H. halys*, and *E. heros* (Johnson 1984; Talamas et al. 2015, 2017; Chen et al. 2021).

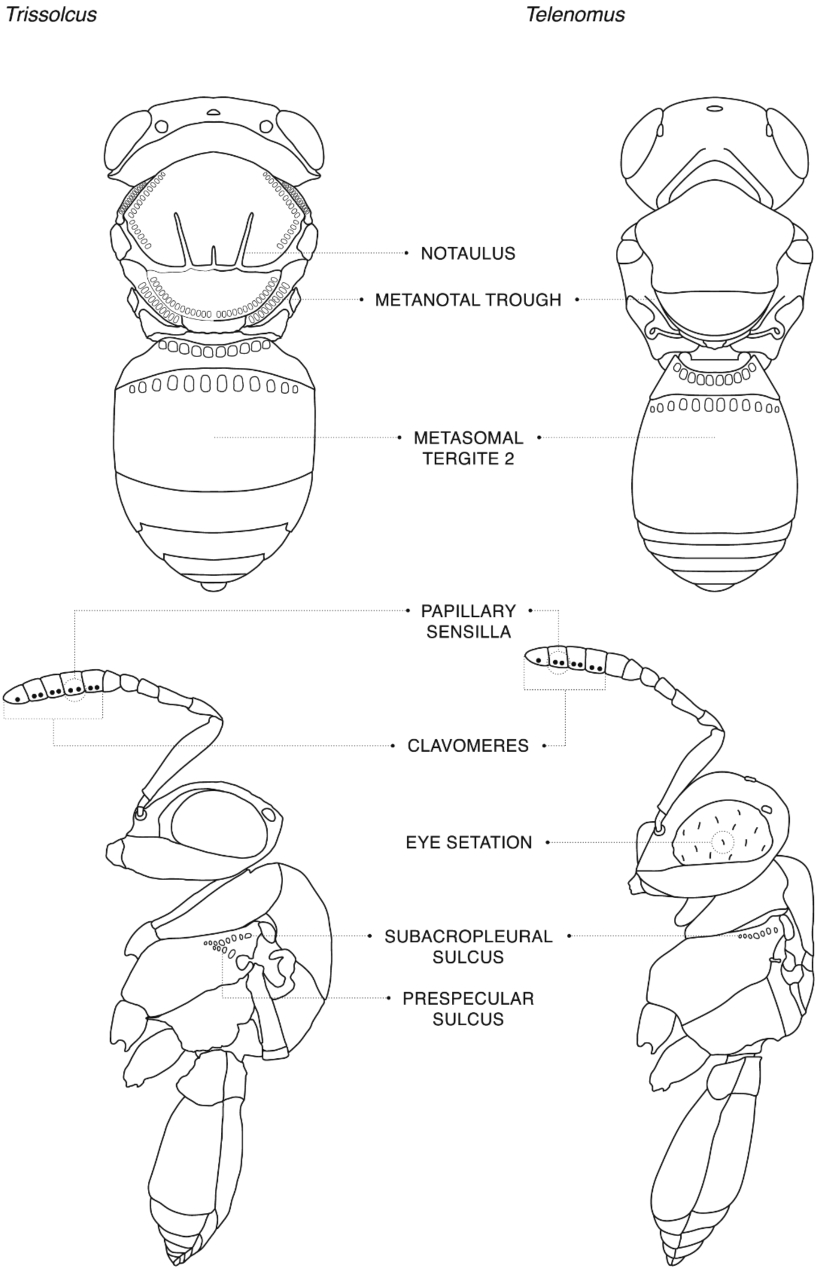

Figure 2.10. Schematic comparison of the main morphological differences between the genus *Trissolcus* (**left**) and *Telenomus* (**right**) in dorsal and lateral view. (*Trissolcus* adapted from Talamas et al. (2015) Credit: Author)

2.3.1.3. Lacewings

Lacewings (Neuroptera: Chrysopidae) are among the most common natural enemies of arthropod herbivores in a wide range of ecosystems and play a key role in the conservation and augmentative biological control of pests in agricultural environments. The common green lacewing, *Chrysoperla carnea* sensu lato (Stephens), is a widespread species complex from the Palearctic region, comprising at least four cryptic green lacewing species (Canard and Thierry 2007). Moreover, *C. carnea* s.l. and other congeneric species are commercially available in Europe (e.g., Biobest 2009; Koppert 2021) and North America (Tauber et al. 2000; McEwen 2001), highlighting their applicability in IPM strategies. Green lacewings have mainly been used for aphid biocontrol in various greenhouse and field crops (Breene et al. 1992; Sarkar et al. 2019; Fang et al. 2022).

The life history of green lacewings includes four stages: egg, three larval instars, a pupal stage, and an adult stage (Fig. 2.11). Eggs of *C. carnea* are oval and pale green when freshly laid, becoming more greyish as they near hatching. Females lay their eggs at the end of slender stalks, up to 1 cm long, on foliage and other substrates, helping protect them from predators and cannibalism.

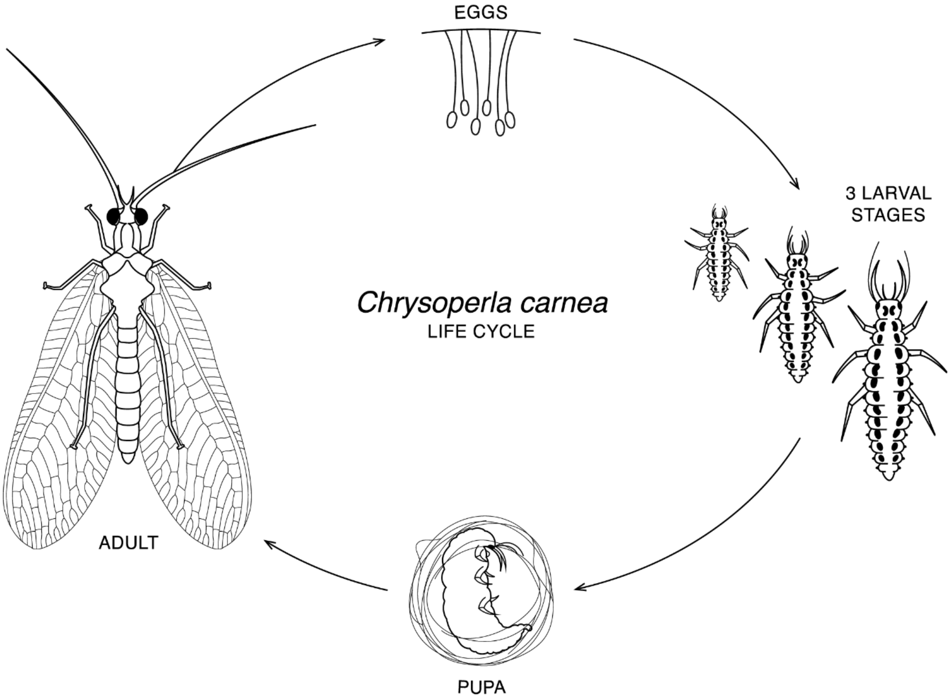

Figure 2.11. Schematic overview of the life cycle of *C. carnea*. (Credit: Author)

The total developmental time of *C. carnea* from egg to adult is 28-32 days at 23 °C (Amarasekare and Shearer 2013). The eggs hatch into first instar larvae; these newly hatched instars typically measure 1-3 mm in length. They have a slender, elongated body with prominent mandibles for feeding. As they grow and moult into the second instar, they typically measure 4-6 mm in length. They become more robust and have a proportionally larger head and mandibles, allowing them to attack a wider range of prey sizes. The third and final instar is the largest larval stage before pupation with larvae 7-10 mm in length. The third instar is the most aggressive and efficient predator among the larval stages, capable of consuming 80% of the early life stages of *Pieris brassicae* L. (Lepidoptera: Pieridae) at 25 °C in laboratory experiments (Huang and Enkegaard 2010). The larval stages prey on a wide range of small arthropods and target various pests, including aphids, lepidopterans, mealybugs, psyllids, leafhoppers, whiteflies, thrips, caterpillars, and spider mites (Easterbrook 1978; Principi and Canard 1984; Stark and Hopper 1988; Huang and Enkegaard 2010; Farrokhi et al. 2017; Golsteyn et al. 2021).

The third-instar larvae pupate within a silken cocoon attached to leaves or in other sheltered places. From the pupae, adults emerge; these are light green with long, slender antennae, golden eyes, and long, delicately veined lacey wings. In contrast to its three larval instars, adults of *C. carnea* are not predaceous but feed on floral and extrafloral nectar, pollen, and honeydew (Principi and Canard 1984; Villenave et al. 2006; Gonzalez et al. 2016; Villa et al. 2016).

2.3.2. Biological control of stink bugs

Biological control is a key component of IPM strategies. It involves managing pests using their natural predators, parasites, or pathogens. Biological control can be divided into three main categories: (1) classical biological control, (2) augmentative biological control, and (3) conservation biological control (Eilenberg et al. 2001; Prokopy and Kogan 2009).

2.3.2.1. Classical biological control

The goal of classical biological control, in its most optimistic form is to reduce the invasive insect to non-pest status by restoring top-down population suppression (Abram et al. 2020). Classical biological control involves the introduction of a natural enemy of a pest from its native habitat to a new environment where the pest has become invasive and lacks natural predators (Eilenberg et al. 2001). To achieve this, researchers carefully select, import, and release natural enemies after thorough evaluation. Over time, the introduced natural enemy establishes a population and provides long-term control of the pest.

In Australia, New Zealand, Hawaii and South America inoculative releases were done involving the scelionid egg parasite *Trissolcus basalis* (Wollaston) from the 1930’s to 1960’s as part of classical biological control programs against *N. viridula* with high parasitism rates and successes (Caltagirone 2003; Panizzi and Lucini 2016; Abram et al. 2020).

Additionally, the native parasitoids of *N.viridula* adults, *Trichopoda pennipes* (Fabricius) and *Trichopoda giacomellii* (Blanchard) (Diptera: Tachinidae) were introduced into Hawaii (USA) and Australia for classical biological control of *N. viridula* (Liljesthröm and Rabinovich 2004; Esquivel et al. 2018).

As invasive species expand their geographic range, so can their natural enemies spread fortuitously. Both *Trissolcus japonicus* (Ashmead) and *Trissolcus mitsukurii* (Ashmead), the main egg parasitoids of *H. halys* in its native range, have already been detected in North America and Europe (Talamas et al. 2015; Abram et al. 2019; Stahl et al. 2019a; Tortorici et al. 2023). These two Asian species are candidates for classical biological control of *H. halys* in its invaded range (Charles et al. 2019; Lara et al. 2019; Haye et al. 2020; Sabbatini-Peverieri et al. 2020, 2021; Giovannini et al. 2022). In addition to being field-released in some areas, *T. japonicus* has now established adventive populations in North America, Switzerland, and Italy, where *T. mitsukurii* is also established, and both species are spreading to other countries in Europe (Lowenstein et al. 2019a; Milnes and Beers 2019; Scaccini et al. 2020; Zapponi et al. 2020, 2021; Bout et al. 2021; Dieckhoff et al. 2021; Rot et al. 2021; Mele et al. 2022).

2.3.2.2. Augmentative biological control

Augmentative biological control (or “augmentation”) comprises the release of mass reared natural enemies with the goal of “augmenting” natural enemy populations or “inundating” pest populations with natural enemies. This approach is employed when indigenous parasitoids or predators either struggle to establish in agricultural environments like fields or orchards or colonize too late in the season to adequately manage pest populations (Collier and Van Steenwyk 2004).

Inundative releases, involve the mass release of natural enemies for immediate pest suppression. The natural enemies are reared in large quantities and released in an agricultural environment to control a pest outbreak. This method often requires repeated applications. It is commonly used in greenhouses or specific crop systems where environmental conditions can be managed to favour natural enemies.

Inoculative releases involve releasing fewer natural enemies at a specific point in the phenology so they can establish a population and provide ongoing control throughout the growing season. This approach relies on the natural enemy's reproductive capacity.

In 2022, Iacovone and co-authors reported on the first large-scale augmentative biological control project carried out in Europe against *H. halys* using the commercially available native egg parasitoid *Anastatus bifasciatus* (Geoffroy) (Hymentoptera: Eupelmidae) at a rate of 1000 individuals/ha in apple and kiwi orchards and vineyards in Italy. At release sites, *A. bifasciatus* showed a significantly higher discovery efficiency (31.4%) and parasitism rate (16.7%) of *H. halys* egg masses than at control sites (1.7% and 1.2%, respectively) without affecting parasitism rates by other naturally occurring species.

2.3.2.3. Conservation biological control

Conservation biological control (CBC) offers opportunities to actively reduce stink bug populations as a component of broader IPM programs. Given the diverse complex of natural enemies, it includes strategies that focus on modifying the agroecological environment or existing agricultural practices to protect and enhance the effectiveness of naturally occurring enemies in the agroecosystem and, therefore, mainly includes cultural practices.

Parasitoids and predators of shield bugs are known to exploit and gain advantages from plant resources. The most commonly applied measures to achieve this biodiversity in orchards are wildflower strips and hedgerows (Kremen et al. 2007; Scheper et al. 2015; Tschumi et al. 2015; Dainese et al. 2019; Albrecht et al. 2020). Such cultural practices are supported by the Common Agricultural Policy (CAP) in the EU (Albrecht et al. 2020). These are proven strategies to enhance beneficial insect populations by providing habitat and nutritional resources for survival, establishment, and reproduction (see section 2.2.1) (Albrecht et al. 2020; Beliën et al. 2021; Fountain 2022).

CBC strategies would be most effective in ecosystems where stink bugs complete at least one full generation, enabling the utilization of all available natural enemies throughout their life stages to impede population growth (Ademokoya et al. 2022).

2.4. Host plant preference

Feeding injury from *H. halys* to apples (Joseph et al. 2014), soybeans, and corn (Venugopal et al. 2015) has often been highest at crop edges adjacent to woodlands or woody orchard edges. A two-year study conducted by Bergh et al. (2021) in fruit orchards in the Mid-Atlantic (USA) confirmed that *H. halys* captures and fruit injury were mostly the highest at edges bordering woodlands and that other border habitats e.g., hedgerows also contributed significantly to captures and injury in some instances (Albrecht et al. 2020). Similarly, Rice et al. (2016) showed that landscapes with higher forest edges had higher rates of stink bug damage in tomatoes by

H. halys. These wooded edges are therefore considered “risky” borders for orchards because they can harbour large *H. halys* populations that can invade crops during the growing season. Similarly, growers with IPM and organic systems for apple and pear cultivation in Belgium have also reported higher damage by the native *P. rufipes* to fruits from orchards near wooded edges and woodlands (Peusens and Beliën 2012).

Insights into which host plants are preferred by economically important pentatomids like *P. rufipes* and *H. halys* in Belgium can therefore be useful to inform the strategic planting of mixed hedgerows and the sowing of wildflower plots by pip fruit growers (see section 4.4). Methods to infer host plant preference of insects include but are not limited to: direct observations and controlled feeding experiments (Siemann and Rogers 2003; Jogesh et al. 2008; Branson and Sword 2009; Fielding and Conn 2011; Fan et al. 2013), microscopical identification of ingested plants and pollen (Joern 1979, 1983; Silberbauer et al. 2004), stable isotope methods (Hood-Nowotny and Knols 2007), biochemical approaches (Stephens et al. 2008), and DNA-based methods (Matheson et al. 2008; Hereward and Walter 2012; García-Robledo et al. 2013; Frei et al. 2019; Wang et al. 2019; Avanesyan et al. 2021; Kheirodin et al. 2021).

2.4.1. Barcoding techniques

Over the last decade, scientific research has highlighted the effectiveness of DNA barcoding as the molecular tool for the rapid and precise identification of species (Antil et al. 2022). It uses short genetic markers or “barcodes” in an organism’s DNA to recognize it as belonging to a particular species, by comparing a DNA sample from the unidentified species to identified sequences present in a DNA barcode reference library. This is based on the principle of the barcoding gap, referring to the observed difference between the largest intra-species genetic distance and the smallest inter-species genetic distance. Ideally, there should be a clear and significant gap, meaning that the smallest genetic difference between two different species (inter-species variation) is still larger than the largest genetic difference within a single species (intra-species variation). If the barcoding gap is wide and clear, reliable species discrimination can be achieved (Antil et al. 2023).

For instance, the mitochondrial Cytochrome c Oxidase I (COI) gene is extensively used as a DNA barcode for the identification of invertebrate species (Bucklin et al. 2011; Gariepy et al. 2014). Due to its high rate of evolution and variability among different species yet relative conservation within a species the COI gene provides a reliable genetic marker for distinguishing closely related taxa. It has also shown utility in the separation of genetic lineages in relation to the reconstruction of invasion routes sometimes in combination with the Cytochrome c Oxidase

II (COII) region (Auger-Rozenberg et al. 2012; Gariepy et al. 2014; Chapman et al. 2015; Yan et al. 2021).

DNA barcoding holds several advantages over traditional identification methods. It can offer taxonomic clarity and a better species resolution. In the context of trophic relationships, DNA barcoding can detect host or prey species that might be overlooked during field observations.

Understanding the dietary preferences of predatory insects and herbivorous pest insects is crucial, particularly in the context of managing invasive pests in agriculture. For instance, using DNA barcoding, Harwood et al. 2007) revealed trophic interactions between *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) that supresses *Aphis glycines* (Matsumura) (Hemiptera: Aphididae), additionally identifying *Neohydatothrips variabilis* (Beach) (Thysanoptera: Thripidae), and an intraguild predator, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) as alternative food sources. Similarly, Ingels et al. (2013) detected hoverfly and lacewing DNA in the gut contents of *H. axyridis*, confirming intra-guild predation.

In the context of this research, barcoding techniques also allow for the identification of ingested plant species to infer feeding behaviours among insect herbivores and provide insight into their role within food webs (Miller et al. 2006; Jurado-Rivera et al. 2009; Valentini et al. 2009; Navarro et al. 2010; Bafeel et al. 2012; Traugott et al. 2013; Wallinger et al. 2013, 2015; Avanesyan 2014; Avanesyan and Lamp 2020). For instance, Avanesyan and Lamp (2020) utilized the gut content of the spotted lanternfly, *Lycorma delicatula* (White) (Hemiptera: Fulgoridae) to identify ingested plants using amplification of the rbcL barcode region and similarly for the grasshopper *Melanoplus femurrubrum* (De Geer) (Orthoptera: Acrididae) using the trnL barcode region (Avanesyan and Culley 2015).

DNA barcoding to reveal insect herbivore diets in its simplest form (assuming the presence of one hostplant in the gut) would typically involve the following key steps: (1) extraction of plant DNA from the gut, (2) amplification of specific plant DNA fragment (a barcode), (3) sequencing of the DNA fragment, and (4) comparison of the DNA fragment with reference databases for species identification (Fig. 2.12).

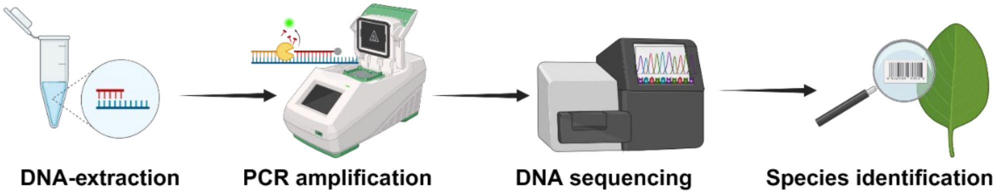

Figure 2.12. Overview of DNA barcoding for species identification. (Credit: Author)

Successful plant DNA recovery from insect gut contents has been demonstrated using various methods, including Qiagen DNA extraction kits and CTAB protocols. Target genes such as trnL, rbcL, matK, Internal Transcribed Spacer 1 (ITS1), and ITS2 have proven effective for amplifying plant DNA from digested material (Zhu et al. 2019). Techniques such as Sanger sequencing (DNA barcoding) and next-generation sequencing have been utilized to identify plant species in the diet of insects, honey sources, plant community compositions, and various species interactions (Hawkins et al. 2015; González-Chang et al. 2016; Pornon et al. 2016; Deiner et al. 2017; Dormontt et al. 2018; Adamowicz et al. 2019; Matesanz et al. 2019; Baksay et al. 2020; Kennedy et al. 2020).

Beyond conventional barcoding, diagnostic PCR assays with species-specific primers have proven effective for identifying the diets of insect herbivores (Kheirodin et al. 2021). These PCR methods are particularly useful for insects with a limited range of host plants. Eventually, the application of high-throughput DNA sequencing technologies (see section 2.4.2) has been pivotal in unravelling the intricate food web dynamics involving generalist insects. When herbivore insects like the Pentatomidae under study are highly polyphagous, the use of next-generation sequencing (NGS) technologies or metabarcoding can be especially useful to infer dietary preferences.

2.4.2. Metabarcoding techniques

DNA metabarcoding is a technique commonly used in ecological and molecular studies that involves sequencing DNA from multiple species in a sample to identify and quantify the diversity of the organisms present therein which is a powerful and efficient tool to infer the dietary preferences of polyphagous insects. Amongst other techniques, it can employ next-generation amplicon sequencing (NGAS) to achieve this. Unlike traditional Sanger sequencing, which processes a single DNA fragment at a time, NGS platforms can perform massive parallel sequencing of single DNA molecules, where millions of fragments are sequenced concurrently. The combined features of high throughput single-molecule DNA sequencing have facilitated a wide range of applications, from whole-genome sequencing, targeted gene sequencing, and transcriptome analysis (RNA-seq) to metagenomics and epigenomics (Taberlet et al. 2012).

During NGAS, the DNA is extracted from the sample of interest; from this extract, a DNA region of interest (a barcode) is amplified using traditional PCR. The PCR products, per sample, are then subjected to NGS where, depending on the sequencing depth, thousands or millions of fragments of interest per sample are sequenced.

DNA metabarcoding techniques on the gut content or faecal samples have been extensively used to reveal predator-prey relationships and herbivore diets in mammals, birds, and fish (Sow et al. 2020; Wang et al. 2022; Zurdo et al. 2023) as well as in predatory and herbivorous arthropods (García-Robledo et al. 2013; Zhu et al. 2019). However, to date, no studies have attempted to detect host plant DNA in herbivorous hemipterans with a piercing-sucking feeding mode like Pentatomidae.

Chapter 3

Distribution and genetic diversity of *Halyomorpha halys* in Belgium

This chapter is adapted from:

Distribution and genetic diversity of the invasive pest *Halyomorpha halys* (Hemiptera, Pentatomidae) in Belgium.

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Abstract

The brown marmorated stink bug, *Halyomorpha halys,* native to East Asia, is an invasive pest of economic importance. It has invaded North America and many European countries and is further expanding its range. In Belgium, it was first observed in 2011. *Halyomorpha halys* is known to cause severe damage in commercial fruit orchards and vegetable crops. A dramatic and unmitigated expansion of *H. halys* in its adventive range could lead to significant economic implications for agricultural production. In this study, occurrence data of *H. halys* since its first observation in Belgium was analysed together with molecular information to map the populations and evaluate the genetic diversity of this pest. The genetic diversity of *H. halys* in Belgium was compared to data from other invaded and native countries reported in previous studies to identify possible invasion routes. The analysis of 1176-bp of mitochondrial DNA cytochrome c oxidase I and II genes (COI and COII) led to the discovery of two novel COI-COII haplotype combinations currently unique to Belgium. The invasion of *H. halys* in Belgium is likely the result of multiple and ongoing introductions from its native region and from already invaded countries in Europe, particularly Italy. The expansion of the brown marmorated stink bug populations in Belgium is recent and ongoing. Presently, it appears to thrive best in northern Belgium.

3.1. Introduction

Halyomorpha halys (Hemiptera: Pentatomidae) or the brown marmorated stink bug is a stink bug of economic importance, native to East Asia and an invasive pest species in Europe, North America, and other regions (Hoebeke and Carter 2003; Leskey et al. 2012c, 2012b; Haye et al. 2015; Hamilton et al. 2018; Zhu et al. 2019). In Europe, *H. halys* has already been detected as far north as The Netherlands (Aukema 2019), Belgium (Claerebout et al. 2019) and the north of Germany (Hartung et al. 2022). Due to its polyphagous nature, *H. halys* can feed on virtually all primary fruit and vegetable crops in its invaded areas (Kuhar et al. 2012; Haye et al. 2015). For example, in northern Italy, it has become a key pest in fruit orchards, with an estimated economic impact of €588 million on the production of pear, apple, peach, and kiwi in 2019 (Bulgarini et al. 2021). *Halyomorpha halys* causes damage through its feeding activity. It inserts its stylets into the plant and injects saliva, pre-digesting the plant tissue before feeding on it (Rice et al. 2014). This feeding behaviour causes scarring, deformities, pitting, and discolouration of the product, rendering it unmarketable or even inedible (Rice et al. 2014; Bulgarini et al. 2021). Since its first record in Belgium in 2011, observations of *H. halys* on public citizen science databases (Waarnemingen.be) only appeared in 2017. Currently it is assumed that *H. halys* has established univoltine overwintering breeding populations in Belgium (Claerebout et al. 2019). An observation of *H. halys* from the Haspengouw region in July 2021 indicated that *H. halys* is already present in commercial pip fruit orchards in Belgium. However, to date, no dramatic population increases, mass occurrences or damage has been reported in fruit production in Belgium (Berteloot et al. 2021). In 2022, in Belgium, 219.000 t of apples and 366.000 t of pears, some of the most vulnerable fruits to *H. halys*, were produced (European Commission, 2022). Apples and pears represent a combined revenue of over €148 million of which pear production is the most significant part with a revenue of €119 million (Verbond van Belgische Tuinbouwcoöperaties, 2022). An unmitigated expansion of *H. halys* in Belgium could therefore have a considerable impact on fruit production, especially in the Flanders region, which produces most of the apples, pears, and soft fruits in the country.

The increased accessibility and affordability of molecular technologies, as well as the expansion of databases containing publicly available DNA sequence data, have aided in the use of molecular tools to assess the genetic diversity and potential origin of invasive species (Cristescu 2015; Hamelin and Roe 2020). Due to its lack of recombination, relative neutrality and shorter coalescence time, mitochondrial DNA (mtDNA) is a highly popular molecular marker for examining genetic diversity and phylogeography of (invasive) animal species (Ficetola et al. 2008; Rollins et al. 2013; Bras et al. 2019; Ryan et al. 2019). Furthermore, for invasive species, the accumulation of easily reproducible genetic data in public databases facilitates the regular addition of new data from the invasion front to build a global pattern of invasion dynamics. For example, for *H. halys*, mtDNA sequences (portions of the mitochondrial gene cytochrome c oxidase subunits I and II - COI and COII, respectively) are already available for both native (China, Japan, Korea) and invaded countries (Gariepy et al. 2014; Xu et al. 2014; Cesari et al. 2015, 2018; Lee et al. 2018; Yan et al. 2021).

In this study, we set out to fill one of the geographical knowledge gaps in Europe on the genetic diversity and distribution of *H. halys*. First, we investigated the genetic diversity of *H. halys* in Belgium by sequencing 99 specimens from 18 locations at two mitochondrial genes: COI and COII. Then, we compared those sequences to previously published *H. halys* sequences to infer the putative invasion routes to Belgium. Lastly, we used public citizen occurrence data to map the distribution of *H. halys* in Belgium from 2020 to 2022 and gain demographic insights into the Belgian populations.

3.2. Materials and Methods

3.2.1. Insect sampling

Halyomorpha halys specimens were collected from 18 locations in Belgium (Supplementary material I) from agricultural sites and private gardens in 2021 and 2022 using baited traps (dual lure from Trécé Inc., Adair Oklahoma, USA) or by beating of the lower part of a tree trunk/shrub. The collected samples were stored dry or in 70% ethanol at -20 °C until used for molecular analysis.

3.2.2. DNA extraction & sequencing

DNA was extracted from two legs using a Chelex extraction method (Walsh et al. 1991). Briefly, the legs were crushed with a sterile pestle in 100 µl of 5% Chelex® 100 (Bio-Rad, USA) solution. They were then incubated at 85 °C for 90 min, and the supernatant was collected after 3 min of centrifugation at 12,000 rpm. The cytochrome oxidase subunit I (COI) and II (COII) barcode regions of the mitochondrial DNA were amplified through PCR. For the COI region, the LCO 1490 (5'- GCTCAACAAATCATAAAGATATTGG-3') and HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') primers (Folmer et al. 1994) were used. For COII, the HhalysCO2F2 (5'-TAACCCAAGATGCAAATTCT-3') and HhalysCO2R2 (5'-CCATATATAATTCCTGGACGA-3') primers (Xu et al. 2014) were used. For both regions, the following PCR cycles were used: initial denaturation at 94 °C for 3 min, 38 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 30 s and extension at 72 °C for 45 s, followed by a final extension step at 72 °C for 7 min. The PCR product quality was checked on agarose gel. Both strands (forward and reverse) for each barcode region were sequenced (Eurofins, Germany GmbH) by Sanger sequencing.

3.2.3. Haplotype diversity analysis

Forward and reverse sequences were trimmed and assembled into a consensus sequence using CodonCode Aligner (version 10.0.2). COI and COII sequences obtained in this study were compared to COI and COII sequences from Yan et al. (2021) and Cesari et al. (2018), retrieved from GenBank and BOLD. Other previously conducted studies on the genetic diversity of *H. halys* only sequenced COI or COII fragments (or only reported unique haplotypes found without specifying which samples are associated with which haplotypes) (Gariepy et al. 2014, 2015; Xu et al. 2014; Valentin et al. 2017; Lee et al. 2018; Kapantaidaki et al. 2019). All individual COI and COII haplotypes found in this study have already been reported in other publications (see results section 3.3) and were given the haplotype name used in those publications (Cesari et al. 2018; Yan et al. 2021). However, for COI, there were discrepancies between the sequences of haplotypes with the same name found in Cesari et al. (2018) and Yan et al. (2021). Haplotypes found in our study were named after the reference haplotypes of the study of Valentin et al. (2017) if a sequence match was found. Haplotype names from Cesari et al. (2018) were distinguished with the letter “c” (Table 3.1). The sequence of haplotype H41 from Yan et al. (2021) perfectly matched the H41 haplotype from Valentin et al. (2017) but corresponded to the sequence of H46 from Cesari et al. (2018). Therefore, the name of the sequence of this haplotype was retained in the present study as H41. Another sequence found in this study without a match in Valentin et al. (2017) or Yan et al. (2021) was already named H41 in Cesari et al. (2018) and was renamed “H41c” to distinguish it. For H40 and H43, the same haplotype names were given to different sequences by Cesari et al. (2018) and Yan et al. (2021). Again, those sequences were compared to the ones of Valentin et al. (2017). The H40 and H43 sequences from Yan et al. (2021) perfectly matched the identical name sequences from Valentin et al. (2017), and these names were retained in the present study. A letter “c” was added to the H40 and H43 sequences from Cesari et al. (2018) to distinguish them. Finally, the H42 and H49 sequences from Cesari et al. (2018) did not match the H42 and H49 haplotypes from (Valentin et al. 2017) (no H42 and H49 haplotypes in Yan et al. (2021) for comparison). To be consistent, the letter “c” was added to the H42 and H49 sequences from Cesari et al. (2018).

Sequences were aligned and analysed in R v4.0.2 (R Core Team 2024), using packages msa (Bodenhofer et al. 2015), adegenet (Jombart 2008) and pegas (Paradis 2010). The relationships among haplotypes within Belgium and between haplotypes from Belgium and other countries were investigated through haplotype networks and genetic diversity indexes (haplotype, Hd, and nucleotide diversity, π). We tested for the presence of a genetic structure in Belgium by performing a Mantel test (Euclidean) on genetic and geographic distance matrices between collected specimens.

Table 3.1. Summary of the discrepancies between sequences of COI-haplotypes with the same name in different studies and the names used in the present study.

Valentin et al. (2017)	Cesari et al. (2018)	Yan et al. (2021)	This study
H40	/	H40	H40
/	H40	/	H40c
H41	H46	H41	H41
/	H41	/	H41c
/	H42	/	H42c
H43	/	H43	H43
/	H43	/	H43c
/	H49	/	H49c

3.2.4. Occurrence data

Occurrences from January 1st, 2017, to December 31st, 2022 were obtained from the publicly available citizen science database (Waarnemingen.be). For *H. halys*, the recorded occurrences on the public citizen science database are quality-controlled, and the taxonomic accuracy of each observation is confirmed or rejected by a specialist through photos. The occurrence data was additionally examined to check the validity of all the occurrences. Only occurrences with a high confidence level in the taxonomic identification of *H. halys* and with spatial coordinates were used. Additionally, specimens collected during this research were identified through morphological identification using the *H. halys* identification key from (Maistrello et al. 2016) and confirmed by our molecular analysis. The occurrence data was visualised with QGIS 3.30.1 (QGIS Development Team 2023).

3.3. Results

3.3.1. DNA sequences

COI: 99 *H. halys* sequences were retrieved out of 99 specimens collected. All specimens yielded a 658-bp DNA sequence. The DNA sequence data and specimen collection information were made accessible on GenBank with accession numbers OR581617-OR581715.
COII: 93 sequences with a length of 518-bp were retrieved from 99 *H. halys* specimens collected. The DNA sequence data and specimen collection information were made accessible on GenBank with accession numbers OR602454-OR602546.

3.3.2. Haplotype diversity of *H. halys*

For the COI fragment individually, nine distinct haplotypes were found, consisting of 14 polymorphic sites (Fig. 3.1a). Three haplotypes were dominant (H01, H03 and H08) and were shared by roughly 80% of the individuals collected. Haplotype diversity was 0.77 ± 0.02 (mean ± SD). The obtained COI sequences from this study were compared with 609 COI sequences from other European countries, Chile, the USA, China, and Japan. H01 and H03 haplotypes are shared with those in invaded areas in Europe and America, as well as with those in the native area of China, but not with Japan. Haplotype H08 is shared only with some invaded areas in Europe (Austria and Italy). The remaining haplotypes are shared with Italy (H40c, H41, H41c, H42c and H49c), Japan (H23 and H41) and Hungary (H41).

For the COII fragment individually, five distinct haplotypes were found, consisting of 5 polymorphic sites (Fig. 3.1b). Haplotype diversity was 0.70 ± 0.01. Our COII sequences were compared with 651 COII sequences from other European countries, Chile, the USA, China, and Japan. Haplotypes h01, h03 and h11 accounted for 91 out of 95 haplotypes from Belgium. Haplotypes h01 and h03 are shared with invaded areas in Europe, the USA and China but not with Japan. Haplotypes h11 and h15 are only shared with some other European countries (Austria, Hungary, and Italy) and Japan. Haplotype h21 is shared only with Italy.

The resulting concatenated 1176-bp sequences rendered 12 distinct COI-COII haplotypes among 95 specimens consisting of 22 polymorphic sites in total (Fig. 3.1c). Two novel COI-COII haplotypes were identified (H03h11 and H08h21) in our samples; these haplotypes are unique to Belgium. The ten other haplotype combinations were previously reported in the literature. The comparison of COI-COII haplotypes from Belgium to previously published sequences confirm the pattern observed for individual loci. The most frequent haplotype H01h01 is shared with all native and invaded countries included in this study (Table 3.2). H03h03, the second most frequent haplotype has been recorded in Austria, Chile, China, Italy, and Hungary. Some of the remaining haplotypes (H03h11, H03h21, H41ch03 and H49ch03) are only shared with Italy (and Austria for H08h11) and are close to haplotypes from China. Others (H23h11, H41h15 and H41ch11) are shared with Italy (and Hungary for H41h15) and/or with (or close to) Japan (H46h15, H42h11, H23h11 and H08h11). Finally, the H08h21 and the H03h11 haplotypes are unique to Belgium and are close to the haplotypes from Italy (within the group of haplotypes found in most invaded countries and China).

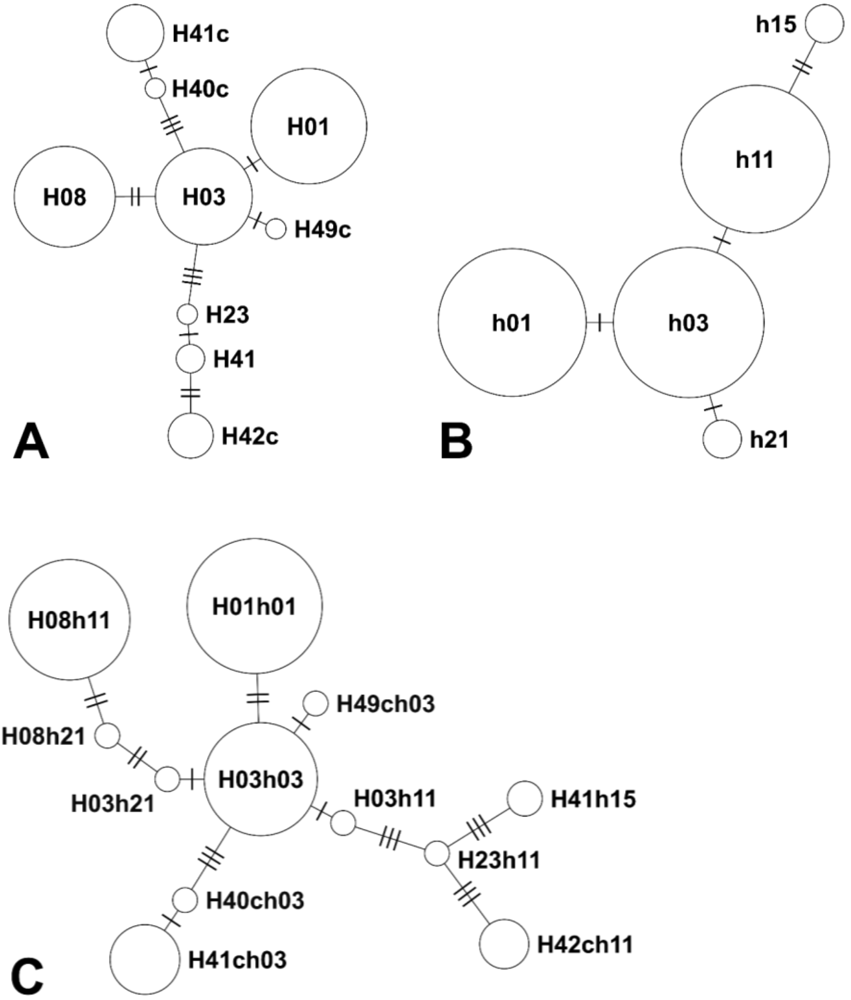

Figure 3.1. Haplotype network using **A.** COI, **B.** COII and **C.** COI-COII fragments of *H. halys* in Belgium. The circle size is proportional to the square root of the frequency of the haplotypes. The tick marks on each line represent a base pair difference.

Table 3.2. Summary table of mtDNA (COI-COII) diversity by country. With N: sample size, Hn: number of haplotypes, h: haplotype diversity and π: nucleotide diversity (only countries with available COI-COII sequences are listed).

Continent	Country	First record	N	Hn	Hd ± SD	π ± SD	Study
Asia	China	native	90	24	0.86 ± 0.02	0.0033 ± 0.0018	Yan et al. 2021
	Japan	native	65	32	0.94 ± 0.01	0.0024 ± 0.0014	Yan et al. 2021
	Turkey	2017 (Güncan and Gümüş 2019)	11	1	0	0	Yan et al. 2021
Europe	Austria	2015 (Rabitsch and Friebe 2015)	15	4	0.69 ± 0.10	0.0021 ± 0.0013	Yan et al. 2021
	Belgium	2011 (Claerebout et al. 2018)	95	12	0.79 ± 0.02	0.0031 ± 0.0018	This study
	Georgia	2015 (Gapon 2016)	31	1	0	0	Yan et al. 2021
	Greece	2011 (Milonas and Partsinevelos 2014)	8	3	0.61 ± 0.16	0.0025 ± 0.0017	Cesari et al. 2018
	Hungary	2014 (Vétek et al. 2014)	90	3	0.11 ± 0.04	0.0003 ± 0.0003	Yan et al. 2021
	Italy	2012 (Maistrello et al. 2014)	16	18	0.72 ± 0.03	0.0028 ± 0.0016	Cesari et al. 2018, Yan et al. 2021
	Romania	2015 (Macavei et al. 2015)	30	1	0	0	Cesari et al. 2018
	Serbia	2015 (Šeat 2015)	13	5	0.61 ± 0.07	0.0014 ± 0.0008	Yan et al. 2021
	Slovenia	2017 (Rot et al. 2018)	15	3	0.51 ± 0.12	0.0012 ± 0.0008	Yan et al. 2021
North America	United States	2001 (Hoebeke and Carter 2003)	24	1	0	0	Yan et al. 2021
South America	Chile	2017 (Faúndez and Rider 2017)	31	2	0.06 ± 0.06	0.0001 ± 0.0002	Yan et al. 2021

The results of our Mantel test indicated a significant but weak correlation (r = 0.14, $p = 0.001$) between the genetic distance and the geographical distances for pairs of individuals. High haplotype diversity was observed in Belgium, Hd = 0.79 ± 0.02, with a nucleotide diversity value of $\pi = 0.0031 \pm 0.0018$ (Table 3.2). This level of genetic diversity was lower than that of native regions Japan and China (Hd = 0.94 ± 0.014 and 0.86 ± 0.023, respectively) but similar to Italy (Hd = 0.72 ± 0.033) (Table 3.2). Other invaded areas of *H. halys* typically had lower haplotype diversity but their estimation may be approximate due to shallower sampling depth (low sample size and/or few sampling locations; Table 3.2).

3.3.3. Distribution of *H. hal*ys in Belgium

The 740 observations from 6 years (2017-2022) were checked for accuracy and completeness. Since the first record in 2011, occurrences in subsequent years initially remained low, without any public citizen database records or specimens collected until 2017. *Halyomorpha halys* was recorded once in 2017 and 2018, 5 times in 2019 and 35 times in 2020. In recent years, the number of observations of *H. halys* has increased dramatically. In 2021, 183 observations were recorded, followed by a substantial increase to 515 in 2022. Up until November 2023, the Belgian public citizen database reported over 2200 observations, a more than tenfold increase compared to 2021. From the occurrences of *H. halys* in 2020-2022, most of the observations were made in northern Belgium, mainly around the urban areas of the cities Gent, Leuven, and Mechelen and the region of Haspengouw (Fig 2a-c).

Figure 3.2. Map of Belgium with recorded occurrences of *H. halys* in **A.** 2020, **B.** 2021, and **C.** 2022. Each dot is an individual record, and density clouds indicate the level of density of occurrences in one area. Data from citizen-science database (waarnemingen.be).

3.4. Discussion

This study uncovered 9 COI, 5 COII and 12 COI-COII distinct haplotypes from 99 *H. halys* specimens collected in Belgium. Among these, two new COI-COII haplotype combinations (H03h11 and H08h21), currently unique to Belgium, were observed.

The principal COI haplotypes present in Belgium were H01 (33%), H08 (24%) and H03 (23%). H01 and H03 are the most frequent haplotypes in China and in most invaded countries (Gariepy et al. 2014, 2015, 2021; Valentin et al. 2017; Cesari et al. 2018; Lee et al. 2018; Yan et al. 2021). As such, their presence in Belgium is to be expected and therefore does not help to determine the origin of *H. halys* in Belgium. Interestingly, however, H08 is the second most abundant haplotype in Belgium. So far, this haplotype has been exclusively detected in certain European countries: Austria, France, Italy, Switzerland and Serbia (Valentin et al. 2017; Cesari et al. 2018; Gariepy et al. 2021; Yan et al. 2021). This suggests that at least part of the *H. halys* population in Belgium originates from previously invaded territories in Europe (i.e., bridgehead effect; (Lombaert et al. 2010). Remarkably, eight out of the nine COI haplotypes found in Belgium are shared with Italy. Furthermore, half of these haplotypes were reported exclusively in these two countries (H40c, H41c, H42c and H49c) suggesting that Italy is a direct region of origin of *H. halys* in Belgium. The rare H23 haplotype was only found once in Belgium and has been reported in Japan and the Western USA (Oregon) (Valentin et al. 2017). It was also detected in a shipment in Australia originating from Italy, suggesting that the H23 haplotype is also present in Italy (Horwood et al. 2019; Gariepy et al. 2021). Introductions may thus have originated from Japan or the USA in addition to Italy.

For the COII fragment, h01, h03 and h11 each accounted for 32% of the abundance. The h01 and h03 haplotypes are native to China and Korea (but have not been reported in Japan yet) and are the most frequent COII haplotypes in invaded countries (Xu et al. 2014; Cesari et al. 2018; Yan et al. 2021). The distribution of the h11 COII haplotype is more restricted and has only been reported in Austria, Italy, Japan, and Korea (Xu et al. 2014; Cesari et al. 2018; Yan et al. 2021). Consistent with Italy as a source of *H. halys* for Belgium, the h21 haplotype was isolated from two specimens collected in Belgium and is only shared with Italy (Cesari et al. 2018).

In this study, the combination of COI and COII fragments did not result in a significantly better geographic resolution to reveal possible origins of the invasion. However, the presence of haplotype H03h21 provides some additional support for Italy as a source of the invasion since this combination is only shared with a sample from the Piedmont region of Italy (Cesari et al. 2018). Additionally, the inclusion of the COII fragment in this study revealed two novel haplotype combinations unique to Belgium: H08h21 and H03h11. The former is likely to originate from Italy since haplotype h21 has been exclusively reported there (Piedmont region, same sample as for H03h21), while H08 has been detected multiple times (Piedmont and Lombardy regions of Italy). H03h11 is more singular because it combines a COI haplotype native to China (H03) and a COII haplotype native to Korea or Japan (h11; see above). Such a combination could arise from recent COI or COII mutations. H03 is only two substitutions from the closest COI haplotype native to Japan (H27), and h11 is only one substitution from three COII haplotypes native to China (h03, h05 and h10). Alternatively, this combination could also exist in the regions of origin (China and/or Japan/Korea) but has not yet been sampled. Belgium's dominant haplotypes generally overlap with those from previously invaded European countries, such as Italy, Switzerland, Austria, and Hungary. In contrast, rarer haplotypes are exclusively shared with Italy and its native region of Japan (H23).

The genetic diversity found in Belgium is surprisingly high (Hd = 0.79, π = 0.0018, N = 95) compared to other invaded countries previously studied (Table 3.2), several distinct haplotypes were isolated from single sampling sites (Supplementary material I). A reduced genetic diversity is usually expected for introduced species because of a limited number of founders associated with early genetic drift. However, genetic bottlenecks associated with introductions can be counteracted if many individuals are introduced at the same time or if repeated introductions occur (Dlugosch and Parker 2008). Similarly, Schuler et al. (2021) found a high haplotype diversity (Hd = 0.68, π = 0.0046, N = 156) in the *H. halys* population of South Tyrol in Northern Italy. The population in Tyrol was established by a secondary introduction from Eastern USA into the Emilia Romagna region of Italy and the spread of the founder population in Switzerland to the neighbouring countries. In contrast, (Valentin et al. 2017) concluded that most invasive populations in North America and Europe were established from a direct introduction of *H. halys* from China with separate introductions into Eastern and Western USA and Canada, as well as into Switzerland and Greece. The high genetic diversity of *H. halys* populations in Belgium and the overlap of haplotypes with previously invaded European countries, such as Italy, Switzerland, Austria, and Hungary but also with Japan indicates that the Belgian populations probably originated from multiple invasions from already invaded European countries, mainly Italy, but also directly from Japan through inadvertent human-mediated transportation (often due to global trade of goods) (Valentin et al. 2017). The weak genetic structure detected by our Mantel test could be related to the recent local expansion of these genetically diverse clusters.

Previous studies have shown that citizen science provides valuable data to characterise the spread of *H. halys* (Maistrello et al. 2016; Stoeckli et al. 2020; Streito et al. 2021). From our data in Belgium, more *H. halys* observations are recorded in the northern part of Belgium compared to the southern part of Belgium, with more urban areas than agricultural, forested, and rural areas. However, on a national scale, spatial biases are commonly associated with human population density, settlements, and road infrastructure (Kelling et al. 2015; Geldmann et al. 2016; Girardello et al. 2019). The bias towards urban areas in citizen-collected occurrence data and rising popularity of tools to make observations (e.g., smartphones) represents a challenge to infer true spatio-temporal patterns from our occurrence data (Bowler et al. 2022). Nevertheless, it also provides natural experimental gradients to examine the impacts of future environmental scenarios including climate change (Lahr et al. 2018). Urban areas are often seen as heat islands, offering more suitable refugia to survive winters or more favourable conditions throughout the seasons to develop and reproduce. Therefore, these urbanised areas often comprise a larger population of insects (Kaiser et al. 2016; Frank and Backe 2022). According to Kistner (2017), the northern part of Belgium lies near the latitudinal border of the climate in Europe suitable for the winter survival and summer reproduction of *H. halys*. In contrast, the southern part of Belgium is currently modelled to be an unsuitable eco-climatic region for *H. halys,* with colder summer and winter temperatures and fewer refugia to survive winter due to the lower urbanisation (Kistner 2017). Niche modelling through climate suitability by Streito et al. (2021) essentially confirms *this*, with the northern part of Belgium having more suitable areas for *H. halys* while the southern part is more marginal to unsuitable for its survival and expansion. Despite the possibility of spatio-temporal bias in our occurrence data, some temporal trends can still be derived from citizen-collected occurrence data (Powney et al. 2019; Outhwaite et al. 2020; Sheard et al. 2021; Zattara and Aizen 2021; Bowler et al. 2022). Our observational data shows an almost 15-fold increase in observations from 2020 to 2022. However, to date, no damage related to *H. halys* has been reported in agricultural production in Belgium (Berteloot et al. 2021). Based on the occurrence data, it is likely that the population expansion of *H. halys* in Belgium is very recent, as a relatively high number of observations were only made in 2021, 2022 and 2023. Streito et al. (2021) define three main phases in the invasion of *H. halys:* (1) the very beginning of the invasion, when populations of *H. halys* are low, and naturalists and official monitoring services who are excellent observers and expecting the emergence of *H. halys* can detect its presence, (2) when abundance increases, non-naturalist citizens are able to detect the species and start to provide information on the dynamics of the invasion and indirectly on the level of populations through citizen science platforms, and (3) when populations have expanded and become large enough, agricultural professionals take over and can predict and assess the population density and damages. From our occurrence data, the *H. halys* population in Belgium is seemingly under expansion, being numerously recorded in the citizen science databases.

Lastly, only the adult stadium of *H. halys* can overwinter by going into a diapause state. Adults are highly mobile and are therefore often successful in finding a place to survive the winter. Adults that exit diapause in the spring can then reproduce as soon as temperatures become favourable, producing offspring throughout the spring and summer, expanding the population. In current climatic conditions populations are likely to decline significantly in the winter due to Belgium being situated at the northern latitudinal border of climatic suitability for *H. halys*, with winters cold enough to kill more individuals than winter temperatures in southern European countries like Italy, possibly delaying the fast expansion of its populations. However, both parts of Belgium are modelled to be suitable for the survival and development of *H. halys* by 2100 (Kistner 2017).

3.5. Conclusions

The results of this study provide haplotype information for *H. halys* from a newly invaded region. The haplotype diversity in Belgium is surprisingly high, with 9 COI, 5 COII and 12 COI-COII haplotypes found. The invasion of *H. halys* in Belgium likely occurred repeatedly and is assumed to be still ongoing through human-mediated transportation from other invaded European countries and directly from its native regions in Eastern Asia. A significant overlap between Belgian and Italian haplotypes points to Italy as the most probable source for a significant proportion of haplotypes currently present in Belgium. By combining the citizen-collected occurrence data with the molecular data, we assume the population expansion of *H. halys* is recent and ongoing.

3.6. Acknowledgements

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Chapter 4

Diet preference of *Halyomorpha halys* and *Pentatoma rufipes* through metabarcoding

This chapter is adapted from:

Unveiling the diet of two generalist stink bugs, *Halyomorpha halys* and *Pentatoma rufipes* (Hemiptera: Pentatomidae), through metabarcoding of the ITS2 region from gut content.

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Abstract

The use of DNA metabarcoding has become an increasingly popular technique to infer feeding relationships in polyphagous herbivores and predators. Understanding host plant preference of native and invasive herbivore insects can be helpful in establishing effective Integrated Pest Management strategies (IPM). The invasive *Halyomorpha halys,* and native *Pentatoma rufipes* are piercing-sucking stink bug pests that are known to cause economic damage in commercial fruit orchards.

In this study, we performed Molecular Gut Content Analysis (MGCA) on field-collected specimens of these two herbivorous pentatomids using next-generation amplicon sequencing (NGAS) of the Internal Transcribed Spacer 2 (ITS2) barcode region. Additionally, a laboratory experiment was set up where *H. halys* was switched from a mixed diet to a monotypic diet, allowing to determine the detectability of the initial diet in a time series of up to 3 days after the diet switch. We detected 68 unique plant species from 54 genera in the diet of two stink bug species, with fewer genera found per sample and a smaller diet breadth for *P. rufipes* than for *H. halys.* Both stink bug species generally prefer deciduous trees over gymnosperms and herbaceous plants. Landscape type significantly impacted the observed genera in the diet of both stink bug species, while season only had a significant effect on the diet of *H. halys*.

This study provides further insights into the dietary composition of two polyphagous pentatomid pests and illustrates that metabarcoding can deliver a relevant species-level resolution of host plant preference.

4.1. Introduction

Evaluating insect-plant feeding dynamics presents a significant challenge in food-web ecology (Schoonhoven et al. 2005). Understanding the relationships between herbivorous insects and their host plants is critical when the insects of interest are agricultural pests, as it can hold substantial implications for pest management (Bergmann et al. 2016; Zhu et al. 2019; Avanesyan and Lamp 2020). Characterizing ecological host ranges of polyphagous herbivores in agricultural habitats can allow the identification of host plant preferences for oviposition, development, or survival, thereby informing more effective control measures (Bergmann et al. 2016; Avanesyan et al. 2021).

Halyomorpha halys (Stål) and *Pentatoma rufipes* (L.) (Hemiptera: Pentatomidae) are herbivorous stink bugs of economic interest, attacking a range of agricultural crops (Hoebeke and Carter 2003; Haye et al. 2015; Powell 2020).

The brown marmorated stink bug, *H. halys*, is native to eastern Asia and has invaded the USA, Europe, and elsewhere. In Europe, it has been present since 2004 and has continued to invade European countries towards northern latitudes (Lee et al. 2013; Haye et al. 2015; Berteloot et al. 2021; Hartung et al. 2022). Over the last decade, *H. halys* has emerged as a major agricultural pest in its invaded areas, leading to substantial economic impacts on various crops, such as pears, apples, corn, and hazelnuts (Leskey et al. 2012b; Costi et al. 2017; Bosco et al. 2018; Stahl et al. 2019b; Bulgarini et al. 2021; Francati et al. 2021).

The forest bug, or red-legged shield bug, *P. rufipes*, is native to the Palearctic region (Powell 2020). It has mainly been linked to damage in apple and pear orchards in Germany, Switzerland, and Belgium (Beliën et al. 2015; Alkarrat et al. 2020; Powell 2020).

When polyphagous stink bugs like *H. halys* or *P. rufipes* invade agricultural cropping systems such as orchards, the damage can rapidly increase (Powell 2020; Berteloot et al. 2021). Organic orchards, in particular, are at high risk of economic losses (Beliën et al. 2015). The feeding activity of both stink bugs causes pitting and deformities in developing fruits like apples and pears, rendering the agricultural product inedible and unmarketable (Rice et al. 2014; Powell 2020).

A well-established practice in IPM is the use of annual or perennial wildflower strips and mixed hedgerows acting as a refuge area for beneficial arthropods, including pollinators, as well as predators and parasitoids, which may assist in the biological control of pests, like stink bugs (Beliën et al. 2021; Fountain 2022). However, such wildflower strips and hedgerows may also offer resources to pests, including stink bugs, supporting them in part of their life cycle. These landscape elements may thus also serve as sources of infestation, increasing the risk of damage to the crop (Garratt et al. 2017).

Therefore, a more profound insight into the diet of two harmful stinkbugs under study can be valuable to IPM as it may allow to identify preferred host plants and develop strategies that avoid the use of suitable host plants for these pests.

Various approaches to investigate insect host plant relationships have been used: these include direct observations of phytophagy in the field (Bakken et al. 2015; Bergmann et al. 2016; Liu 2019; Hadden et al. 2023), laboratory feeding trials (Cooperband et al. 2019), biochemical approaches (Stephens et al. 2008), isotope techniques (Hood-Nowotny and Knols 2007) and microscopical identification of ingested plant parts (Joern 1979, 1983). The latter technique is unsuitable for pentatomids like *P. rufipes* or *H. halys,* given their piercing-sucking mode of feeding (McPherson et al. 2000). Observational methods are relatively simple to perform and require no complex equipment. However, they greatly depend on sampling conditions (e.g., weather or field conditions). Moreover, results can be obfuscated by seasonality, cryptic behaviour, and probing (Wiman et al. 2014; Lowenstein and Walton 2018).

In contrast to more traditional techniques, molecular approaches to detect host plants in herbivore insects use polymerase chain reaction (PCR) and Sanger sequencing techniques to amplify plant barcode genes from insect body DNA samples (Matheson et al. 2008; Hereward and Walter 2012; García-Robledo et al. 2013; Frei et al. 2019; Wang et al. 2019; Avanesyan et al. 2021; Kheirodin et al. 2021).

In recent years, high throughput DNA metabarcoding techniques, including next-generation amplicon sequencing (NGAS), have helped in furthering the understanding of the diet of polyphagous insect herbivores (Moorhouse-Gann et al. 2018; Avanesyan and Lamp 2020; Cao and Dietrich 2021; Gariepy et al. 2021). During NGAS, amplicons are generated through PCR using specific primers to target a specific region of interest (e.g., a barcode). This PCR product, containing the amplicons, is then subjected to Next-Generation Sequencing (NGS) technology. The resulting metabarcoding data can then be used for various applications, including molecular gut content analysis (MGCA) (Masonick et al. 2019). Next-generation sequencing techniques, like amplicon sequencing, can greatly improve host plant detection, especially when the plant-herbivore relationships are of a polyphagous nature. Multiple plant barcode amplicons can be detected in one sample with a high throughput (Bowser et al. 2013; Gerwing et al. 2016)· Furthermore, in comparison to traditional methods (e.g., observations), DNA metabarcoding through NGAS can provide a greater taxon depth, more accurate identification of host plants, and higher throughput with a predictable cost and time frame (Smart et al. 2017; Bell et al. 2021). Complementary to metabarcoding techniques, the host plant can be recorded during insect sampling to validate molecular results.

Several molecular markers (barcodes) have been widely used for plant species identification, including rbcL, matK, trnH-psbA, and the internal transcribed spacer (ITS) of nuclear ribosomal DNA. More recent studies find that the ribosomal internal transcribed spacer 2 (ITS2) provides high-resolution results at family, genus, and species level even in identifying plant material from arthropod guts (Chen et al. 2010; Cheng et al. 2016; Moorhouse-Gann et al. 2018; Zhu et al. 2019). For instance, the use of ITS2 in pollen DNA metabarcoding is already an established technique to reveal bee foraging strategies (Richardson et al. 2015; Sickel et al. 2015; Smart et al. 2017; Bänsch et al. 2020; Milla et al. 2021). Nevertheless, attempts to detect host plant DNA in polyphagous sap-sucking insects, such as Hemiptera, have been limited to date (Zhu et al. 2019; Cao and Dietrich 2021) or completely absent for specialized feeders like Pentatomids.

In this study, we introduce a novel protocol to assess the diet composition of *P. rufipes* and *H. halys* using NGAS of the ITS2 barcode region. First, we collected specimens of both pentatomids in different habitats, including orchards and gardens in rural and urban environments, to identify gut content under natural conditions. Second, we conducted a time series feeding trial. Here, *H. halys* was fed a known mixed diet of two host plant species before being switched to a monotypic diet with a different plant. The primary objective of the feeding trial was to determine how long after a host switch the first host plant could still be detected. As a secondary outcome, the feeding trial also served as a validation step for the taxonomic results generated from the field samples. This mixed diet time series was included in the NGAS.

4.2. Materials and Methods

4.2.1. Rearing

Halyomorpha halys adults were collected in the field in Gentbrugge, Belgium. The collected adults were then further reared in a climatic chamber (PHCBI MLR-352H-PE, Japan) set at 24 ± 0.5 °C, 65 ± 5% RH, with a photoperiod of 16:8 h (L:D) in clear polypropylene boxes (40 x 30 x 20 cm) provided with 4 x 8 cm meshed vents on each side. Bean pods (*Phaseolus vulgaris* L. cv. ‘Helda’) and sunflower seeds (*Helianthus annuus* L.) were provided as a food source. Water was supplied via moistened synthetic cotton (Roltasoft Hartmann, Germany). Crumpled paper towels were placed in the rearing boxes to provide shelter and surfaces for laying eggs. Food and water were replaced twice per week.

4.2.2. Field-captured specimens

From 2019-2022, both male and female adults of *H. halys* and *P. rufipes* were collected in commercial organic orchards (apple, pear, and mixed crops), private orchards, and urban gardens in Flanders, Belgium. Twenty-five locations were sampled throughout northern Belgium (Fig. 4.1). Specimens of *P. rufipes* were hand-caught by scanning trees and wildflower plots in and around the sampling area for 30 min, spending 1 minute per tree or per herbaceous plot; host plants were recorded. Specimens of *H. halys* were collected using pyramid-shaped live traps and sticky traps baited with pheromones (Pherocon Trécé BMSB) hung in trees and checked twice per week. A general description of sampling locations can be found in Supplementary table I.

Collected specimens were placed into clean 1.5 mL tubes (Eppendorf) and frozen on dry ice to be stored in the lab at -80 °C. Prior to DNA extraction, each stink bug body was dipped in 1 mL of a 1% bleach solution. The alimentary canal was dissected from crop till rectum, flash frozen in liquid nitrogen in a 1.5 mL tube with two stainless steel beads (5 mm Ø), and lysed using a TissueLyser II (Qiagen Inc. Valencia CA, USA). Total DNA was purified from the dissected guts using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's protocol. Purified genomic DNA samples were submitted to LGC genomics (GmbH, Berlin, Germany).

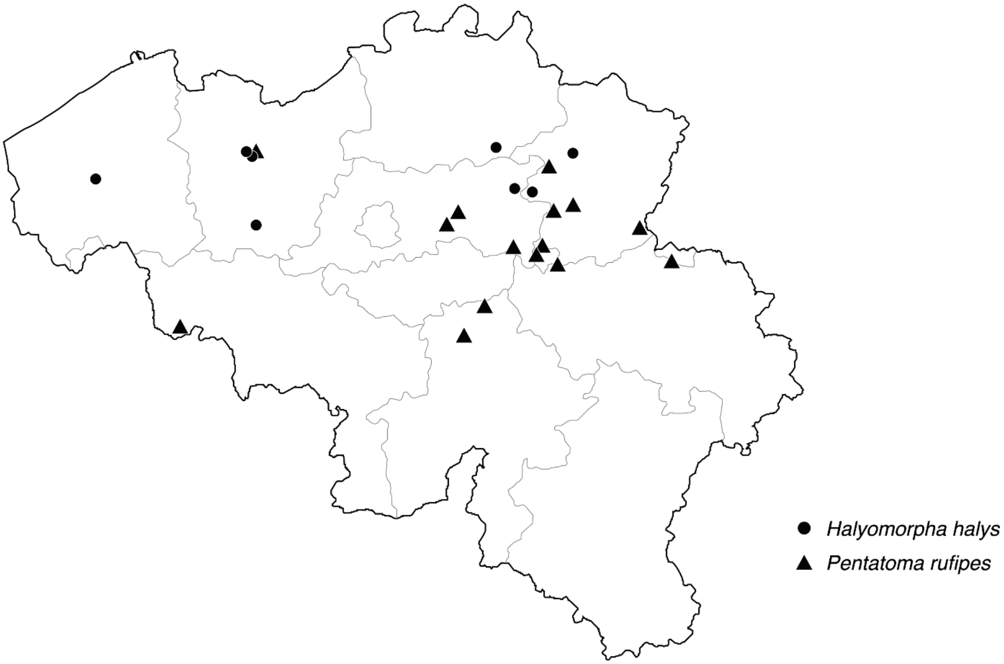


Figure 4.1. Map of Belgium with sampling locations for *H. halys* and *P. rufipes*.

4.2.3. Laboratory feeding trial

The laboratory feeding trial was conducted in a climatic chamber at 24 ± 0.5 °C, 65 ± 5% RH, with a photoperiod of 16:8 h (L:D). Newly emerged male and female adults (< 24 h) were starved for 48 h to remove any food from their digestive system. They were then fed a mixed diet of bean pods and sunflower seeds for 24 h in polystyrene insect breeding dishes with meshed covers (100 x 40 mm; SPL Life Sciences Co., Korea), with each breeding dish containing six adults. After 24 hours of initial feeding, the *H. halys* adults were offered a potted apple seedling (*Malus domestica* Borkh. cv. 'Golden Delicious') of approximately 8 weeks old and 20 cm tall to feed on in custom polystyrene (500 x 300 mm) arenas with mesh covered tops, again containing six individuals per arena. At discrete time intervals of 0 (not offered an apple seedling), 6, 12, 24, 48, and 72 hours in the arena with an apple seedling, four individuals were randomly collected and frozen at -20 °C to be later subjected to DNA extraction of the digestive system. Lastly, these DNA samples from the laboratory feeding trial also serve as positive DNA extraction controls for the field-collected samples.

4.2.4. PCR Amplification and Library Preparation

For the ITS2 region a first amplification was performed on 1-10 ng of DNA extract (total volume 1µl) using the ITS-S2F (5'-ATGCGATACTTGGTGTGAAT-3') (Chen et al. 2010) and the ITS-u4 (5'- RGTTTCTTTTCCTCCGCTTA-3') (Cheng et al. 2016) which each contained an additional Illumina TruSeq adaptor sequence. For the second PCR (indexing), 1 µl of each amplification obtained in the first PCR was used, separately amplified in a 20 µl reaction volume using standard i7- and i5- sequencing adapters. For both PCR reactions, 15 pmol of each primer was used in 20 µL volume of 1x MyTaq buffer containing 1.5 units MyTaq DNA polymerase (Bioline GmbH, Luckenwalde, Germany) and 2 µl of BioStabII PCR Enhancer (Sigma-Aldrich Co.). The PCR conditions used for the amplification were: initial denaturation at 96 °C for 60 s, 35 cycles of denaturation at 96 °C for 15 s, annealing at 58 °C for 30 s, and extension at 70 °C for 90 s, and final hold at 8 °C. The second PCR for indexing was similar to the conditions of the first amplification, with a modified annealing temperature of 3 cycles at 50 °C and seven cycles at 58 °C. Negative PCR controls were included in the sequencing workflow by the sequencing service provider (LGC Genomics, Germany GmbH). DNA concentrations of amplicons were assessed by agarose gel electrophoresis. Approximately 20 ng of indexed amplicon DNA of each sample was subsequently pooled (up to 96 samples per pool). The pooled libraries were purified with one volume of Agencourt AMPure XP beads (Beckman Coulter, Inc., IN, USA) to remove primer dimers and other small mispriming products, followed by an additional purification on MiniElute columns (QIAGEN GmbH, Hilden, Germany). Size selection was performed by preparative gel electrophoresis on an LMP-Agarose gel. Sequencing was done on an Illumina MiSeq (Illumina, Inc., CA, USA) using V3 Chemistry (2x300 bp) (LGC Genomics, Germany GmbH).

4.2.5. Sequence analysis

Raw data were first preprocessed and demultiplexed with the MiSeq Reporter Generate FASTQ workflow (Illumina). The demultiplexed data was further analyzed with QIIME2 (Bolyen et al. 2019). Adapter and primer sequences were trimmed from the reads using the cutadapt trim-paired plugin (Martin 2011; Bolyen et al. 2019). Trimmed demultiplexed reads were quality filtered and denoised with DADA2 in QIIME2 (Callahan et al. 2016; Bolyen et al. 2019), wherein forward and reverse reads were truncated to 250 bp and 185 bp, respectively, to trim low-quality 3’-tails (Phred score < 30). In brief, during the DADA2 filtering step, paired-end reads are joined and quality-filtered, after which chimeric sequences and singletons are removed, and the joined reads are eventually de-replicated into Amplicon Sequence Variants (ASVs). We then clustered ASVs into operational taxonomic units (OTUs) at 97% similarity using vsearch-cluster-features-de-novo (Rognes et al. 2016; Bolyen et al. 2019). Taxonomic assignments were performed with the QIIME2 feature-classifier plugin using the BLAST+-based global alignment (Camacho et al. 2009) and the VSEARCH-based local alignment (Rognes et al. 2016) search against a custom ITS2 plant database, created using the DB4Q2 workflow from (Dubois et al. 2022). A taxonomic classifier was also trained using our custom ITS2 plant database and the Naïve Bayes algorithm of the QIIME2 fit-feature-classifier plugin with default settings (Pedregosa et al. 2011; Bolyen et al. 2019). The trained classifier was then used to assign taxonomy to the OTUs. Finally, a consensus taxonomy was manually assigned at the species level when the percentage identity was ≥ 97% and when two or more techniques provided matching taxonomies at the species level. Conflicting taxonomic results were manually checked by blasting or eventually marked as unassigned.

4.2.6. Data analysis

The resulting OTU table and taxonomy were merged with the metadata of the samples into one data file using R (version 4.3.2.) in R Studio (version “Ocean Storm”) (R Core Team 2024; RStudio Team 2024). Field samples that contained less than 10,000 reads after DADA2 filtering, were omitted from further analysis. When OTUs represented less than 1% of the reads of a sample, they were excluded from the dataset. For the bar plot visualizations OTUs were aggregated at genus-level taxonomy to facilitate interpretation.

Following Deagle et al. (2019), taxon counts at species level per sample were analysed using relative read abundance (RRA). The RRA for each taxon within each sample was calculated relative to each stink bug. Using the OTU tables with sequence reads converted to the RRA metric for each stink bug species, we performed a non-metric multidimensional scaling (nMDS) ordination based on a Bray-Curtis dissimilarity matrix with k-means clustering to identify distinct clusters within each shield bug species based on landscape type or season. To quantify the explanatory power of the landscape type and season on the observed molecular diet composition, we permutational multivariate analysis of variance (PERMANOVA) with 999 permutations for our relative read abundance OTU matrix at the species level, using the landscape type and the season of sample collection as explanatory variables for each stink bug species. For a comprehensive step-by-step protocol detailing the materials and methods used in this study, we refer to the following DOI link: https://dx.doi.org/10.17504/protocols.io.e6nvwdewdlmk/v1

4.3. Results

We sequenced the plant ITS2 region on the Illumina MiSeq from the gut content of 168 samples from two stink bug species, *H. halys,* and *P. rufipes*, collected from 24 field locations. Additionally, 24 samples from a controlled feeding trial with *H. halys* were sequenced. This resulted in 13,419,235 reads from 192 samples (Table 4.1). All 192 samples detected at least one plant species. After sequence analysis and quality filtering, 179 samples were retained for the final analysis, from which we recovered 8,034,929 reads (60% of input) with an average of 45,914 ± 25,588 reads per sample and a median OTU length of 353 bp.

4.3.1. Field samples

Overall, 7,471,579 reads were recovered from the 168 gut samples from the field-collected stink bugs; herein 2710 ASVs were identified, and these were clustered into 421 OTUs. After filtering, 71% of OTUs were assigned taxonomy at the species level, while 29% could not be successfully identified at the genus level and were marked as ‘unassigned’.

We identified a total of 68 plant species (> 97% percentage identity) belonging to 54 genera, of which 16 genera were shared between the two stink bug species. For *P. rufipes,* 6 unique genera were found, whereas 32 genera were unique for *H. halys* (Table 4.2). For 91% of the *P. rufipes* samples collected, the recorded host plant was successfully retrieved through the amplicons present in the sample.

The most detected genus in the field samples across both species was *Acer* (46/178). For *H. halys,* we found a median of 3 unique genera per sample; herein, *Prunus* was the most detected genus (32/82). For *P. rufipes,* we found a median of 2 unique genera per sample, with *Acer* being the most detected genus (20/73), closely followed by *Quercus* (18/73). The ten most detected genera for each stink bug are presented in Figure 4.2. The host plant composition at genus level of all samples for each stink bug species can be seen in Figures 4.3 and 4.4. Genera that comprised < 5% in a given sample were aggregated as "other" for these figures.

Landscape type was a significant factor ($p < 0.001$), with some explanatory power for OTU composition in *H. halys* (6.5% of variation) and *P. rufipes* (6.4% of variation) samples. The season in which the samples were collected was significant for *H. halys* ($p = 0.004$) but not for *P. rufipes* ($p = 0.286$) (Table 4.3). The nMDS ordination analyses revealed limited clustering by landscape type where samples from orchards and gardens cluster separately with some overlap between the landscape type clusters (Fig. 4.5).

4.3.2. Laboratory experiment

After quality filtering, 582,433 reads were retained across the samples from the laboratory experiment (Table 4.1), of which 98% percent correctly identified one of the plant species they were offered during the experiment. At least one of the plant species in the original diet (bean pods and sunflower seeds) was still detectable in each sample at each interval. The second diet offered (apple seedlings) was detected in 8/20 samples and the new diet was detected in at least one sample per time interval after the diet switch (Fig. 4.6).

4.4. Discussion

In this study, we used a DNA metabarcoding approach with NGAS to infer the diets of field-collected *P. rufipes* and *H. halys*. Our results demonstrate the capacity of the ribosomal ITS2 barcode to identify host plants at the species level in the diet of polyphagous stink bugs, revealing a median taxon depth of 2 genera per sample for *P. rufipes* and a mean of 3 genera for *H. halys*.

The laboratory experiment substantiates the sensitivity of the technique to detect feeding events that happened up to 72 hours after a host plant switch, while also partially validating the observed plant taxonomy in the gut from field-collected adult insects. Hepler et al. (2021) found that there is variation in the persistence between host plant genes in the gut of *H. halys.* In their study, the DNA of *Phaseolus* was easily detectable for two chloroplast markers three days (for *trnF*) and 14 days (for *trnL*) after a host plant switch. Thus, primer sets developed to amplify different DNA regions may differ in their efficiency to successfully detect template DNA at lower concentrations. Moreover, primer pairs may also differ in their efficiency in the amplification of certain taxa, e.g., *Phaseolus*. Additionally, chloroplast DNA regions may be more resilient to digestion and more readily detectable in gut contents due to their higher abundance per cell compared to the nuclear ITS2 barcode region. In hindsight, with the ITS2 primer pair used in this study, initial diet DNA may have been detected in the gut after a period longer than 72 hours following a diet switch as demonstrated by Fluch et al. (2024).

In the field samples, we found that landscape type is a significant predictor of diet composition for both stink bugs (Table 4.3). The nMDS ordination analysis also shows clustering per landscape, with some overlap between the two landscape types. Urban gardens and parks often share plant genera with forests and commercial orchards (e.g., *Acer, Betula,* and *Fagus*) explaining the overlap in clusters from both landscape types. In contrast, popular ornamental plant genera like *Syringa*, *Clerodendrum*, *Hamamelis,* and *Catalpa* are more unique to gardens or public parks. *Malus*, *Pyrus,* and other genera with fruit trees or crops are likely more common in or near orchards. Species from genera like *Acer, Betula, Fagus,* and *Prunus,* ubiquitous in both rural and urban environments, can be of particular importance for *H. halys* to establish a population, as well as for *P. rufipes* to sustain a population.

From the host plants found in both stink bugs (Table 4.2), it is clear that the diet breadth of *H. halys* almost entirely includes that of *P. rufipes*. *Halyomorpha halys* forages on more species, some specific to urban areas, and seems to be more polyphagous than *P. rufipes*. Lee et al. (2013) listed over 100 host plants for *H. halys*, including members of the genera *Acer*, *Alnus, Brassica, Castanea, Catalpa, Crataegus, Cucumis, Fraxinus, Glycine, Helianthus, Humulus, Malus, Phaseolus, Prunus, Pyrus, Robinia, Viburnum, Vitis,* and *Wisteria*. In addition to Lee et al. (2013), Bergmann et al. (2016) also identified members of the genera of *Cercis, Liquidambar, Syringa,* and *Tilia* amongst 88 species of host plants used by all life stages of *H. halys* in North America. While species of all these genera were also found in our samples, we also identified members of the genus *Fagus, Betula, Cornus, Corylus*, *Frangula* and *Salix,* to be frequent in our samples of *H. halys* (Fig. 4.4 & Table 4.2). In the case of *P. rufipes*, the lower taxon depth per sample could be due to localized populations that have more adapted to feeding on particular host plant species with a locally higher abundance (Dulwich 1985; Powell 2020). The presence of common genera like *Quercus*, *Acer*, *Fagus*, and *Prunus* could indicate critical species for populations of *P. rufipes* to survive and reproduce. The genera in our MGCA align with those listed as preferential for *P. rufipes* in Powell (2020). The seasonality of the sampling is a significant predictor of the RRA of taxa detected in *H. halys* samples but not in *P. rufipes* samples. However, while statistically significant, seasonality showed limited explanatory power and lacked clear clustering in the nMDS analyses. Polyphagous pentatomids like *P. rufipes* and *H. halys* usually do not feed equally across all potential host plants and can temporarily become oligophagous and even monophagous when locally adapted to abundant plant species or seasonally available resources (Dulwich 1985; Martinson et al. 2015). It is known that *H. halys* is a landscape-level pest that moves across habitats, can switch from one host plant to another throughout the seasons, and so invades a perimeter of susceptible crops (Martinson et al. 2015; Venugopal et al. 2015; Philips et al. 2017). It is able to track resources as they become available through the season. We assume that our sampling size is too small to reveal such ecological phenomena. Larger-scale studies, including more samples throughout the seasons, across landscapes, and life stages, could improve understanding of where and when *H. halys* will occur in different landscapes and aid in developing IPM approaches for *H. halys* (Philips et al. 2017).

While DNA metabarcoding provides high taxonomic resolution and the results of our study using NGAS confirm host plant preferences for both stink bug species in literature (Lee et al. 2013; Bergmann et al. 2016; Powell 2020). It comes with several caveats. The results obtained by NGAS include many steps, such as DNA extraction and PCR amplification, which can lead to over- or underestimation of actual preferences (Deagle et al. 2019; Peel et al. 2019). Amplification products are not a perfect representation of the original DNA composition in the gut. Nevertheless, the relative read abundance of metabarcoding amplicons can still be used as an estimate of relative preference when it is applied with respect to the investigated taxa and research questions (Danner et al. 2017; Smart et al. 2017; Deagle et al. 2019).

Most plant genera detected in the gut of *H. halys* (86% of reads) and *P. rufipes* (99% of reads) were deciduous trees or shrubs. Herbaceous plants were found in fewer quantities; however, they are still substantial in proportion for *H. halys* (11% of reads) and less so for *P. rufipes* (0.5%). Gymnosperms comprise 3% of the reads found for *H. halys* and only 0.1% for *P. rufipes*. For *H. halys*, this finding is consistent with Bergman *et al*. who found that in contrast to Asian populations, North American gymnosperms were only identified as partial hosts for *H. halys*. Here, we found mostly singular occurrences of the genera *Abies, Juniperus, Keteleeria, Picea*, and *Pinus* in separate samples with RRAs ranging from 5% (*Pinus*) up to 80% (*Juniperus*) (Fig. 4.4.). For *P. rufipes,* two samples from the same location (with *Pinus* in the vicinity; see Supplementary table I) contained *Pinus* with rather low relative read abundances of 3.4% and 4.2% (Categorized as “Other” in Fig. 4.3).

The general preference of *H. halys* and *P. rufipes* for deciduous trees or shrubs and less so for gymnosperms and herbaceous plants suggests that mixed hedgerows and woodlands containing certain deciduous species in and around orchards are more attractive to these stink bugs than (semi-) natural or managed herbaceous stands, like flower strips. For *H. halys*, this is congruent with a two-year study conducted by Bergh et al. (2021) in fruit orchards in the Mid-Atlantic (USA) showing that *H. halys* captures and fruit injury were the highest at orchard edges bordering woodlands and other border habitats like hedgerows. Similarly, Rice et al. (2016) found that landscapes with larger forest edges had higher rates of stink bug damage in tomatoes by *H. halys*. Such hedgerows and forest edges are therefore considered “risky” borders for orchards as they can harbour large *H. halys* populations that can invade crops during the growing season (Bergh et al. 2021).

From this study, practical implications for landowners experiencing economic damage from these pests are to avoid the over-use of attractive host plants in their mixed hedges and in woodlands around their agricultural fields, for example, members from the *Acer*, *Prunus*, *Quercus*, *Fagus*, *Corylus*, and *Betula* genera as found in our study and earlier studies (Lee et al. 2013; Bergmann et al. 2016; Powell 2020).

Sown-in flower strips can support pollinators, generalist predators, and parasitoids by providing essential floral resources, which improve natural pest control and pollination, thereby promoting crop productivity and biodiversity without further attracting stink bugs (Clapp et al. 2014; Albrecht et al. 2020; Montgomery et al. 2020; Fountain 2022; this study). However, they could also pose a potential drawback by attracting voles due to increased food supply and protection from birds of prey (Granatstein and Sánchez, 2009).

In addition to flower strips, hedgerows further enrich orchard ecosystems by offering vertical structure and year-round habitat, supporting beneficial insects and contributing to carbon sequestration, soil and water regulation, windbreak, and shading (Clapp et al. 2014; Montgomery et al. 2020). Despite these advantages, they often include preferential host plants that can cause additional attraction for stink bugs, which may in turn be a threat to nearby fruit crops. Including gymnosperms within hedgerows can provide additional benefits, as they can provide year-round resources for beneficial insects and provide hedgerow-level ecosystem services without additionally attracting stink bugs to orchards (Clapp et al. 2014; this study). However, some gymnosperms e.g. *Juniperus* also pose a risk by acting as hosts for rust fungi, which can spread to nearby fruit trees (Lee et al. 2016).

Contrarily, preferential host plants in hedgerows can also be used in the framework of attract-and-kill methods as trap plants, additionally baited with pheromones to increase attraction of the stink bugs. These baited plants and a perimeter can be sprayed with insecticides to cull stink bug populations in orchards without having to spray the fruit trees themselves (Morrison III et al. 2019).

4.5. Conclusions

This study effectively employed DNA metabarcoding of the ITS2 region using NGAS techniques to elucidate the dietary preferences of *P. rufipes* and *H. halys* in Belgium. The findings confirm that both species exhibit a preference for deciduous trees and shrubs, especially for genera such as *Acer*, *Prunus*, and *Fagus*. These preferences are further shaped by local plant communities, emphasizing the impact of environmental factors on diet preference.

Importantly, this research has practical implications for IPM strategies. Detailed insights into stink bug dietary preferences can inform the strategic planting of certain plant species so as not to further attract stink bugs into orchards or to deploy attract-and-kill strategies, thereby minimizing economic damage. This approach not only improves strategies to reduce damage by stink bug pests but also promotes biodiversity and ecosystem services, aligning with sustainable agricultural practices in IPM.

Future research should aim to expand on these findings by including larger sample sizes and more diverse environmental settings to better understand the dynamics of host plant usage throughout different seasons and across various landscapes. Such studies could be vital in developing more effective and sustainable IPM strategies that leverage the natural host plant preferences of stink bugs to manage their populations more effectively.

4.6. Acknowledgements

We thank Rik Clymans and Alexandre Kuhn for their valuable sampling efforts in this study.

Table 4.1. Overview of the samples collected and retained, as well as reads obtained after sequencing and retained after quality control with DADA2.

Species	Variable	*df*	F-value	p-value	R^2
Halyomorpha halys	Landscape	1	6.6106	0.0001***	0.0652
	Season	2	1.9148	0.0040**	0.0378
Pentatoma rufipes	Landscape	1	4.6681	0.0001***	0.0646
	Season	1	1.1992	0.2860	0.0482

Table 4.3. Summary of the explanatory power of the categorical variables (Landscape type and season) in the PERMANOVAs (999 permutations).

		Samples collected	Samples retained	Reads obtained	Reads retained
Field collected samples	*Halyomorpha halys*	95	82	6,376,484	3,871,203
	Pentatoma rufipes	73	73	6,320,345	3,600,376
Laboratory experiment	*Halyomorpha halys*	24	24	723,406	582,433
Total		192	178	13,419,235	8,054,012

Table 4.2. Overview of all plant taxa with relative read abundances > 1% per sample in this study.

Stinkbug	Clade	Order	Family	Genus	Species
Halyomorpha halys & *Pentatoma rufipes*	Angiospermae	Cucurbitales	Cucurbitaceae	*Cucumis*	*C. sativus*
		Dipsacales	Adoxaceae	*Sambucus*	*S. nigra*
		Fagales	Betulaceae	*Betula*	*B. nana*
				Corylus	*C. avellana*
			Fagaceae	*Castanea*	*C. crenata*
				Fagus	*F. sylvatica*
				Quercus	*Q. aliena, Q. robur, Q. rubra*
			Juglandaceae	*Juglans*	*J. regia*
		Rosales	Rosaceae	*Malus*	*M. domestica*
				Prunus	*P. cerasifera, P. serotina*
				Pyrus	*P. amygdaliformis, P. communis, P. cordata*
				Sorbus	*S. aucuparia*
			Urticaceae	*Urtica*	*U. dioica*
		Sapindales	Sapindaceae	*Acer*	*A. cappadocicum, A. platanoides, A. pseudoplatanus*
		Poales	Poaceae	*Lolium*	*L. perenne*
	Gymnospermae	Pinales	Pinaceae	*Pinus*	*P. nigra*
Pentatoma rufipes	Angiospermae	Caryophyllales	Caryophyllaceae	*Stellaria*	*S. media*
		Fabales	Fabaceae	*Trifolium*	*T. repens*
		Fagales	Fagaceae	*Quercus*	*Q. faginea, Q. frainetto*
		Poales	Poaceae	*Holcus*	*H. lanatus*
		Proteales	Platanaceae	*Platanus*	*P. x acerifolia*
		Ranunculales	Ranunculaceae	*Ranunculus*	*R. acris*
		Rosales	Rosaceae	*Pyrus*	*P. calleryana*
				Rubus	*R. plicatus*
Halyomorpha halys	Angiospermae	Apiales	Apiaceae	*Pimpinella*	*P. saxifraga*
		Asterales	Asteraceae	*Helianthus*	*H. annuus*
		Brassicales	Brassicaceae	*Brassica*	*B. oleraceae*
		Cornales	Cornaceae	*Cornus*	*C. sanguinea*
		Dipsacales	Adoxaceae	*Viburnum*	*V. tinus*
		Ericales	Ericaceae	*Rhododendron*	*R. maximum*
		Fabales	Fabaceae	*Cercis*	*C. siliquastrum*
				Glycine	*G. max*
				Medicago	*M. sativa*
				Phaseolus	*P. vulgaris*
				Robinia	*R. pseudoacacia*
				Wisteria	*W. sinensis*
		Fagales	Betulaceae	*Alnus*	*A. glutinosa*
				Carpinus	*C. betulus*
		Lamiales	Bigoniaceae	*Catalpa*	*C. bignonioides*
			Lamiaceae	*Clerodendrum*	*C. trichotomum*
			Oleaceae	*Fraxinus*	*F. excelsior*
				Ligustrum	*L. x vicaryi*
				Syringa	*S. vulgaris*
		Malpighiales	Salicaceae	*Salix*	*S. alba*
		Malvales	Malvaceae	*Tilia*	*T. platyphyllos*
		Rosales	Cannabaceae	*Humulus*	*H. lupulus*
			Rhamnaceae	*Frangula*	*F. alnus*
			Rosaceae	*Crataegus*	*C. monogyna*
				Cydonia	*C. oblonga*
				Prunus	*P. avium, P. lusitanica*
		Sapindales	Sapindaceae	*Acer*	*A. campestre*
		Saxifragales	Hamamelidaceae	*Hamamelis*	*H. japonica*
		Vitales	Vitaceae	*Vitis*	*V. vinifera*
	Gymnospermae	Cupressales	Cupressaceae	*Juniperus*	*J. durangensis*
		Pinales	Pinaceae	*Abies*	*A. nordmanniana*
				Picea	*P. glauca*
				Pseudotsuga	*P. mensziesii*
				Keteleeria	*K. fortunei*

Figure 4.2. The top 10 most detected genera in field collected samples of *H. halys* (left) and *P. rufipes* (right).

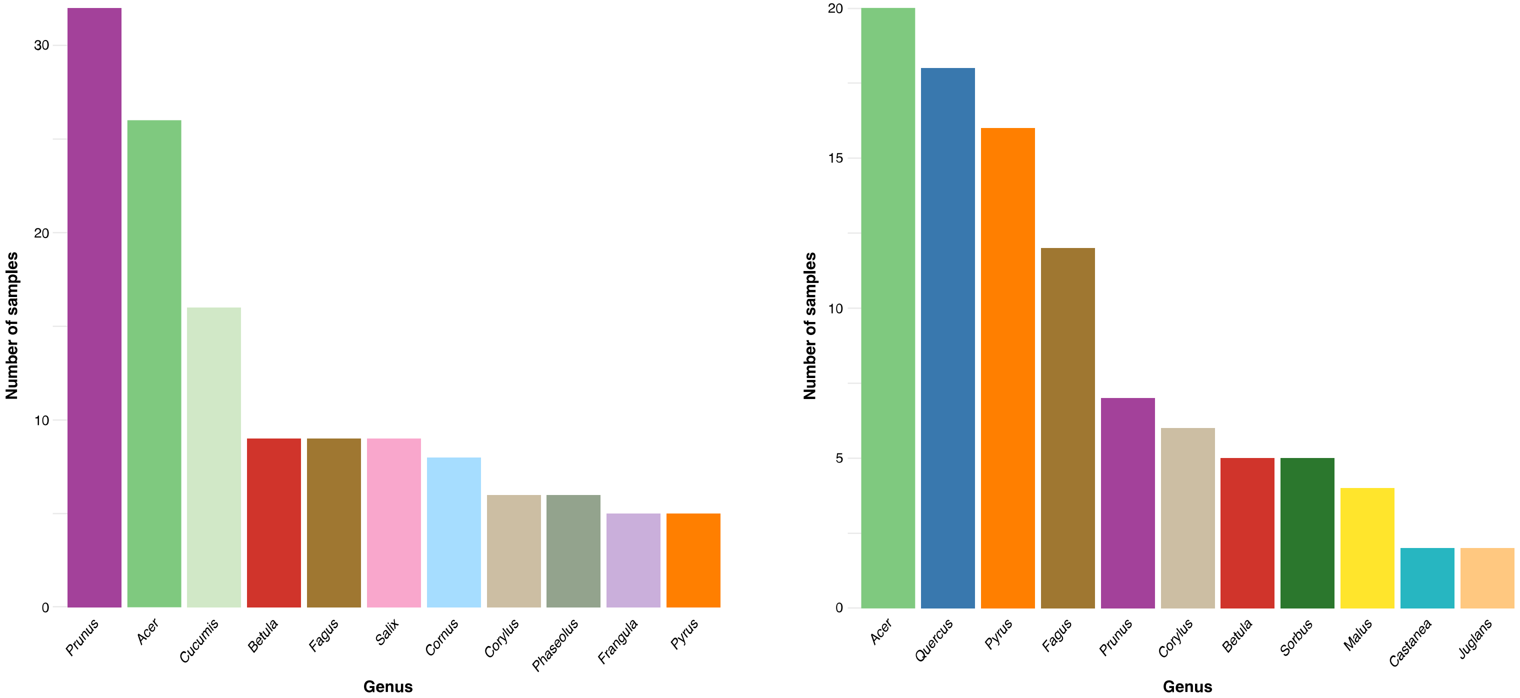


Figure 4.3. Barplot with all samples (x-axis) of *P. rufipes* grouped per host plant they were found on (Genera with < 5% of relative read abundance in a sample are classified as “Other”).

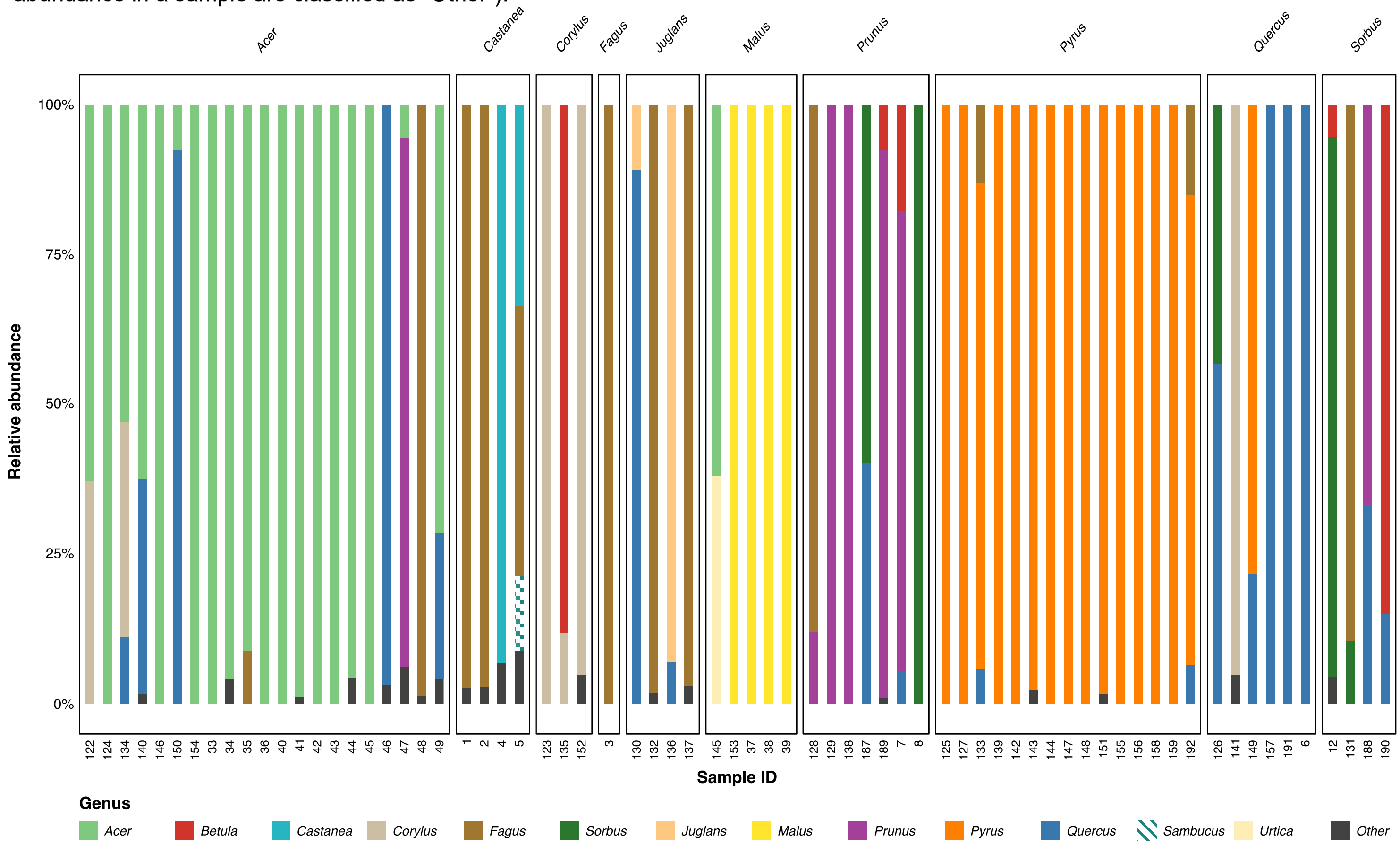


Figure 4.4. Barplot with all samples of *H. halys* (x-axis) (Genera with relative read abundance < 5% in a sample are classified as “Other”).

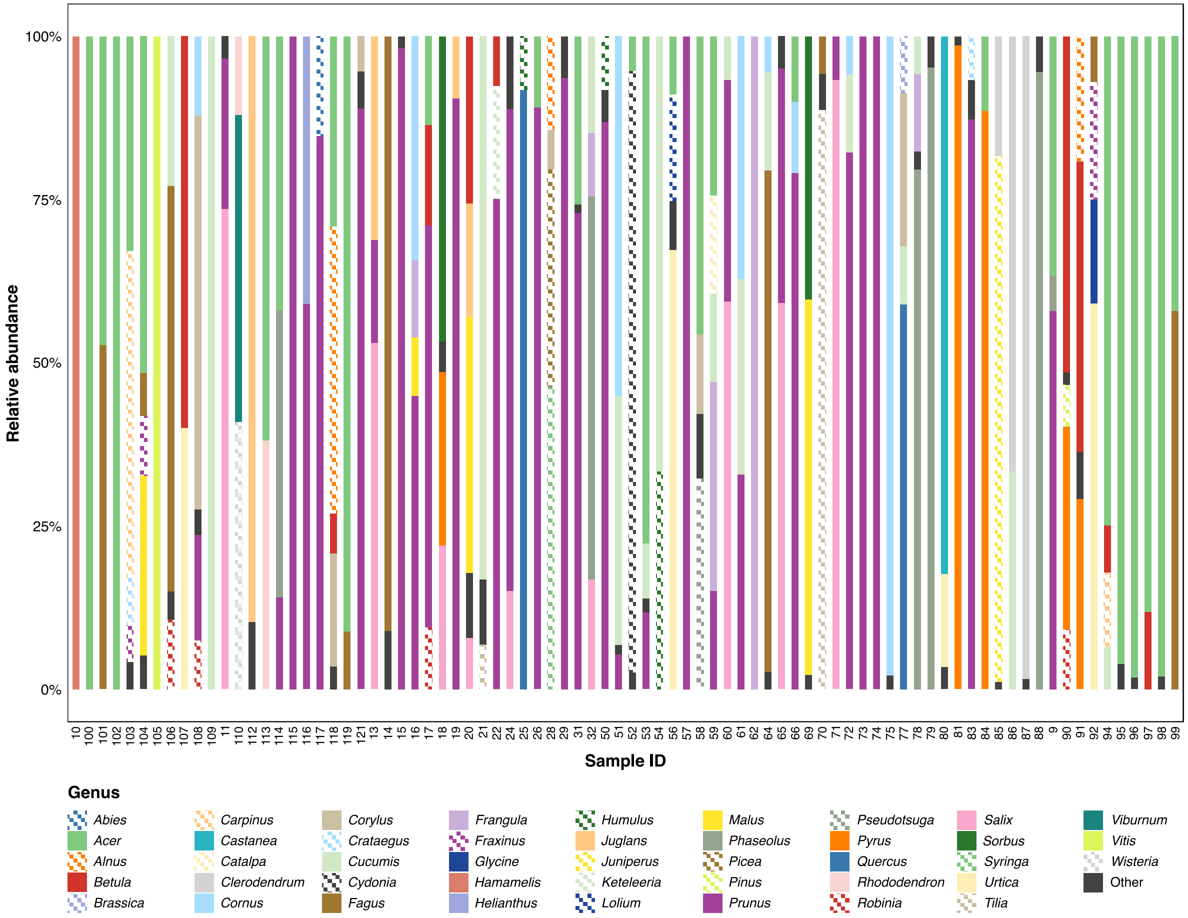


Figure 4.5. Non-metric multidimensional scaling (nMDS) ordinations based on relative read abundance (RRA) per season (A-B) and landscape type (C-D) for each stink bug species.

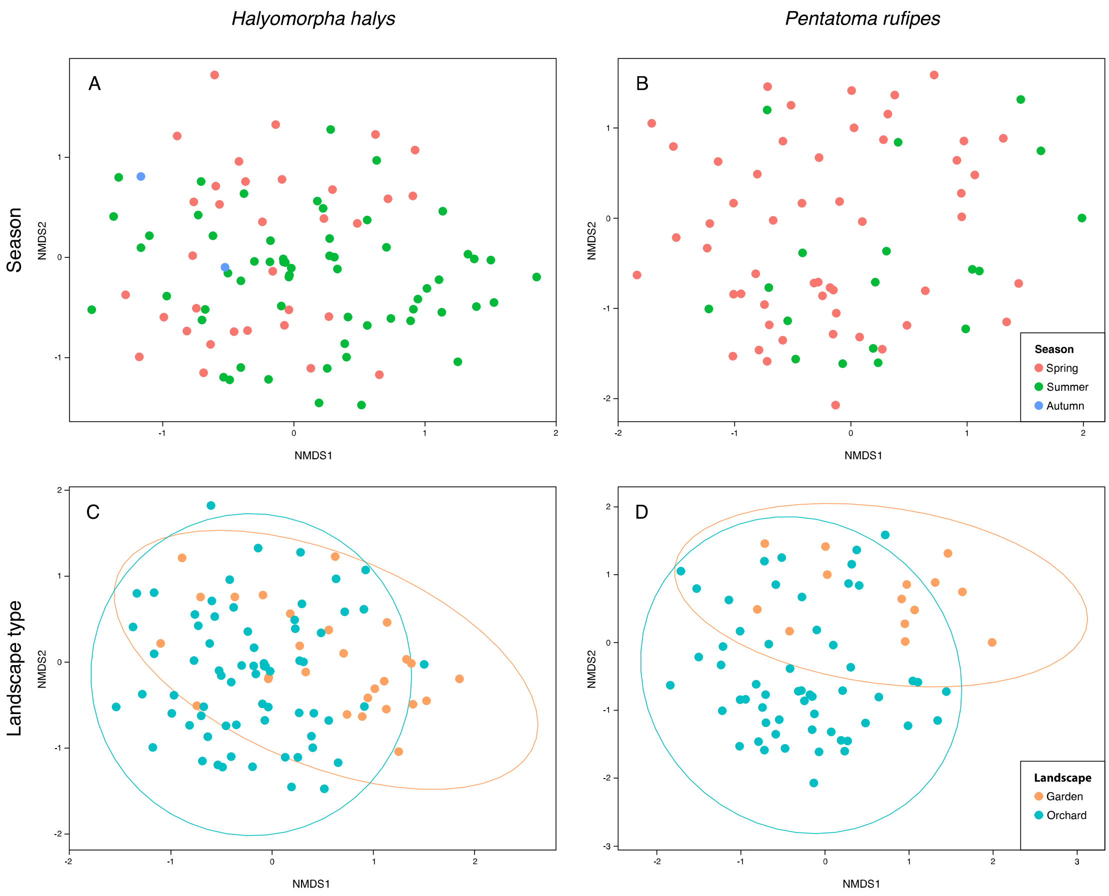
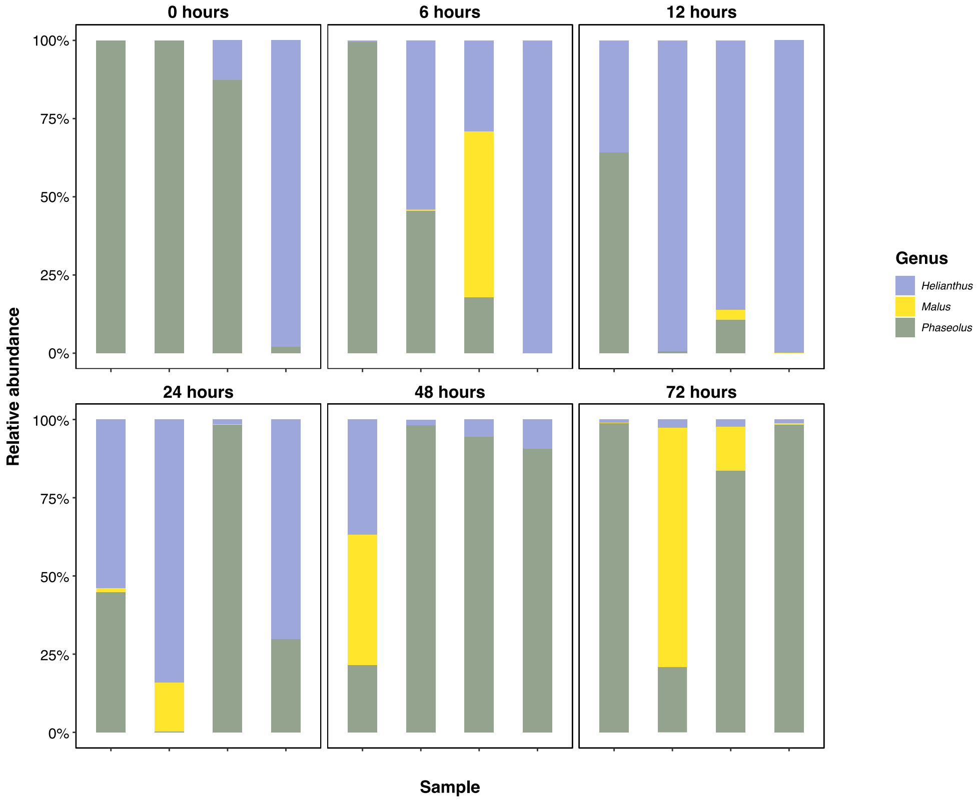


Figure 4.6. Barplot with the relative abundances of the detected diet species in the *H. halys* samples from the laboratory experiment per time interval.



Supplementary table I: Descriptions of the sampling locations.

Location	Type	Landscape	Description
Zottegem	Orchard	rural	IPM orchard with forest edges
Koersel	Orchard	rural	IPM blueberry orchard with forest edges
Gent	Garden	urban	Public park with forest trees and ornamental plants
Merelbeke	Garden	urban	Garden with forest trees and ornamental plants
Staden	Orchard	rural	Private orchard with hedges, Wildflower strips
Gent (Coupure Links faculteitstuin)	Garden	urban	Private garden with forest trees and ornamental plants
Westerlo	Garden	rural	Private garden with forest trees and forests in neighbourhood, ornamental plants
Ezemaal	Orchard	rural	Orchard
Gingelom	Garden	urban	Private garden with ornamental plants
Kessel-Lo	Garden	urban	Private garden with ornamental plants
Lummen	Garden	urban	Private garden with ornamental plants
Nieuwenhoven	Orchard	rural	Orchard
Vlijtingen	Garden	urban	Private garden with ornamental plants
Kerkom-bij-Sint-Truiden	Orchard	rural	Orchard
Heverlee	Garden	urban	Private garden with ornamental plants
Sint-Lambrechts-Herk	Garden	urban	Private garden with ornamental plants
Neufchateau	Orchard	rural	IPM orchard with forest edges
Lesdain	Orchard	rural	IPM orchard with forest edges
Upigny	Orchard	rural	IPM orchard with forest edges
Rhisnes	Orchard	rural	IPM orchard with forest edges
Rosoux-Crenwick	Orchard	rural	IPM orchard with forest edges
Halen	Orchard	rural	Organic orchard with mixed hedges and forest edges, Wildflower strips
Assent	Orchard	rural	Organic orchard with mixed hedges and forest edges, Wildflower strips
Gentbrugge	Orchard	urban	Private orchard with hedges, wildflowers, ornamentals and small forest edge

Chapter 5

Predation efficacy of *Chrysoperla carnea* on two economically important stink bugs

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Predation efficacy of *Chrysoperla carnea* on two economically important stink bugs

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Abstract

Larvae of the green lacewing *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) are generalist predators feeding on a range of pests in agroecosystems. Little is known about the suitability of stink bugs as prey for lacewing larvae. In the present laboratory study, the predatory efficacy of *C. carnea* on two economically important stink bugs, *Halyomorpha halys* (Stål) and *Nezara viridula* (L.) (Hemiptera: Pentatomidae), was evaluated at three constant temperatures (16, 21, and 26 °C) in different arenas (10 cm diameter dishes with bean pod slices or caged small bean plants). We found that second and third instars of *C. carnea* readily prey on the first instars of both stink bug species. However, only third instars of *C. carnea* could successfully kill and feed on second instars of either pentatomid. The complexity of the arena, as well as the life stage of the prey and predator influenced the predation efficacy of *C. carnea* on stink bug nymphs, whereas temperature did not. The observed predation rates on the early instars of both stink bugs suggest that augmentative releases of *C. carnea* larvae may have some potential in greenhouse crops, particularly against *N. viridula* infestations, whereas in open field crops their natural populations may also contribute to pest suppression within the broader predator guild.

5.1. Introduction

The role of generalist predators as effective regulators of arthropod pests in agricultural ecosystems is well-established (Rosenheim et al. 1999; Bordini et al. 2021). Generalist predators can also contribute substantially to the biological control of invasive species that often lack specialist natural enemies in their newly colonised habitats (Chang and Kareiva 1999; Morrison III et al. 2016a).

The brown marmorated stink bug, *Halyomorpha halys* (Stål), and the southern green stink bug *Nezara viridula* (L.) (Heteroptera, Pentatomidae) are both invasive stink bugs with populations established outside of their native regions (Haye et al. 2015; Esquivel et al. 2018).

Halyomorpha halys is native to East Asia and is an invasive pest in the USA, Canada, South America (Chile), Europe, and the Caucasus region (Haye et al. 2015; Gapon 2016; Faúndez and Rider 2017; Streito et al. 2021). It is highly polyphagous and has been reported to feed on pome fruit (e.g., apple, pear), vegetable crops (e.g., pepper, tomato, sweet corn), and numerous ornamentals in its invaded areas (Kuhar et al. 2012; Leskey et al. 2012c; Haye et al. 2015; Maistrello et al. 2017). For example, in northern Italy, it has become a key pest in fruit orchards, with an estimated economic impact of €588 million on the fruit production of pear, apple, peach, and kiwi in 2019 (Bulgarini et al. 2021).

Nezara viridula is believed to have originated in the Ethiopian region of East Africa (Todd 1989). It is now widely distributed throughout tropical and subtropical regions of Eurasia, Africa, Australia, and the Americas and has expanded its range in temperate areas of Europe over the last decades (McPherson et al. 2000; Esquivel et al. 2018). The insect has established populations as far north as Great Britain, Belgium, The Netherlands, and Slovakia and will likely increase further in abundance and establish in new regions of Europe (Salisbury et al. 2009; Dethier and Chérot 2014; Vétek and Rédei 2014; Aukema 2019; McPherson 2018). The southern green stink bug has become one of the most economically important pest species of the Hemiptera, inflicting damage to crops in the open field as well as in greenhouses (Panizzi 2004; Gard et al. 2022). In Europe, it is a key pest of greenhouse vegetable crops such as tomatoes, sweet pepper, cucumber, and eggplant (Vandekerkhove and De Clercq 2004; Gard et al. 2022). For example, in 2019 the economic impact of *N. viridula* was estimated €25,000–35,000 per ha for eggplant production under plastic tunnels in France (Gard et al. 2022).

When highly polyphagous non-native herbivores such as *H. halys* and *N. viridula* invade cropping systems, economic damage can quickly accrue (Panizzi 2004; Akotsen-Mensah et al. 2018). To effectively manage these stink bug pests, broad-spectrum insecticides are still widely used, particularly pyrethroids (Vandekerkhove and De Clercq 2004; Leskey et al. 2012b; Takeuchi and Endo 2012). However, given the non-target effects of such pesticides, this approach has led to ecological disturbance in the affected agroecosystems and has thus complicated integrated pest management strategies against stink bugs and other pests alike (Douglas and Tooker 2016; Alfaro-Tapia et al. 2021; Schneeweiss et al. 2022).

Several generalist predators have been reported to attack nymphs and egg masses of stink bugs in laboratory and field settings (De Clercq et al. 2002; Ehler 2002; Abram et al. 2014; Rice et al. 2014; Bulgarini et al. 2021). In laboratory experiments, Ehler (2002) found that the green lacewing *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) attacked *N. viridula* nymphs and, to a lesser extent, also eggs. Lacewings were also shown to attack *H. halys* eggs in the laboratory (Abram et al. 2014). From field surveys, Rice et al. (2014) have reported that, among others, members of the Chrysopidae family attack *H. halys* in North America. Bulgarini et al. (2021) quantified predation by *C. carnea* on *H. halys* eggs and on nymphs of the first and second instar but they only tested second instar larvae of the predator.

Chrysoperla carnea (sensu lato) is among the most common natural enemies of arthropod herbivores in a wide range of agricultural ecosystems and plays a key role in the conservation and augmentation biological control of various arthropod pests, including aphids, lepidopterans, mealybugs, psyllids, leafhoppers, whiteflies, thrips, caterpillars, and spider mites (Principi and Canard 1984; Stark and Hopper 1988; Easterbrook et al. 2006; Huang and Enkegaard 2010; Farrokhi et al. 2017; Golsteyn et al. 2021). In contrast to its three larval instars, adults of *C. carnea* are not predaceous but feed on floral and extrafloral nectar, pollen, and honeydew (Principi and Canard 1984; Villenave et al. 2006; Gonzalez et al. 2016; Villa et al. 2016). *Chrysoperla carnea* is commercially available in Europe (e.g., Biobest 2009; Koppert 2021) and North America (Tauber et al. 2000; McEwen 2001) and has been used mainly for aphid biocontrol in various greenhouse and field crops.

Predation of the more vulnerable stages of stink bugs, such as the egg and early nymphal stages, may be significant in regulating their populations (Pote and Nielsen 2017). The present laboratory study aimed at quantifying the predation efficacy of second and third instar *C. carnea* on the early nymphal stages of *H. halys* and *N. viridula,* under varying conditions of temperature and arena complexity.

5.2. Materials and Methods

5.2.1. Insect rearing

Halyomorpha halys adults were collected in the field in Gentbrugge, Belgium, in July 2020. The collected adults were then further reared in clear polypropylene containers (40 x 30 x 20 cm) provided with 4 x 8 cm meshed vents on each side in climatic chambers (PHCBI MLR-352H-PE, Japan) set at 24.0 ± 0.5 °C, 65 ± 5% RH, and a photoperiod of 16:8 h (L:D). Bean pods (*Phaseolus vulgaris* L. *cv.* 'Helda') and sunflower seeds (*Helianthus annuus* L.) were provided as a food source. Water was supplied via moistened synthetic cotton (Roltasoft Hartmann, Germany). Crumpled paper towels were placed in the rearing containers to provide shelter and surfaces for egg laying. Food and water were replaced twice per week. Egg masses were collected during the replenishment of food and water; during trials, egg masses were collected daily. Life stages were kept in separate rearing boxes. An in-house culture of *N. viridula* at Ghent University, originating from individuals collected from different sites in Europe (France, Spain and Italy), was kept under identical conditions but in smaller rearing containers (30 x 20 x 9 cm).

A culture of *C. carnea* (sensu lato) was started using individuals obtained from Biobest Group NV, Westerlo, Belgium (Chrysopa System). The lacewings were reared in climatic chambers set at 23.0 ± 0.5 °C, 65 ± 5% RH, and a photoperiod of 16:8 h (L:D) in vented polystyrene insect breeding dishes (10 x 2 cm; SPL Life Sciences Co., Korea). Adults were fed with commercial bee pollen grains (Weyn's Honing, Belgium) and synthetic cotton drenched in a solution of 9:1 water and honey held by a bottlecap. Food was replenished twice per week. Eggs were laid on the breeding dish lids and collected twice weekly by transferring the lids to a new breeding dish containing thawed *Ephestia kuehniella* (Zeller) eggs, supplied by Koppert Biological Systems (Berkel en Rodenrijs, The Netherlands) as food for emerging larvae. Life stages were kept in separate breeding dishes.

5.2.2. Predation bioassays

Following preliminary experiments, we excluded combinations of the second larval instar of *C. carnea* with the second nymphal instar of either stink bug due to size mismatch. Combinations with stink bug eggs were also excluded as they proved not to be suitable prey in our preliminary trials, corroborating the findings of Bulgarini et al. (2021) in the case of *H. halys*. Thus, our predation bioassays included combinations where first and second instars of *N. viridula* and *H. halys* were exposed to third instars of *C. carnea* and the first instars of either stink bug were exposed to second instars of the lacewing.

Predation bioassays were conducted in two arena types and under three different temperatures for a total period of 48 hours. The two experimental arenas used were (1) insect breeding dishes (100 x 40 mm; SPL Life Sciences Co., Korea) with a 3 cm piece of bean pod (*P. vulgaris* cv. ‘Helda’) as food for the stink bug nymphs and (2) a microcosm arena consisting of a custom polystyrene cylinder (250 x 100 mm) with a mesh-covered top containing a bean plant (*P. vulgaris* cv. ‘Prelude’) with one developed leaf. For the microcosms, bean plants with similar leaf surfaces were selected and were one week old (ca. 15 cm high). Temperatures tested were 16, 21, and 26 ± 1 °C reflecting a cool, average, and hot summer day in northwestern Europe. The different combinations of predator and prey life stages as explained above were tested at the three temperatures (16, 21, and 26 °C) in both arenas (breeding dish and microcosm with bean plant), resulting in 36 combinations; for each combination, 12 replicates were done (N = 432). Each replicate contained a single *C. carnea* larva and 30 stink bug nymphs. In addition, survival of both stink bug prey was recorded in control treatments without a predator, with one control for each replicate (N = 432).

The first and second instar prey nymphs were less than 24 h in their respective instar. Stink bug nymphs were introduced into the arena using a fine paintbrush (da Vinci, Nürnberg, Germany; Thickness: 1.7 mm) and placed on either the bean pod (in breeding dishes) or on the bean plant (in microcosms). Nymphs were left to settle for 2 h before the predator was introduced into the arenas.

Early-instar lacewing larvae of the second or third stadium (< 24 h in their respective stadium) were taken from the rearing stock and individually starved for a 24 h period before testing during which time they had access to water but not food. The larvae were weighed 2 h before testing using a Sartorius Genius ME215P microbalance (Sartorius, Goettingen, Germany; precision: 0.1 mg). Lacewing larvae with an *a priori* established weight range of 0.90 ± 0.15 mg for second and 2.10 ± 0.15 mg for third instars were selected for the experiments. Predators were released in the arena by dropping them on the bottom of the breeding dish or on the leaf of the bean plant with a fine paintbrush.

Prey counts were performed 24 h and 48 h after the start of the test by visually inspecting each nymph using a flex arm stereomicroscope (Leica A60, Wetzlar, Germany). Both dead and live prey individuals were counted. After 24 h, prey numbers were replenished with 24- to 48-h-old nymphs. Lacewing larvae were weighed after 24 h and again at 48 h, and their survival and moulting were recorded.

For both stink bug species 25 first instar and 25 second instar nymphs (< 24 h in their respective instar) (N = 100) were weighed using a microbalance in order to compare the fresh weights of these prey stages within and between stink bug species.

5.2.3. Alternative diet bioassay

An additional experiment was conducted to compare the fresh weight gain of *C. carnea* larvae feeding on the first instars of *N. viridula* and *H. halys* with that on *E. kuehniella* eggs as a standard alternative diet used in the mass rearing of the lacewing. Since only the third instar of *C. carnea* could kill and feed successfully on first and second instars of either pentatomid, only third instars of the predator were tested in this experiment. Individual third instar *C. carnea* larvae were placed in an insect breeding dish (100 x 40 mm) with an *ad libitum* amount of thawed *E. kuehniella* eggs at a temperature of 21 °C, i.e. the median of the tested temperature range in our predation bioassays. Analogous to the predation experiment, larvae were starved for 24 h and weighed 2 h before the experiment started and again after 24 and 48 hours; only larvae within an initial weight range of 2.10 ± 0.15 mg were used for the experiment. Twelve replicates were performed.

5.2.4. Statistical analysis

All statistical analyses were performed with R version 4.1.3. (R Core Team 2024; RStudio Team 2024)). To compare the cumulative percentage of predation after 48 h among treatments, a Generalized Linear Model (GLM) with a Quasi-Poisson logit link function with interaction terms was applied to infer the relation between predation efficiency (total number of killed prey after 48h) and the factors (1) prey species, (2) prey stage, (3) predator stage, (4) arena type, and (5) temperature. Model performances were compared by assessing goodness-of-fit through analysis of deviance and comparing adjusted R^2 values. Multicollinearity was checked via variance inflation. Obtained beta-coefficients were transformed back for interpretation. The correlation between predation and weight gain was checked with Pearson's correlation coefficient. To compare the weights of the first two nymphal instars of *N. viridula* and *H. halys*, we used a two-way analysis of variance (ANOVA) with stink bug species and nymphal instar as factors. Post-hoc comparisons were conducted using Tukey's Honest Significant Difference test. Finally, the weight gain data from the third instar lacewings used in the alternative diet bioassay with *E. kuehniella* eggs was compared to those of their counterparts fed with first instars of both stink bugs at 21 °C in breeding dish arenas via a one-way analysis of variance (ANOVA) and Tukey's Honest Significant Difference test.

5.3. Results

Prey survival in the controls was higher than 99%. Less than 1% of tested predators died or advanced to the next stadium during the 48 h duration of the experiment. The few lacewing larvae (N = 3) that moulted during the course of the experiment were excluded from the statistical analysis. The percentage of prey items killed during the first 24 h (day 1) and the second 24 h (day 2) of the experiment for each treatment is presented in Table 5.1 and 5.2 for *N. viridula* and *H. halys,* respectively. The cumulative percentages of prey items killed during the 48-hour experiment per treatment are shown in Figure 5.1.

The final predation efficacy GLM was:

Predation ~ prey species + prey life stage + predator life stage + arena + prey species × prey life stage + prey species x predator life stage

The final GLM with interaction terms and R²-adjusted = 0.840, outperformed the Ordinary Least Squares (OLS) linear models with significant interaction terms ($R^2 = 0.688$) and without interaction terms ($R^2 = 0.637$), as well as the GLM without interaction terms (adjusted $R^2 = 0.812$). The Analysis of Deviance indicated a significant improvement in the goodness-of-fit for the final GLM with two interaction terms compared to the GLM without interaction terms and the OLS linear models ($p < 0.0001$). The temperature regime had no significant effect ($p = 0.097$) on the percentage of prey killed after 48 h and was therefore omitted as a factor from the model via backward selection. Larvae killed more prey during the first 24 h after starvation compared to the second 24 h of the experiment (Table 5.1 & 5.2). The predation GLM with its coefficients and their standard errors can be found in Table 5.3. The factor prey species was highly significant ($p < 0.0001$), with *N. viridula* being more vulnerable to predation than *H. halys*. The life stage of the stink bugs also affected predation ($p < 0.0001$), with first instars of the prey being more susceptible to predation by *C. carnea* than second instars. The predator stage impacted the predation percentage on stink bug nymphs significantly as well ($p < 0.0001$), with the third larval stadium being more efficient as a predator than the second larval stadium in all tested treatments. The GLM operates from a standard setting where the predation percentage is a combination of factors with the following categories: (1) Predator life-stage: L2, (2) Prey species: *H. halys,* (3) Prey life stage: first instar nymph, (4) Arena: microcosm. In this setting, the GLM predicts a mean of 9.2 ± 0.1% first instar *H. halys* nymphs killed by second instar *C. carnea* larvae in a microcosm arena over 48 h (vs. observed at 21 °C: 9.2 ± 1.4%, Fig. 5.1). When the model parameters are changed, for example, to the third larval instar of the predator presented with first instars of *N. viridula* in a breeding dish arena, the predicted mean response variable of our model is 61.6 ± 1.4% of prey nymphs killed over 48 h (vs. observed at 21 °C: 60.8 ± 4.7%, Fig. 5.1).

The fresh weight of *H. halys* nymphs averaged 1.2 ± 0.1 mg and 2.6 ± 0.1 mg for the tested first and second instars, respectively. First and second instars of *N. viridula* weighed 0.5 ± 0.1 mg and 2.7 ± 0.1 mg, respectively. Whereas fresh weights overall differed significantly between groups (F = 2601, df = 3, 96; $p < 0.0001$), weights of the second instars of both stink bug species were similar (Tukey test, $p = 0.2361$).

Over the entire dataset, fresh weight gain of the lacewing larvae was strongly positively correlated with their predation rates (r(427) = 0.86, $p < 0.0001$).

Third instars of *C. carnea* fed with *E. kuehniella* eggs gained significantly (F = 56.28; df = 2, 33; $p < 0.001$) more weight in 48 h (8.3 ± 0.5 mg) than their counterparts feeding on the first of *H. halys* (4.9 ± 0.3 mg) or *N. viridula* (4.4 ± 0.2 mg) (Tukey test, $p < 0.0001$ for both contrasts).

5.4. Discussion

The present laboratory bioassays quantified the predatory capacity of second and third instar *C. carnea* larvae on the first and second nymphal instars of *N. viridula* and *H. halys* in a simple dish arena and a more complex microcosm arena. Our findings add to earlier studies showing that lacewing larvae can have some impact on the early instars of pentatomids. Ehler (2002) reported that field-collected green lacewing larvae (no instar defined) succeeded in killing on average 6 second instars of *N. viridula* in petri dish arenas over a 48-h period. (Bulgarini et al. 2021) showed that after 48 h, second instars of *C. carnea* decreased survivorship of first instar *H. halys* by 20%, resulting in ca. 3-4 nymphs killed when at least 18 nymphs were offered on a young bean plant in a 20-cm high container at 26 °C. In our experiments, a similar number of first instars of both *H. halys and N. viridula* were killed by second instar *C. carnea* larvae after 48 h in an experimental set-up with 30 nymphs per microcosm.

Third instars of *C. carnea* proved to be more effective predators of first instar nymphs of both stink bug species. For instance, third instar *C. carnea* killed on average 2-3 times as many first instar *N. viridula* and *H. halys* as compared to second instar larvae (Table 5.1 & 5.2). Additionally, our experiments show that third instar *C. carnea* are able to subdue second instars of both stink bug species, killing ca. 10-12 individuals of *N. viridula* nymphs versus ca. 3-4 individuals of *H. halys* nymphs over a 48-h period in a dish arena. The observed results align with those reported by (Principi and Canard 1984; Klingen et al. 1996; Huang and Enkegaard

2010) indicating a substantially higher predation efficiency in third instar lacewings, compared to second instars, on various insect prey.

Our data show that *H. halys* nymphs are less vulnerable to predation by *C. carnea* larvae than *N. viridula* nymphs. On average, 28% more *N. viridula* nymphs were killed compared with those of *H. halys* when all factors were held constant in our model and data. Several factors may explain this difference in predation rates among the pentatomid prey. First instars of *H. halys* have an overall larger body size, weighing twice as much as *N. virdula* first instars. Larger body size or greater weight can result in a longer handling time by *C. carnea* larvae for *H. halys* prey than for their *N. viridula* counterparts, resulting in less prey consumed over the experimental period. The second instars of *H. halys* and *N. viridula* are more similar in size and weight. Nevertheless, the second instars of *H. halys* were still less vulnerable to predation by *C. carnea* larvae than those of *N. viridula*. Differences in the kill rate of second instar nymphs may be explained by the different motility and anti-predator behaviour of the pentatomids. *Halyomorpha halys* nymphs of the second instar (and beyond) have a strong walking capacity (Lee et al. 2014), making them better at avoiding and escaping the attacks of *C. carnea* compared to second instars of *N. viridula*.

Earlier studies on the predatory behaviour and feeding preferences of *C. carnea* suggest a predilection for certain prey types based on their mobility, size, and detectability. Specifically, *C. carnea* tends to prefer aphids over thrips, possibly due to the immobility, larger size, and chemical cues of the aphids (McEwen 2001; Shrestha and Enkegaard 2013).

Further, deterrency and antinutritional factors might also play a role in the survival of stink bug nymphs attacked by a generalist predator like *C. carnea*. Pentatomidae are known for their chemical defences that may be released when disturbed, acting as a deterrent for predators (Aldrich 1988; Noge et al. 2012; Harris et al. 2015; Weber et al. 2017; McPherson 2018). The production of these compounds can vary between species and life stages (Borges and Aldrich 1992). For instance, some members of the subfamily Pentatominae (including *N. viridula*) excrete 4-oxo-(E)-2-decenal when agitated, but only do so during the first instar (Borges and Aldrich 1992). In other species, this compound is emitted by all nymphal instars but is absent in the secretions of adults (Pareja et al. 2007). The chemical composition of *H. halys* nymphal secretions has not been thoroughly studied in comparison to those of *N. viridula* (Pote and Nielsen 2017). Thus, variation in the production of these defensive compounds could also have led to differences in vulnerability to predation by green lacewing larvae among nymphal instars and stink bug species.

When third instar lacewing larvae were fed with eggs of *E. kuehniella,* their weight gain was significantly higher than in the treatments with stink bug nymphs. *Ephestia kuehniella* eggs are likely more suitable prey than stink bug nymphs, allowing more efficient extraction of nutrients. As an immobile prey type, eggs of *E. kuehniella* may require less time and energy handling as compared to stink bug nymphs. Further, this type of factitious food has been reported to be of superior nutritional value to various insect predators, including *C. carnea* larvae, versus certain aphids, which can be considered the main natural prey of those predators (El-Arnaouty et al. 2000; Specty et al. 2003). However, it should be noted that we only measured weight gain over a 48-h period and experiments monitoring the full development of *C. carnea* larvae on the studied stink bug nymphs would be needed to gain a more profound insight into the nutritional value of this pentatomid prey.

In our study, a significant arena effect was observed in terms of the number of stink bug nymphs killed by the predator. Predators are known to spend more energy and time searching for prey items in more complex arenas, affecting the predator's functional response (Juliano et al. 2022).

The temperature range implemented in our experiments (16 – 26 °C), on the other hand, did not significantly affect predation by the lacewing larvae on *N. viridula* and *H. halys* nymphs. This is surprising, given that killing and prey consumption rates often respond to increases in temperature, due to higher metabolic rates and increased activity of arthropod predators (Rosenblatt and Schmitz 2016; González-Tokman et al. 2020). Our data do indicate that our *C. carnea (sensu lato)* strain from a commercial source displays good predation capacity at more favourable temperatures of 21-26 °C, as well as at temperatures as low as 16 °C, suggesting its potential for augmentative use against stink bug outbreaks under various climatic conditions. Augmentative releases of green lacewings may, for instance, help in slowing down population build-up of *N. viridula* in greenhouse crops early in the growing season as well as assist in suppressing localized infestations during warmer periods of the year. Inundative or inoculative releases of green lacewings and other generalist predators, like *Podisus maculiventris* (Say) (De Clercq et al. 2002) in greenhouse crops may thus add to the action of more specialist natural enemies of *N. viridula* like the egg parasitoid *Trissolcus basalis* (Wollaston) (Gard et al. 2022).

Spot releases of *C. carnea* may also help reducing localized *H. halys* nymphal populations in certain cropping systems, like greenhouse vegetables, although the effect may only be restricted to the gregarious and less agile first instars of the pest (Bulgarini et al. 2021; this study). On the other hand, augmentative releases of generalist predators like green lacewings to suppress populations of stink bugs in open-field crops and orchards may not be cost-effective.

Besides *C. carnea*, multiple other generalist predators of *N. viridula* and *H. halys* nymphs have been reported including Coccinellidae, Malachidae, Anthicidae, Lygaeidae, Nabidae, Reduviidae, Opiliones and Araneae (Ehler 2002; Bulgarini et al. 2021). All of these could play a role in the conservation biological control of stink bug populations in the open field. Here, measures could be taken towards attracting and supporting natural populations of green lacewings and other omnivorous predators, including the introduction of selected flowering plants (Principi and Canard 1984; Landis et al. 2000; Tauber et al. 2000; Gonzalez et al. 2016; Uyttenbroeck et al. 2016; Albrecht et al. 2020; Fountain 2022) and mixed hedgerows (Albrecht et al. 2020; Beliën et al. 2021).

In the present study, green lacewing larvae were offered solely stink bug prey in small experimental arenas and during a limited time frame. In order to fully appreciate the potential of this generalist predator in the prevention or suppression of pentatomid outbreaks, further studies could monitor the full development of *C. carnea* when exclusively fed on stink bug prey, focus on the prey preference of *C. carnea* towards stink bugs when also offered other, more suitable prey types like aphids, investigate potential interactions with other natural enemies in the crop system, as well as conduct larger-scaled experiments in more realistic settings like greenhouses.

Table 5.1. Coefficients with their standard errors of the predation GLM with Quasi-Poisson distribution.

	Estimate (β)	$e^{(\beta)}$	Std. Error (SE)	$e^{(sd)}$	t-value	Pr (>| t |)
(Intercept)	2.22	9.17	0.07	1.07	32.64	< 0.0001
Prey species (to *Nezara viridula*)	0.41	1.51	0.08	1.09	4.92	< 0.0001
Prey life stage (to N2)	-1.01	0.36	0.08	1.08	-12.58	< 0.0001
Predator life stage (to L3)	0.88	2.41	0.08	1.08	11.5	< 0.0001
Arena (to Breeding dish)	0.36	1.43	0.04	1.04	10.01	< 0.0001
Prey species x Prey life stage	0.48	1.62	0.09	1.1	5.14	< 0.0001
Prey species x Predator life stage	0.25	1.28	0.1	1.1	2.56	0.0107

Table 5.1. Mean ± standard error (N=12) percentage of killed first or second instar nymphs (N1, N2) of *N. viridula* by second or third larval instars (L2, L3) of *C. carnea* during the first 24 h (Day 1) and the second 24 h (Day 2) of the experiment at three temperatures and in two types of arenas.[1]

C. carnea L2				*C. carnea* L3							
N. viridula N1				*N. viridula* N1				*N. viridula* N2			
Breeding dish		Microcosm		Breeding dish		Microcosm		Breeding dish		Microcosm	
Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
13.9 ± 2.2	5.6 ± 1.0	9.4 ± 1.8	5.3 ± 1.6	34.4 ± 3.1	27.8 ± 1.8	26.1 ± 3.3	11.9 ± 2.0	20.0 ± 2.3	11.9 ± 1.6	15.8 ± 2.7	10.3 ± 1.3
14.4 ± 1.5	6.1 ± 1.2	8.9 ± 2.1	2.8 ± 1.3	33.1 ± 2.9	27.8 ± 2.4	22.8 ± 3.9	23.3 ± 4.9	19.2 ± 1.5	20.8 ± 3.6	13.3 ± 2.0	11.9 ± 2.6
16.9 ± 1.8	5.6 ± 1.1	8.9 ± 1.7	3.1 ± 1.0	34.7 ± 2.7	28.3 ± 2.6	21.9 ± 3.6	20.8 ± 3.5	21.9 ± 2.4	12.2 ± 1.3	18.9 ± 3.3	8.1 ± 1.4

Table 5.2. Mean ± standard error (N=12) percentage of killed first or second instar nymphs (N1, N2) of *H. halys* by second or third larval instars (L2, L3) of *C. carnea* during the first 24 h (Day 1) and the second 24 h (Day 2) of the experiment at three temperatures and in two types of arenas.[1]

C. carnea L2				*C. carnea* L3							
N. viridula N1				*N. viridula* N1				*N. viridula* N2			
Breeding dish		Microcosm		Breeding dish		Microcosm		Breeding dish		Microcosm	
Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
13.9 ± 2.2	5.6 ± 1.0	9.4 ± 1.8	5.3 ± 1.6	34.4 ± 3.1	27.8 ± 1.8	26.1 ± 3.3	11.9 ± 2.0	20.0 ± 2.3	11.9 ± 1.6	15.8 ± 2.7	10.3 ± 1.3
14.4 ± 1.5	6.1 ± 1.2	8.9 ± 2.1	2.8 ± 1.3	33.1 ± 2.9	27.8 ± 2.4	22.8 ± 3.9	23.3 ± 4.9	19.2 ± 1.5	20.8 ± 3.6	13.3 ± 2.0	11.9 ± 2.6
16.9 ± 1.8	5.6 ± 1.1	8.9 ± 1.7	3.1 ± 1.0	34.7 ± 2.7	28.3 ± 2.6	21.9 ± 3.6	20.8 ± 3.5	21.9 ± 2.4	12.2 ± 1.3	18.9 ± 3.3	8.1 ± 1.4

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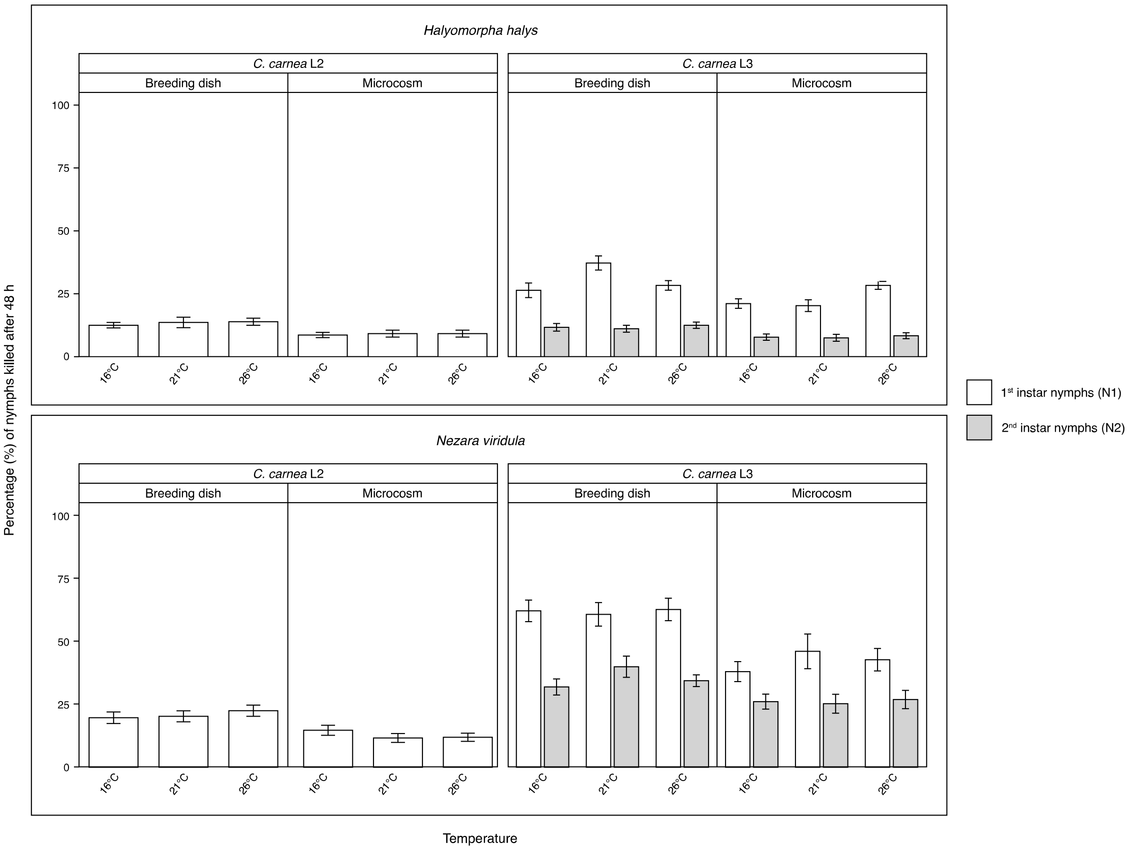
[1] In all combinations, the initial number of prey nymphs offered per predator was 30

Figure 5.1. Cumulative percentage (mean ± SE) of first or second instar nymphs (N1, N2) killed by second or third larval instars (L2, L3) of *C. carnea* after 48 h at three temperatures and in two types of arenas for both *H. halys* (top) and *N. viridula* (bottom). In all combinations, the initial number of prey nymphs offered per predator was 30.



Chapter 6

Egg parasites of economically important stink bugs in Belgium

This chapter is adapted from:

First record in Belgium of *Trissolcus basalis* (Hymenoptera, Scelionidae), an egg parasitoid of economically important stink bugs (Hemiptera, Pentatomidae).

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Abstract

Parasitic wasps of the family Scelionidae are gaining increasing attention for their use in biological control, due to their parasitism of eggs of key pentatomid pests in agriculture like *Halyomorpha halys* (Stål) and *Nezara viridula* (L.).

In this study cryo-killed sentinel egg masses of *H. halys* were distributed in agricultural and urban environments to attract native and non-native egg parasitoids. Additionally, egg parasites were sampled through the collection of naturally laid eggs of native stink bugs and opportunistic net sweeps in orchards.

Sentinel eggs of *H. halys* were unable to attract egg parasitoids. In contrast, naturally laid egg masses by several species of Pentatomidae were fully or partially parasitised in 31% of the collected egg masses. Egg parasitoids found in this study all belonged to the genus *Trissolcus* or *Telenomus.*

6.1. Introduction

Pentatomid eggs are attacked by a complex of parasitoid species belonging to the genera *Trissolcus*, *Telenomus* (Hymenoptera: Scelionidae), *Ooencyrtus* (Hymenoptera: Encyrtidae), and *Anastatus* (Hymenoptera: Eupelmidae) (see section 2.3.1.2.) (Moraglio et al. 2021b).

These parasitic wasps, in particular members of the Scelionidae family are gaining increased attention in biological control research due to their parasitism of eggs of key pentatomid pests in agriculture (Lowenstein et al. 2019a; Stahl et al. 2019b; Haye et al. 2020; Dieckhoff et al. 2021; Moraglio et al. 2021a, 2021b; Tortorici et al. 2023).

The occurrence, distribution, biology, and host ranges of European scelionids have been poorly documented, with the exception of some species whose hosts are harmful crop pests. Notable examples include *Trissolcus basalis* (Wollaston) on *Nezara viridula* (L.) (Colazza and Bin 1995; Gard et al. 2022) and *Trissolcus semistriatus* (Nees von Esenbeck) and *Trissolcus belenus* (Walker) on *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae) (Davari and Parker 2018).

The global interest in egg parasitoids capable of attacking the harmful stink bug *Halyomorpha halys* (Stål) has spurred taxonomic research. Recent studies have revised the systematics of Palaearctic and Nearctic *Trissolcus* spp. (Talamas et al. 2017; Tortorici et al. 2019) and some of the Palearctic *Telenomus* spp. (Tortorici et al. 2024) to provide insights in parasite – stink bug host relationships and morphological characters to identify these difficult to distinguish Scelionidae.

Additionally, adventive (self-introduced) populations of the non-native *Trissolcus japonicus* (Ashmead) were discovered in Switzerland, Italy, Germany and Serbia (Stahl et al. 2019a; Sabbatini-Peverieri et al. 2020; Dieckhoff et al. 2021; Moraglio et al. 2021b; Mele et al. 2022; Konjević et al. 2024), as well as the non-native *Trissolcus mitsukurii* (Ashmead) in France, Italy, Slovenia and Serbia (Sabbatini-Peverieri et al. 2020; Bout et al. 2021; Rot et al. 2021; Mele et al. 2022; Konjević et al. 2024); both are the main egg parasites of *H. halys* in its native range. In Italy, the release of *T. japonicus* to control *H. halys* was authorized and started in the summer of 2020 (Moraglio et al. 2021b). As well as in France, the release of *T. japonicus* was approved in 2022 as a biological control agent for *H. halys* (ANSES, 2022). It is expected that *T. japonicus* will continue to expand its range in Europe as predicted by bioclimatic envelope models (Avila and Charles 2018; Haye et al. 2024).

The range expansion of *H. halys* and reported damage in pip fruit by the native *Pentatoma rufipes* (L.) has heightened the importance of understanding European scelionid species and their role in the biological control of native and invasive stink bugs (Stahl et al. 2019b; Moraglio et al. 2021a, 2021b; Sabbatini-Peverieri et al. 2021).

This chapter aims to investigate the presence of scelionid species parasitizing eggs of native Pentatomidae as well as the invasive *H. halys* in Belgium.

6.2. Materials and Methods

6.2.1. Sentinel egg survey

Egg masses of *H. halys* laid on paper towels were collected daily from an in-house rearing (see section 5.2.1). To sterilize the egg masses, they were frozen at -80 °C until deployment in the field. Each egg mass was attached to the inside of a breeding dish (50 x 15 mm; SPL Life Sciences Co., Korea) with the mesh removed to allow access to the egg mass by parasitoids. Breeding dishes were glued to clothespins to allow attachment to plants or structures. A total of 211 sentinel egg masses were deployed from July to September in 2022 and 2023. Sentinel egg masses were distributed in hedgerows and fruit trees of commercial organic orchards in Halen and Assent for 48 - 72 hours.

Egg masses were then collected from the field and reared individually in breeding dishes (100 mm x 40 mm) in a climatic chamber, at 24 °C, 16:8 h L:D and 70% RH (PHCBI MLR-352H-PE, Japan) to assess parasitism. All emerged parasitoids were transferred into 70% ethanol for later identification.

6.2.2. Field surveys and sweep net sampling

In addition to the sentinel egg campaign, opportunistic sweep net samplings were conducted during other sampling campaigns. This involved using sweep nets to catch egg parasitoids of stink bugs. Additionally, naturally occurring egg masses of relevant stink bugs were collected in the field.

From July to September 2020-2023 wild egg masses of relevant stink bugs encountered during sampling were collected in orchards and gardens. Egg masses were taken back to the lab for incubation as described for sentinel eggs.

Sweep nets were used in two organic pip fruit orchards located in Halen and Assent, Belgium. During each session, the net (40 cm, mesh size 100 µm) was swept through the vegetation in the wildflower borders. This process involved performing approximately 50 sweeps per flowering strip, with the net held approximately 1 meter above ground level. Sweeping sessions were concluded by transferring the contents of the sweep nets to collection bags. Upon arrival at the laboratory, these bags were stored dry at -20 °C for later identification purposes.

6.2.3. Morphological identification

Parasitoids were morphologically identified using the keys of Talamas et al. 2017 and Tortorici et al. 2019. All morphological identifications and difficult to identify specimens were morphologically confirmed and identified by Dr. E.J. Talamas (Florida Department of Agriculture and Consumer Services, Division of Plant Industry). Photographs were taken using a microscope (Keyence VHX-970F) or macrophotography camera (Macropod Pro 3D Micro Kit with Canon 6D mk II).

For *T. basalis*, specimens in 70% ethanol were deposited at the Ghent University Museum, Zoology Collections with collection numbers: UGMD_104422 and UGMD_104423.

Collections from the Royal Belgian Institute of Natural Sciences, The Entomological Conservatory at Gembloux and online databases including waarnemingen.be (accessed 20 October 2021), fauna-eu.org (accessed 20 October 2021) and gbif.org (accessed 20 October 2021) were checked to confirm any previous records of *Trissolcus* and *Telenomus* in Belgium.

6.2.4. Molecular identification

Molecular identification to confirm morphological identification was carried out uniquely for *T. basalis*. Molecular identification was done with twenty frozen adult individuals of both sexes from the F2 generation, originating from a single mated female. The adults were pooled for DNA extraction and mitochondrial cytochrome c oxidase I (COI) fragment sequencing. DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen). The DNA samples were quantified using a NanoDrop2000 spectrophotometer (Thermo Scientific). At least 20 ng of genomic DNA was used per PCR. The 5’-COI region was PCR-amplified using the primers [LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO-2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer et al. 1994). The PCR was performed in a 50 µl reaction volume: 1 µl DNA, 29.5 µl molecular grade water, 10 µl 5X Green GoTaq Flexi PCR buffer, 2.5 µl dNTPs (25mM each), 5 µl $MgCl_2$, 1.5 µl of each primer (1µM each), 0.2 µl GoTaq G2 Flexi DNA polymerase (5u/µl) (Promega Corp., Wisconsin, USA). Thermocycling conditions were optimized to shorten reaction times and included initial denaturation at 94 °C for 300 s, followed by 35 cycles of 94 °C for 30 s, annealing at 41 °C for 45 s and extension at 72 °C for 60 s; then further 600 s at 72 °C for final extension. All PCR products were purified using the E.Z.N.A Cycle Pure Kit (Omega Bio-tek Inc, Georgia, USA) following the manufacturer’s instructions and sent for sequencing to an external service (LGC Genomics GmbH, Berlin, Germany). The obtained forward and reverse sequences were inspected and a consensus sequence was made using the BioEdit Software V7.2.0. The consensus sequence was compared with sequences present in the GenBank database by similarity search using the BLAST (Basic Local Alignment Search Tool) available through the NCBI platform, to confirm the taxonomic identity. The COI sequence generated was deposited in GenBank (MZ087751).

6.3. Results

None of the *H. halys* sentinel egg masses were parasitized. From all 41 naturally occurring egg masses collected 13 (~ 31%) were parasitized with full or partial emergence of the egg mass and in 2 of the 14 sweep samples relevant parasitoids were found. In total, seven unique species of egg parasitoids were found, all belonging to the genera *Trissolcus* or *Telenomus* (see section 2.3.1.1.) (Table 6.1).

Table 6.1. Overview of the egg parasitoids collected during this study.

Host	Year	Collector	Location	Species
N. viridula	2020	Rik Clymans	Gentbrugge	*Telenomus turesis*
N. viridula	2020	Lore Vervaet	Sint-Amandsberg	*Telenomus turesis*
R. nebulosa	2020	Lore Vervaet	Sint-Amandsberg	*Trissolcus basalis*
P. prasina	2020	Gertie Peusens	Vlijtingen	*Trissolcus cultratus*
P. prasina	2020	Gertie Peusens	Vlijtingen	*Telenomus truncatus*
R. nebulosa	2020	Rik Clymans	Gentbrugge	*Trissolcus cultratus*
A. custos	2021	Rik Clymans	Gentbrugge	*Trissolcus belenus*
R. nebulosa	2021	Olivier Berteloot	Halen	*Trissolcus basalis*
P. prasina	2023	Olivier Berteloot	Assent	*Telenomus truncatus*
P. prasina	2023	Olivier Berteloot	Assent	*Trissolcus cultratus*
A. custos	2023	Olivier Berteloot	Staden	*Trissolcus belenus*
A. custos	2023	Olivier Berteloot	Staden	*Trissolcus belenus*
R. nebulosa	2023	Olivier Berteloot	Halen	*Telenomus truncatus*
sweep net	2023	Olivier Berteloot	Halen	*Trissolcus semistriatus*
sweep net	2023	Olivier Berteloot	Assent	*Trissolcus colemani*

6.3.1. *Trissolcus basalis*

Trissolcus basalis (Wollaston) is part of the *basalis* group within the *Trissolcus* genus. The species in the *basalis* group are defined by a smooth metapleuron (no setation) and the absence of a hyperoccipital carina. Within this group, *T. basalis* can be identified from Nearctic congeners by the combination of some additional characters. An incomplete netrion sulcus and a mesopleuron with shallowly impressed episternal foveae and metapleuron without a well-defined paracoxal sulcus. The mesoscutal humeral sulcus is present as a smooth furrow and the second metasomal tergite is striated longitudinally (Talamas et al. 2017) (Fig. 6.2 - 6.5, specimen from Sint-Amandsberg).

6.3.1.1. Distribution

Although *T. basalis* is considered to be globally distributed (Colazza and Bin 1995; Talamas et al. 2017). Its distribution within Europe has been rather sparsely documented, with records from Cyprus, Montenegro, Portugal, Spain, Italy, Hungary, France and Germany (Awan et al. 1990; Colazza and Bin 1995; Tortorici et al. 2019; Awad et al. 2021) (Fig. 6.1).

Figure 6.1. Map of Europe with highlighted countries where *T. basalis* was recorded (according to Talamas et al. (2017); Tortorici et al. (2019); Awad et al. (2021)).

Figure 6.2. Lateral habitus of *T. basalis*, specimen from Sint-Amandsberg. (Credit: E.J. Talamas)

Figure 6.3. Dorsal habitus of *T. basalis*. (Credit E.J. Talamas)

Figure 6.4. Ventrolateral habitus of *T. basalis*. (Credit E.J. Talamas)

Figure 6.5. Anterior head of *T. basalis*. (Credit E.J. Talamas)

6.3.1.2. Host associations

Aelia acuminata (L.), *Aelia cognata* Fieber, *Aelia germari* Küster, *Agonoscelis rutila* (Fabricius), *Calidea dregeii* Germar, *Carpocoris fuscispinus* (Boheman), *Coleotichus blackburniae* (White), *Cuspicona simplex* Walker, *Dolycoris baccharum* (L.), *Eurydema ornata* (L.), *Eurygaster austriaca* (Schrank), *Eurygaster integriceps* Puton, *Euschistus servus*, *Euthyrhynchus floridanus*, *Graphosoma semipunctatum* (Fabricius), *Halyomorpha annulicornis* (Signoret), *Nezara viridula*, *Odontotarsus grammicus* (L.), *Oechalia schellenbergi* Guérin-Méneville, *Plautia affinis* (Dallas), *Piezodorus hybneri* (Gmelin) and *Raphigaster* sp. (Johnson 1984; Talamas et al. 2015). The parasitoid has also been reported to emerge from frozen sentinel eggs of *H. halys* eggs, but these records are considered to be rare events (Balusu et al. 2019).

6.3.2. *Trissolcus semistriatus*

Trissolcus semistriatus (Nees von Esenbeck), belongs to the *basalis* group as well and is most similar to *T. basalis*. It can be distinguished from *T. basalis* by the complete netrion sulcus (incomplete in *T. basalis*), distinct and regular cells of the postacetabular sulcus and, in most cases, by the distinct cells of the mesoscutal humeral sulcus (always indicated by a smooth furrow in *T. basalis* and very rarely so in *T. semistriatus*). Additionally, the angular form of the occipital carina in dorsal view, with a short carina extending toward the median ocellus, is found only in this species (Tortorici et al. 2019) (Fig. 6.6, specimen from Halen).

6.3.2.1. Distribution

Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, France, Greece, Hungary, Iran, Iraq, Ireland, Italy, Japan, Kazakhstan, Lebanon, Macedonia, Morocco, Russia, Serbia, Slovakia, South Korea, Spain, Sweden, Turkey, Ukraine and United Kingdom (Tortorici et al. 2019).

6.3.2.2. Host associations

Aelia rostrata Boheman, *Brachynema germarii* (Kolenati), *Carpocoris* sp., *D. baccarum, E. ornata*, *Graphosoma lineatum* (L.), *G. semipunctatum*, *Holcostethus vernalis* (Wolff) and *Rhaphigaster* sp. (Tortorici et al. 2019).

Figure 6.6. Lateral habitus of *T. semistriatus,* specimen from Halen. (Credit E. J. Talamas)

6.3.3. Trissolcus belenus

Trissolcus belenus (Walker), belongs to the *basalis* group and thus lacks the presence of a hyperoccipital carina. The distinction of *T. belenus* and *T. colemani* from *T. semistriatus* is more nuanced. *Trissolcus belenus* can be identified by the presence of setae on the first laterotergite (without setae in *T. colemani*) and the absence of both the notauli on the mesoscutellum (Tortorici et al. 2019) (Fig. 6.7, specimen from Gentbrugge).

Figure 6.7. Lateral habitus of *T. belenus*, specimen from Gentbrugge. (Credit: Author)

6.3.3.1. Distribution

Belgium, China, Italy, Morocco, Portugal, Russia, Sweden, Switzerland, United Kingdom, Ukraine (Tortorici et al. 2019).

6.3.3.2. Host associations

A. rostrata, *Arma custos* (Fabricius) *Carpocoris* sp., *Dolycoris* sp., *G. lineatum*, *Palomena prasina, P. rufipes*, *Picromerus bidens* (L.) *Piezodorus* sp. and frozen sentinel eggs of *H. halys* (Tortorici et al. 2019; Moraglio et al. 2021b; Zapponi et al. 2021).

6.3.4. Trissolcus colemani

Trissolcus colemani (Crawford) belongs to the *basalis* group and is identified by a combination of characters more than by the presence of a distinct feature. The foveate mesoscutal humeral sulcus separates it from all the species treated here with the exception of *T. belenus*. *Trissolcus colemani* and *T. belenus* are very similar in general appearance and these two species can be separated most reliably by the setation of laterotergite 1: present in *T. belenus* and absent in *T.*

colemani. The anteroventral extension of the metapleuron in *T. colemani* is very slender compared to *T. belenus* (Tortorici et al. 2019) (Fig. 6.8, specimen from Assent).

6.3.4.1. Distribution

Belgium, China, Greece, India, Iran, Italy, Morrocco, Pakistan, Russia, Sweden, United Kingdom (Tortorici et al. 2019).

6.3.4.2. Host associations

Dolycoris indicus Stål*, A. acuminata, Aelia* sp.*, B. germarii, Dolycoris* sp.*, G. semipunctatum* and *Graphosoma* sp. (Tortorici et al. 2019).

Figure 6.8. Lateral habitus of *T. colemani*, specimen from Assent. (Credit E.J. Talamas)

6.3.5. *Trissolcus cultratus*

Trissolcus cultratus (Mayr) belongs to the *flavipes* group, containing the major egg parasites of *H. halys.* The hyperoccipital carina is present in members of this group (in contrast to the *basalis* group). *Trissolcus cultratus* can be distinguished from its group members by the coarse arched rugae on the frons between the anterior ocellus and the antennal scrobe. This species also lacks a well-developed orbital furrow near the malar sulcus. Lastly to distinguish it from the similar *Trissolcus exerrandus* Kozlove & Lê, it has few to no setae on the eyes while *T. exerrandus* has setose eyes (Talamas et al. 2017) (Fig. 6.9, specimen from Vlijtingen).

Figure 6.9. Lateral habitus of *T. cultratus*, specimen from Vlijtingen. (Credit: Author)

6.3.5.1. Distribution

Austria, Belgium, China, Czech Republic, France, Germany, Hungary, Japan, Poland, Russia, South Korea, Sweden, Switzerland and United Kingdom (Talamas et al. 2017).

6.3.5.2. Host associations

P. rufipes, *P. prasina*, *R. nebulosa* and *H. halys* (Zapponi et al. 2020; Moraglio et al. 2021b).

6.3.6. Telenomus turesis

Telenomus turesis Walker belongs to the *podisi* group and diagnostic characters have only recently been clarified in combination with molecular analysis (Tortorici et al. 2024). The first metasomal tergite has one pair of sublateral setae (in contrast to *Telenomus gifuensis* Ashmead) and the frons of the head is wide with the minimum distance between the inner margin of the compound eyes being more than the eye with in frontal view. The compound eyes have dense setation throughout (in contrast to *Telenomus truncatus*). In females a hyperoccipital carina is present directly posterior to the lateral ocellus but weakly sharp and the sculpture on the surface behind the vertex is smooth. Median and posterior femora are yellow to pale brown (Fig. 6.10, specimen from Sint-Amandsberg)

Figure 6.10. Lateral habitus of *Te. turesis*, specimen from Sint-Amandsberg. (Credit: E.J. Talamas)

6.3.6.1. Distribution

Belarus, Belgium, Bulgaria, China, France, Germany, Italy, Iturup (Japan/Russia), Kazakhstan, Moldova, Pakistan, Russia, Ukraine.

6.3.6.2. Host associations

Acrosternum sp., *A. acuminata*, *Aelia furcula* Fieber, *A. rostrata*, *A. custos*, *Carpocoris* sp., *C. fuscispinus*, *D. baccarum*, *G. lineatum*, *H. halys*, *Holcostethus strictus* (Fabricius), *P. prasina*, *Palomena viridissima* (Poda), *P.* bidens, *Piezodorus lituratus* (Fabricius) and *E. austriaca*, *E. integriceps*, *Eurygaster maura* (L.), and *Eurygaster testudinaria* (Geoffroy) (Tortorici et al. 2024).

6.3.7. Telenomus truncatus

Telenomus truncatus (Nees von Esenbeck) belongs to the *podisi* group as well. However, in contrast to *Te*. *turesis* it has compound eyes with sparse and short setation throughout. Additionally, females have a sharp hyperoccipital carina directly posterior to the lateral ocellus. The surface behind the vertex is smooth and the median and posterior femora are dark brown with yellowish tips (Fig 6.11) (Tortorici et al. 2024).

Figure 6.11. Lateral habitus of *Te. truncatus*, specimen from Assent. (Credit E.J. Talamas)

6.3.7.1. Distribution

Armenia, Azerbaijan, Belgium, France, Georgia, Germany, Italy, Kazakhstan, Moldova, Russia, Turkmenistan, Ukraine.

6.3.7.2. Host associations

Carpocoris sp., *D. baccarum*, *G. lineatum*, *H. halys*, *P. prasina*, *P. viridissima*, *P. bidens*, *P. lituratus*, *R.nebulosa*, and *E. integriceps* (Tortorici et al. 2024).

6.4. Discussion

Four out of five species of the genus *Trissolcus* and both species of the genus *Telenomus* documented here represent the first records of these egg parasitoid species in Belgium, indicating the presence of established breeding populations. *Trissolcus cultratus* was recorded earlier in Belgium (Waarnemingen.be, species 998891).

Our record of *T. basalis* from Belgium possibly constitutes the most northern record at present in Europe. Other European specimens were collected in more southern countries or regions. Records from France were from the Aquitaine and Provence-Alpes-Côte-d'Azur, two southern regions (USMENT00896070-00896071, 00896037-00896040, 00896055-0089604060, and 0089629, examined by Talamas et al. (2017). The specimens from Germany (SMNS_Hym_Sce_000805-000806, examined in Awad et al. (2021) were collected in the most southern state, Baden-Württemberg.

It is possible that only in recent decades *T. basalis* has colonized Belgium and other parts of northwestern Europe or has become ubiquitous enough to be detected in this area due to the northward habitat expansion of its prime host *N. viridula*. Additionally, the warming of the climate in northwestern Europe could have played a role in the gradual northward habitat expansion of *T. basalis* following its main host *N. viridula*.

During this study, the cryo-killed sentinel egg masses from *H. halys*, were unsuccessful in attracting exotic or native egg parasitoids. Previously frozen sentinel eggs are known to result in a lower incidence of parasitism, and thus not enough egg masses could be placed at sufficient locations to attract native or non-native egg parasites of *H. halys* (Jones et al. 2014; McIntosh et al. 2018).

Moreover, the parasitism rate of *H. halys* eggs with successful native parasitoid emergence is reported to be rather low (Abram et al. 2017; Moraglio et al. 2020; Haye et al. 2024). Several studies have shown that invasive insect pests initially suffer lower parasitism and are attacked by fewer parasitoid species in their invaded ranges than in their native ranges (Cornell and Hawkins 1993), but that, over time, these species tend to recruit an increasing number of native parasitoids. Additionally, naturally occurring *T. cultratus* populations in China have been found to successfully parasitize *H. halys* eggs and even act as a hyperparasitoid of *T. japonicus* (Konopka et al. 2017; Haye et al. 2024).

The deployment of fresh *H. halys* eggs during this research was undesirable since this would increase the chances of introducing *H. halys* in agricultural zones aiding in their spread and successful establishment in Belgium.

In contrast to the unsuccessful parasitism of sentinel eggs by native egg parasites, parasitism rate of naturally laid eggs by native parasites was 31% with partial or full emergence. Similarly, differences in parasitism rates were found in Haye et al. (2024) where parasitism rates by native parasitoids on native stink bug eggs (*P. prasina* and *P. rufipes*) were higher than parasitism of native egg parasites on eggs of *H. halys* in Switzerland.

The collection of naturally laid stink bug eggs is a more random and less standardized approach. However, it offers the advantage of discovering parasitoids across all relevant stink bugs. It still often allows for the identification of the host stink bug species via the morphology of the egg mass, including the discovery of instances of hyperparasitism (e.g. *T. cultratus* as a hyperparsitoid of *T. japonicus*) (Haye et al. 2024).

In contrast, using sweep nets provides a more standardized sampling technique; however, it often results in capturing only a single individual of a species. This method also requires prior expertise to effectively distinguish the relevant parasitoids in mixed insect samples. Parasitoids collected through sweep netting cannot be directly linked to their host stink bugs unless there is prior knowledge of their hosts from literature. This can limit the potential for discovering new host-parasitoid associations.

The prevalence and habitat preference of these egg parasitoids remains poorly documented in Belgium for native Scelionidae and undocumented for non-native species. The range expansion of the introduced scelionids will likely reach Belgium within the foreseeable future. To date, however, *T. japonicus* and *T. mitsukurii*, the two key egg parasitoids of *H. halys* in its native area, remain undetected in Belgium. Whereas, this might aid in the biological control of *H. halys*, the arrival of *T. japonicus* could also influence populations of native stink bugs like *P. rufipes*, *P. prasina*, *R. nebulosa and A. custos,* as egg masses of these stink bugs were accepted by *T. japonicus* as a host at rates comparable to the target host *H. halys* (Sabbatini-Peverieri et al. 2021). Not all of these native pentatomids are pests, with *A. custos* even being a predatory species belonging to the subfamily of Asopinae.

In summary, the discovery of the Palearctic Scelionidae in this study, presents a promising opportunity for natural pest control of the harmful stink bugs under study. The identification of these species revealed a pool of natural enemies capable of contributing to the control of harmful stink bug populations in Belgium. Given the economic importance of some of their pentatomid hosts, it will likely be beneficial to support or attract populations of these parasitic wasps in agricultural fields and commercial fruit orchards in a conservation biological control approach or to rear and release them in the framework of augmentative strategies (Rahat et al. 2005). In this context, it may be warranted to further investigate the physical and chemical cues by which these parasitoid wasps locate and recognize their hosts (Bin et al. 1993; Mattiacci et al. 1993) or plant resources (Martorana et al. 2017). This knowledge will be instrumental in designing Integrated Pest Management (IPM) strategies against stink bug pests in Belgium and its neighbouring countries.

Chapter 7

General discussion and future perspectives

7.1. Stink bug ecology insights drive biological control strategies

Balancing the growing need for agricultural products while mitigating negative impacts on human health, biodiversity, and ecosystems is one of the foremost global challenges (Godfray et al., 2010). To address this, the framework of IPM was adopted as a mandatory strategy for the protection of agricultural crops within the European Union (European Union, 2009). IPM is known as a knowledge-intensive approach to pest control that requires an intimate understanding of the factors influencing the management of pests within agricultural systems, including landscape elements, crop biology, pest behaviour, and interactions between insects and plants (Karlsson Green et al. 2020; Ryalls et al. 2024).

The increasing pressure from native pests like the stink bug *Pentatoma rufipes*, which already causes significant economic damage to pip fruit (Beliën et al. 2015), coupled with the looming threat of recent invaders like *Halyomorpha halys*, poses a formidable challenge to fruit production in Belgium and its neighbouring countries. Targeted research to develop and refine control strategies against these stink bugs within the holistic approach of Integrated Pest Management (IPM) is crucial to safeguard the interests of Belgian pip fruit growers and minimize their potential losses (Beliën et al. 2015).

The present research bundles several research outcomes that can substantiate the development of such control strategies against stink bugs, with a focus on fruit crops. It is the first study to monitor and examine the invasion of *H. halys* in Belgium, elucidating its putative invasion routes and summarizing the observations of the past years to evaluate its population dynamics (Chapter 3). Furthermore, this study reveals the relationships between *P. rufipes* and *H. halys* and their host plants, using next-generation amplicon sequencing of the ITS2 barcode region (Chapter 4). Finally, it expands the applicability of the green lacewing *Chrysoperla carnea* as a biological control agent for stink bugs (Chapter 5) and gives a brief overview of egg parasitoids of pentatomid pests present in and around orchards in Belgium (Chapter 6). The findings from these chapters can serve as input for sustainable management strategies tailored to the landscape or the grower to suppress populations of stink bug pests in Belgian pip fruit.

For invasive pests like *H. halys*, monitoring is required to evaluate their entry routes, population growth, and dispersal patterns that could lead to their further spread and invasion into cropping systems (Dlugosch and Parker 2008; Cesari et al. 2018). The introduction and spread of *H. halys* in Europe and North America have already prompted research efforts to identify its invasion pathways based on the underlying genetics of populations originating from native and introduced areas (Gariepy et al. 2014, 2015; Cesari et al. 2015, 2018; Lee et al. 2018;

Kapantaidaki et al. 2019). In Europe, Belgium represented a hiatus, as no COI or COII gene sequences had yet been amplified from specimens collected in Belgium before this study.

During the present research (2020-2023), *H. halys* individuals were obtained via monitoring with pheromone traps (elaborated in section 2.3). These specimens, destined for the molecular gut content analysis (Chapter 4), were also subjected to amplification of the COI and COII barcode regions (Chapter 3). Additionally, the distribution of *H. halys* was inferred using observations from a citizen science database.

The genetic analysis uncovered 9 COI, 5 COII, and 12 COI-COII distinct haplotypes from the Belgian specimens and revealed two new combined haplotypes that are unique to Belgium to date. The data also showed a relatively high haplotype diversity of the Belgian *H. halys* population. Together with the overlap of haplotypes found in the literature (Cesari et al. 2018; Yan et al. 2021), this suggests that the invasion is manyfold and that the source areas of these invasions are mainly Italy, Hungary, Switzerland, and, to a lesser extent, native regions like China and Japan.

In general, pathways of movement and invasions of insects to new areas outside their native area happen through both passive and active forms of transport. Passive transport includes being carried by the wind, water currents, or animals, while active transport involves the movements of the insects themselves (Skendžić et al. 2021).

Far more frequently is the transport of invasive insects mediated by human activities via the international trade system and human travel (see section 2.1.2.2 for *H. halys*). Stowaway transportation on passenger planes is, therefore, an important and growing driver of introductions in alien invasive species in general, as is transport by ship, often making seaports the main epicentres of invasions (Early et al. 2016). For example, between 2000 and 2014, USDA-APHIS recorded 128 interceptions of *H. halys* at ports in the USA. Most of these (98) were linked to maritime cargo. Additionally, in Maistrello et al. (2016) the detection of *H. halys* along main roads or railway lines, together with the many occurrences inside means of transport and transit areas strongly support the hypothesis that human-assisted spread plays a major role in its invasions.

Indeed, the observational data in chapter 3, show that most of the records of *H. halys* were made in northern Belgium, around cities and other urban areas like Gent, Leuven, and Mechelen, but also in the region of Haspengouw. This corroborates other published data where *H. halys* shows a strong association with urban development in its initial establishment and (Wallner et al. 2014; Maistrello et al. 2016; this study).

The breadth of the genetic diversity of *H. halys* found in European haplotype studies, including this study indicates that the invasions are not impacted by genetic bottlenecks or founder effects, two of the most dominant processes influencing the genetic diversity of a species in its new range (Kerdelhué et al. 2014).

Research on the population genetics of *H. halys* in North America has shown that the invasion is primarily driven by the dominant COI haplotype H1 (originating from the Beijing/Hebei regions of China) (Gariepy et al. 2014; Xu et al. 2014). The Canadian population likely originated from the already-established populations in the USA (Gariepy et al. 2014). However, samples collected later, revealed additional haplotypes not previously identified, suggesting further introductions may have occurred in the USA.

In Europe, the invasion was initially restricted to Switzerland, but in the meanwhile *H. halys* has been observed in many European countries (see section 2.1.2.2). Until 2012, the Swiss population had three unique COI haplotypes, none of these COI haplotypes were shared with the North-American samples, implying the invasions were separate and distinct events for North-America and Europe (Gariepy et al. 2014). However, later collections from France, Switzerland, and Italy and this study found the H1 haplotype in Europe matching the dominant North American haplotype (Haye et al. 2015). This suggests that like in North America, in Europe, the introductions are ongoing as well and that the haplotype diversity likely will change over time as invasion events keep occurring from different locations where *H. halys* is native or introduced (Haye et al. 2015). The presence of the H1 haplotype in Europe and specifically Belgium as well could indicate introductions from the USA or the same Asian source.

Climate change may also facilitate the establishment of introduced invasive species. Species that are currently unable to survive in a new environment due to climatic limitations may increasingly be able to survive and become established. Moreover, invasive species that can tolerate new climatic conditions may have a greater potential to overcome biotic constraints and establish permanent populations under climate change (Byers et al. 2002; Skendžić et al. 2021). For example, for the green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) climate warming is directly associated with increased population densities, higher development rates, and outbreak frequencies. Combined with the lack of corresponding effects on the natural enemies of *M. persicae* and the resulting reduced predation pressure, temperature increases are directly correlated with increased economic losses by *M. persicae* (Bale and Hayward 2010). Similarly, Kistner (2017) showed that climate change could increase the number of generations produced annually by *H. halys*, making the invasive insect multivoltine in the northern latitudes of Europe, where it is currently considered univoltine. Climatic conditions in southern Europe e.g., Italy, *H. halys* already allow the development of two generations per year (Maistrello et al. 2017; Bosco et al. 2018). Lastly, climate change causes many plant species to expand their distribution, potentially providing more food sources for herbivorous insects (Skendžić et al. 2021).

Consequently, the multitude of ongoing introductions, high genetic diversity, the presence of populations in urban areas, and possibly more favourable climatic conditions in the future may have an advantageous impact on the invasive success and the establishment of successful populations of *H. halys* in Belgium and other European countries. Higher genetic diversity in populations makes them more likely to be able to adapt to new or changing environments than populations with less genetic variation (Futuyma and Kirkpatrick 2017). Together with its pathways of invasion via human transportation and its different patterns of diffusion, this could hamper pest management strategies to prevent outbreaks of *H. halys*.

Another crucial trait linked to the invasion success of insects is their diet breadth. Introduced insects must first find a suitable host plant to feed on, and if their native host is unavailable, they may need to adapt to a related species (Vermeij 1996). Specific host plant preferences can limit the ability of insects to find a suitable host in new areas, making generalist or polyphagous insects more successful invaders (Ward and Masters 2007). Generalist species, like the spotted wing drosophila, *D. suzukii*, which feeds on over 80 host plant species, exemplify how diet breadth aids in successful colonization (Poyet et al. 2014; Kenis et al. 2016).

To date, few studies have provided comprehensive overviews of the polyphagous diet of *H. halys* (Lee et al. 2013; Bergmann et al. 2016) and only Powell (2020) provides a brief list of the diet preferences of *P. rufipes,* based on observations. Molecular assays to detect plant material in the gut have been developed for various phytophagous insects like beetles, weevils, and grasshoppers (Navarro et al. 2010; Staudacher et al. 2011; García-Robledo et al. 2013; Avanesyan and Lamp 2020). However, the first assay for a herbivorous stink bug species was only recently developed for *H. halys* using the *trnL* barcode region (Hepler et al. 2021). An improved understanding of the diet preferences of harmful stink bugs in and around orchards can therefore augment the design, implementation, and management of floral and hedge plantings.

In Chapter 4, we set out to gain more insight into the highly polyphagous diet of *H. halys* and *P. rufipes*, specifically in Belgium. Both shield bugs were sampled during 2020-2023, and DNA extracts from the guts were subjected to next-generation amplicon sequencing of the ITS2 region. The results from Chapter 4 corroborate and provide additional resolution to the results from two prior studies on *H. halys* and its host plant associations (Lee et al. 2013; Bergmann et al. 2016), but are the first based on a high-throughput molecular assay.

One limitation of this study is that metabarcoding is not inherently quantitative because of possible biases in PCR amplification and differences in gene copy numbers that can distort the relative abundance of DNA sequences, and therefore cannot reveal accurate host plant preferences nor reveal possible probing events by the stink bugs. To address this limitation, researchers have started incorporating spike-in controls, which are known quantities of DNA from reference organisms and mock communities with predetermined species compositions and abundances (Gamacho-Sanchez 2023; Sickel et al. 2023; Parmentier et al. 2024). These additions allow for calibration of the metabarcoding process, helping to correct for amplification biases and providing a more accurate, quantitative assessment of species abundances in the samples.

Another limitation was that no clear seasonal component could be detected from the field-collected samples. The principal cause for this was some drawbacks in the sampling of the stink bugs. During the sampling years *H. halys* was not overly ubiquitous in Belgium, nor did it massively occur in cropping systems yet. Another factor in the sampling is that *H. halys* stops responding to pheromonal stimuli after entering overwintering sites, therefore baited trapping between October and May is largely ineffective (elaborated in section 2.3). As for *P. rufipes*, no semiochemicals are known to attract them, and thus no lures exist to date. This limits sampling to hand catches and net sweeping. Additionally, populations of *P. rufipes* often reside at the top of the canopy and diapause as second instar nymphs under tree bark or other difficult-to-sample overwintering spots (Powell 2020). This could explain the lack of empirical evidence for a seasonal component in the host plant preferences of the stink bug species under study. Nevertheless, seasonal host switching by polyphagous herbivores has been observed in various insects (Mody et al. 2007) including stink bugs like *N. viridula* (Velasco and Walter 1992) and *H. halys* (Bergmann et al. 2016; Zobel et al. 2016).

From the results in Chapter 4, it is clear that diet breadth is, however, highly dependent on the landscape type of the sampling. Factors impacting plant communities in different landscape types can be influenced by the land use, like unmanaged forests and grasslands, in contrast with gardens and public parks. Some plant species, often ornamental or fruit-bearing, are more specific to environments controlled by humans, such as gardens or orchards, respectively.

The host plants uncovered in Table 4.2 reveal that both stink bugs have a preference for deciduous trees and shrubs. The brown marmorated stink bug uses gymnosperms as a partial host only, and for *P. rufipes*, gymnosperms were only detected in two samples in near-lowest threshold read abundances. Some of the herbaceous plants found in stink bug guts, e.g., *Urtica dioica* L. and *Trifolium* spp., were marked specifically as highly attractive for generalist predators and parasitoids, respectively (Albrecht et al. 2020; Fountain 2022). However, these herbaceous plant species were only found occasionally in a small subset of the field samples, mostly in lower read quantities and thus likely not as preferential for stink bugs as deciduous trees and shrubs belonging to genera like *Prunus*, *Acer*, *Betula*, *Fagus*, *Quercus*, *Corylus*, *Salix*, *Sorbus,* and some other genera (see Fig. 4.2).

Organic and IPM pip fruit orchards which are intensively managed agroecosystems are often highly diverse habitats with a multitude of host plants available as part of wildflower strips or planted hedgerows with shrubs and trees. Woody species planted in such hedgerows include *Quercus* spp. (e.g., *Quercus robur* (L.), *Prunus* spp. (*Prunus serotina*, Ehrhart), *Salix* spp. (*Salix alba,* L.), *Rubus* spp. (*Rubus idaeus,* L.), *Carpinus* spp. (*Carpinus betulus,* L.), *Fraxinus* spp. (*Fraxinus excelsior,* L.), *Acer* spp. (*Acer pseudoplatanus,* L.), *Viburnum* spp. (*Viburnum tinus* L.), *Tilia* spp. (*Tilia cordata,* Miller), *Sorbus* spp. (*Sorbus aucuparia,* L.), Rosa spp. (*Rosa canina,* L.), *Crataegus monogyna* (Jacquin), *Alnus glutinosa* (Gaertner), *Corylus avellana* (L.), *Frangula alnus* (Miller), and *Juglans regia* (L.) amongst others (Boutin et al. 2008; Litza 2022; PCFruit 2024).

Many of these species which often constitute hedgerows or forests bordering orchards were found to be preferential host plants of *H. halys* and *P. rufipes* in this study (table 4.2) and could thus increase the attractiveness of the orchard to stink bug pests or serve as sources of infestation. Moreover, from literature, indeed such quantitative inconsistencies and variability have been observed in the role these hedgerows play in pest control, showing that woody borders are related to increased fruit damage by stink bugs (Rice et al. 2016; Bergh et al. 2021) (see section 2.4).

Consequently, the results from Chapter 4 have practical implications for IPM and organically managed orchards with hedgerows or forest borders. With *H. halys* being a perimeter-driven threat, causing more damage at the exterior of orchard blocks (Leskey et al. 2012b), for both organic and IPM orchards, physical techniques can lower pressure from stink bugs by using exclusion nets protecting the rows closest to these woody borders (Candian et al. 2018, 2021; Marshall and Beers 2021, 2024). These exclusion nets are a readily available tool for crop protection and are an environmentally friendly alternative to pesticides (Castellano et al. 2008; Chouinard et al. 2017). They also protect against leafrollers like codling moth (Tasin et al. 2008) and aphids (Dib et al. 2010) in apple orchards. Exclusion nets have also been shown to be effective against *D. suzukii* in cherry, raspberry, strawberry and blueberry (Cormier et al. 2015;

Leach et al. 2016; Rogers et al. 2016). Potential drawbacks of using exclusion nets include the initial cost of the nets and infrastructure, labour costs for installation and maintenance, reduced or limited access for pollinators, the importance of installation timing, and the creation of microclimates that can sometimes lead to fungal infections (Fornasiero et al. 2023).

Furthermore, the recommended taxa for these hedgerows to provide ecosystem services like pest control and pollinator resources should be reconsidered, possibly including gymnosperms in hedgerows and preferentially avoiding the over use of taxa in Table 4.2, as these can further attract pentatomid pests. The practice of sowing in wildflower strips between tree rows can still provide oviposition sites, overwintering opportunities, and food resources for natural enemies and pollinators, without further attracting stink bugs but can in turn allow populations of voles to expand due to increased resources and shelter from birds of prey (Granatstein and Sánchez, 2009).

Contrarily, the preferential host plants found in this study (Table 4.2) that are often part of hedgerows can be used in the framework of attract and kill (AK) methods as trap plants, additionally baited with pheromones to attract the stink bugs to the hedgerows. These baited hedgerows and a perimeter can then be sprayed with insecticides to reduce the impacts of dispersing stink bug populations from these woody borders into specific vulnerable crop fields like pip fruit orchards without having to spray the crops themselves (Morrison III et al. 2019; Park et al. 2024).

However, such AK strategies that apply insecticides on pheromone-baited hedgerows along the field edges could likely, in turn, be harmful to some pollinators and natural enemies, that are attracted by these hedgerows (Lowenstein et al. 2019a; Ribeiro et al. 2021; Ademokoya et al. 2022). Moreover, AK strategies as discussed above are unfeasible for *P. rufipes* as there are no known attractants for this shield bug species. Therefore, AK strategies can be part of a broader IPM approach against stink bugs, but variables like pest pressure, cost-benefit and harmful effects to beneficial insects need to be considered.

Spinosad is the only recommended and allowed pesticide against stink bugs in Belgium (Fytoweb.be). Residues of spinosad degrade quickly in the field, with little residual toxicity at 3-7 days post-application (Cleveland et al. 2002; Thompson and Sparks 2002). Research has however shown mixed results concerning its lethality against certain natural enemies. For instance, after 14 days, it increased mortality with 48% at a dose of 1.2 ppm in earwigs, but mortality remained negligible for lacewing larvae up to 1000 ppm (Medina et al. 2001; Viñuela et al. 2001; Cisneros et al. 2002). Williams et al. (2003) reviewed the responses of 162 insect predator populations in 103 field-type studies and concluded that most insect predators were found to suffer insignificant sub-lethal effects following exposure to spinosad. Whereas parasitoids often show sub-lethal effects including loss of reproductive capacity and reduced longevity mainly through contact with residues and ingestion which were more harmful than topical application. However, this review did not include members of the Scelionidae. In the context of local or orchard insecticide applications, carbamates, pyrethroids, and the neonicotinoid acetamiprid, legal to use against lepidopteran or aphid pests in orchards, will additionally cause high mortality to stink bug populations as well (Leskey et al. 2012a).

In contrast, for Scelionidae like *T. japonicus*, contact with residues of neonicotinoids, pyrethroids, and even spinosad applied at lower rates than recommended field application rates are lethal (Saber et al. 2005; Lowenstein et al. 2019b). Moreover, egg parasitoids will exhibit reduced parasitism on pentatomid eggs treated with pesticides (Turchen et al. 2016). Thus, while in the context of IPM, pesticide applications (e.g., spinosad) can be used in the described AK techniques, they seem to be detrimental to populations of the main egg parasitoids of stink bugs.

Understanding the effective contribution of natural enemies to help control pests is fundamental to determining the success of IPM schemes in open systems like pip fruit orchards (Johnson et al. 2021; Collier 2022; Ryalls et al. 2024). Generalist predators, like lacewings, have been extensively studied as biological control agents of arthropod pests (Principi and Canard 1984; Easterbrook et al. 2006; Shrestha and Enkegaard 2013; Farrokhi et al. 2017; Golsteyn et al. 2021).

During laboratory experiments performed in this research (Chapter 5), we tested the predatory capacity of second-instar and third-instar larvae of *C. carnea* on first-instar nymphs of *N. viridula* and *H. halys* and the predatory capacity of third-instar larvae on second-instar nymphs of both shield bugs. The data obtained from the experiments reveal that, indeed, second and third instars of the *C. carnea* species complex are effective predators of the first nymphal instars of *N. viridula* and *H. halys*, corroborating and adding to the findings of Ehler (2002) and Bulgarini et al. (2021).

Although the wide commercial availability of *C. carnea* allows its use in augmentative biological control and is thus perhaps a less suited strategy in open fields like orchards, it may, for instance, be a cost-effective strategy for the suppression of *N. viridul*a in greenhouse crops, where inundative or inoculative releases of lacewings can add to the action of more specialist natural enemies like the egg parasitoid *T. basalis* (Gard et al. 2022).

In the conservation biological control (CBC) of stink bug populations as a part of a broader IPM approach, nymphal predation by generalist predators such as *C. carnea* could also be an impactful component. However, its overall impact has not been well defined (Ehler 2002; Ademokoya et al. 2022). The CBC of stink bugs could benefit from measures towards conserving or attracting populations of *C. carnea* (and congeneric species) to help control the populations of *N. viridula* and other pests like mites, aphids, or thrips in agricultural environments like greenhouses (Principi and Canard 1984; Turquet et al. 2009; Luna-Espino et al. 2020). For instance, “banker plants” could be used providing pollen for the survival, oviposition, and development of naturally occurring or introduced lacewing in greenhouses (Pijnakker et al. 2020), similar to sown in wildflowers in orchards. Likewise, food sprays with mixtures of protein hydrolysates with honey or sugar may support ovipositing adults or factitious foods like *E. kuehniella* eggs can be used as supplementary food for larvae, though a cost-benefit analysis is warranted before implementing this strategy (Tauber et al. 2000; Bezerra et al. 2017). Further, overwintering shelters can host diapausing adults near greenhouses. These strategies can allow for early season augmentation of *C. carnea* and could replace repeated inundative releases in greenhouses (Pijnakker et al. 2020).

Similarly, in open field agricultural systems like orchards, such measures to enhance local populations of lacewings like the introduction of selected flowering plants might also assist in suppressing harmful stink bug populations and other pests in pip fruit production (Principi and Canard 1984; Landis et al. 2000; Gonzalez et al. 2016; Alcalá Herrera et al. 2019; Cahenzli et al. 2019; Albrecht et al. 2020; Fountain 2022).

As noted above, generalist predators like *C. carnea* have a wide host range, and studies on the feeding preferences and behaviour of *C. carnea* suggest a predilection for certain prey types, such as aphids over thrips, possibly due to their immobility, larger size, and detectability, as well as their chemical cues (McEwen 2001; Shrestha and Enkegaard 2013). This prey preference may limit the effectiveness of *C. carnea* against more agile pests such as *H. halys*, where the effect may only be restricted to the gregarious and less agile first instars of the pest (Bulgarini et al. 2021).

Thus, augmentative and CBC strategies against stink bugs using *C. carnea* can be a valuable tool for IPM in greenhouses, but their utility is still context-dependent. Consequently, augmentative biological control strategies against stink bug pests that include the release of lacewing larvae in orchards and open field crops may not always be cost-effective.

The absence of a single predator capable of efficiently attacking all life stages of *H. halys, N. viridula* and other stink bugs implies that the biological control of these pests will only be provided by a community of predators and parasitoids, rather than by an individual species (Pote and Nielsen 2017). Alongside *C. carnea*, multiple other predators of *N. viridula* and *H. halys* nymphs have been reported belonging to Coccinellidae Malachidae, Anthicidae, Lygaeidae, Nabidae, Reduviidae, Opiliones, and Araneae (Ehler 2002; Bulgarini et al. 2021).

Besides generalist predators, the potential of more specialist natural enemies like egg parasites as biological control agents of pentatomid pests has already been extensively investigated (Colazza and Bin 1995; Haye et al. 2020, 2024; Scaccini et al. 2020; Malek et al. 2021; Japoshvili et al. 2022; Tortorici et al. 2023). Some of these have been used successfully for inundative and classical biological control of a variety of stink bugs, including *T. basalis* against *N. viridula* and *T. japonicus* against *H. halys* (Gard et al. 2022; Simaz et al. 2023). This has led to the commercial availability of *T. basalis* for augmentative releases against *N. viridula* (e.g., Koppert; Nezapar; Biobest: *Trissolcus* System).

Through the collection of naturally occurring stink bug egg masses, egg parasitoids of economically important stink bugs in Belgium were discovered and characterized. Chapter 6 offers a brief overview of some of these egg parasitoids.

However, some limitations were encountered during this research. The unsuccessful attempts to rear *P. rufipes* or attempts to bring caught individuals to egg laying have hampered the deployment of sentinel egg masses of *P. rufipes* to find egg parasitoids of this species specifically. Moreover, sentinel egg masses from *H. halys*, deployed in habitats with known occurrences of egg parasitoids, were unsuccessful in attracting egg parasitoids.

From the opportunistically performed sweep nets and the collected naturally laid egg masses of native stink bugs in Belgium during this study, an assortment of Palearctic scelionid species emerged: *T. basalis, T. belenus, T. cultratus, T. semistriatus, T. colemani, Te. turesis* and *Te. truncatus*. These native scelionids, parasitize on eggs of the stink bugs under study including *P. rufipes*, *H. halys*, *N. viridula,* and additionally *P. prasina* and *R. nebulosa* (see section 6.3) (Tortorici et al. 2019; Haye et al. 2024).

The prevalence of these egg parasitoids tends to be habitat-specific (Cottrell et al., 2023; Tillman et al., 2023). Wildflower strips tailored to the requirements of these parasitoids may enhance the biological control of stink bugs by attracting higher densities of these parasitoids and prolonging their lifespan through the provision of floral food sources (Lee and Heimpel 2008; Blaauw and Isaacs 2015; McIntosh et al. 2020). For instance, McIntosh et al. (2020) showed that coriander (*Coriandrum sativum* L.), buckwheat (*Fagopyrum esculentum* Moench) and dill (*Anethum graveolens* L.) improve median survival of *T. japonicus* by providing nectar resources.

Lastly, the absence of *T. japonicus* in the weedy habitats in Switzerland (Haye et al. 2024) and in earlier studies where *T. japonicus* was more frequently found in woodland or lightly wooded urban habitats rather than near ground crops may indicate a predilection by these egg parasites for woody habitats (Herlihy et al. 2016; Quinn et al. 2019a, b, 2021). This indication of preference for woody habitats by scelionid egg parasites like *T. japonicus* may for instance be linked to the similar preferences exhibited by their hosts demonstrated in this study.

7.2. General conclusions & future perspectives

The use of molecular markers (COI and COII), supplemented with the analysis of public citizen observations in Chapter 3, has marked the beginning of our understanding of the invasion patterns and distribution of *H. halys* in Belgium. However, many questions and uncertainties remain.

The proximity of Belgium to the Northen latitudinal limit, where the climate still allows *H. halys* to survive the winter and reproduce once during the summer, makes it particularly vulnerable to the impacts of climate change as further warming of the climate can lead to multivoltine populations and better survival in the winter. How will the future dynamics of *H. halys* populations, invasions into agroecosystems (especially pome fruit), and dispersal patterns evolve when winters and summers become increasingly warmer in Europe (Kistner 2017)?

Future studies should focus on further identifying the primary points of entry for *H. halys*, the genetic structure, and the spread and reproductive success of founding populations on a continental or sub-continental scale (e.g., western Europe) in relation to climate. The haplotype data collected in Chapter 3 will contribute to a continental-scale study on the invasion of the brown marmorated stink bug in Europe (D. Musolin, personal communication). Such research is essential for refining IPM strategies to manage and limit further enrichment of the genetic diversity of *H. halys*.

Given that *H. halys* is now established in western Europe, including Belgium, it is more practical to focus on IPM strategies to control its populations, rather than focusing on invasion prevention. Quarantine measures and inspections by phytosanitary officers at ports or other points of entry could still be useful in the prevention of introductions of *H. halys* into presently non-invaded countries like the United Kingdom.

Chapter 4, indirectly explores the rationale of intercropping wildflower strips between tree rows in orchards and the planting of hedgerows. The results, however, indicate that mixed hedgerows in particular are attractive to both *P. rufipes* and *H. halys*. Nevertheless, herbaceous plant species, such as wildflowers appear to be less attractive for the stink bugs under study. Therefore, hedgerows should be subjected to greater scrutiny as a cultural practice, while wildflowers perhaps warrant less attention in this context.

Considering that *H. halys* will become more widespread in Belgium, the shortcomings encountered in this study can be resolved. Future research should include upscaled sampling campaigns with more locations, also in the south of the country, and a more substantial number of samples per season or per month, given enough budget. This could reveal seasonal preferences or differences related to plant phenology in the diet of *H. halys* and *P. rufipes* across various landscape types in Belgium. Future studies identifying alarm, sex, or aggregation pheromones in *P. rufipes* could enable the adaptation or cross-application of current monitoring, trapping, and AK techniques developed for *H. halys*. Additionally, including mock community samples and spike-ins could provide more quantitative information on the host plant preference in metabarcoding studies of the gut content of both shield bugs.

In addition to metabarcoding, which mainly identifies species through genetic markers, metabolomics and proteomics could detect tissue-specific metabolites or proteins in the gut contents (Oliveira et al. 2022). This depth of analysis would allow for the identification of not only the plant species consumed by stink bugs but also the specific plant tissues that were ingested such as leaves, stems, fruits, or flowers. Such approaches would provide more detailed insights into feeding behaviours and dietary preferences, enhancing our understanding of plant-insect interactions in general and potentially informing pest management strategies against harmful stink bugs more accurately.

Experiments to quantify the predatory efficacy of *C. carnea* on stink bugs in the presence of perhaps more favourable prey (e.g., aphids) or experiments in greenhouses and open field settings could more accurately reflect its role for the control of stink bugs in agricultural settings. Additionally, future studies could aim to further quantify beneficial conservation biological control practices to attract and sustain native and adventitious generalist predators and egg parasitoids. For example, experiments to determine which herbaceous plants provide the highest attraction and nectar resources for lacewings and other generalist predators, or for certain native (e.g., those found in Chapter 6) or exotic egg parasites of stink bugs.

To summarize, in addition to Chapter 3, continuous genetic analysis and the monitoring of dispersion of *H. halys* populations remains crucial to identify entry pathways, movement, and population densities. Finally, leveraging the results from Chapters 4-6 and perhaps future studies will substantiate IPM practices to encourage the presence of beneficial insects like lacewings and egg parasitoids in orchards without further attracting stink bugs.

Summary

Stink bugs (Pentatomidae) cause significant economic damage in fruit production though their specific pierce and sucking mode of feeding, which results in deformities, pitting and necrosis in the crops they feed on. The native red-legged shield bug, *Pentatoma rufipes* is known to cause such damage in apples and pears, particularly in organic orchards with woody borders or near forest edges. Moreover, the recent invasion of the brown marmorated stink bug, *Halyomorpha halys* a highly polyphagous stink bug, native to East Asia, poses an additional threat for pip fruit growers as this highly polyphagous pentatomid is also known to massively invade cropping systems and cause significant economic damage.

This dissertation investigates the diet of these two economically harmful stink bugs in relation to their attraction to mixed hedgerows and wildflower strips in organic pip fruit orchards. Additionally, it examines the distribution and genetic diversity of the invasive *H. halys* in Belgium. The potential of the green lacewing, *Chrysoperla carnea* (Neuroptera: Chrysopidae), as a biocontrol agent for *H. halys* and *Nezara viridula* is also explored. Finally, this research documents egg parasites of harmful stink bugs present in Belgium.

To infer the genetic diversity of *H. halys*, a molecular analysis was performed using the COI and COII barcode regions. The distribution of the Belgian population of *H. halys* was inferred through citizen science occurrence data. Through the genetic analysis, and the haplotypes present, we discovered that the populations of the brown marmorated stink bug in Belgium is relatively diverse, similar to Italy. Invasions of *H. halys* are recent and ongoing. Invasions are mostly originating from other invaded European countries, especially Italy, but to a lesser extent also originate from its native countries, China and Japan (Chapter 3).

Individuals from both *P. rufipes* and *H. halys* were sampled in orchards, gardens and forest areas in Belgium. Our molecular gut content analysis using next generation amplicon sequencing of the ITS2 region uncovered that *H. halys* has a broader diet preference than *P. rufipes*. Both shield bugs have a preference for deciduous trees including genera like *Acer, Fagus, Prunus, Betula* and *Corylus.* This implicates that hedgerows and forest edges that include such genera in pip fruit orchards could provide an additional factor of attraction or serve as an additional source of these harmful stink bugs towards the orchards. Herbaceous plants are less consumed by these stink bugs and therefore the practice of sowing in wildflower strips between tree rows in orchards does likely not provide additional attraction for these harmful stink bugs in pip fruit orchards (Chapter 4).

The potential of the green lacewing, *C. carnea* was evaluated by determining the predation capacity of different life stages of the chrysopid larvae on the first two life stages of *H. halys* and

N. viridula at different temperatures in a simple and a more complex arena. Both the second and third larval stage consumed first instar nymphs of both stink bugs. The third larval instar *of C. carnea* showed higher predation capacity than the second larval instar, and was also able to consume second instar nymphs of both stink bugs albeit to a lesser extent. Predation by the lacewing larvae was overall higher on nymphs of *N. viridula* compared to *H. halys*. This difference was attributed to life stage interspecific differences, first instars of *H. halys* are almost twice as heavy as first instars of *N. viridula*. Second instars of *H. halys* are more mobile than second instar *N. viridula* nymphs. Field experiments are needed to fully understand the potential of *C. carnea* in open field settings (Chapter 5).

Sentinel eggs of *H. halys* were placed in orchards and gardens to attract egg parasitoids. Additionally opportunistic sweep nets were taken in orchards and naturally laid eggs of harmful pentatomidae were collected. None of the frozen sentinel egg masses of *H. halys* were found to be parasitized. However, 31% of the naturally laid eggs that were collected during this study were parasitized. From these parasitized egg masses 5 different species of egg parasitoids were identified, 2 additional egg parasitoids were identified from opportunistic net sweeps in orchards. All egg parasites were morphologically identified and belonged to the family Scelionidae (Hymenoptera: Platygastridae). The species documented were *Trissolcus basalis*, *Trissolcus belenus*, *Trissolcus colemani*, *Trissolcus cultratus, Trissolcus semistriatus*, *Telenomus truncatus* and *Telenomus turesis* (Chapter 6).

Samenvatting

Schildwantsen (Pentatomidae) kunnen aanzienlijke economische schade in de fruitteelt veroorzaken doordat ze met hun stekende en zuigende monddelen zich voeden aan landbouw gewassen. Dit leidt tot misvormingen en necrose in de vruchten en zaden van de gewassen die ze aantasten. De inheemse roodpootschildwants, *Pentatoma rufipes*, richt dergelijke schade aan bij appels en peren, voornamelijk in biologische boomgaarden met gemengde hagen of in de buurt van bosranden. Bovendien vormt de recente invasie van de bruingemarmerde schildwants, *Halyomorpha halys*, een zeer polyfage schildwants afkomstig uit Oost-Azië, een extra bedreiging voor pitfruit telers, aangezien deze sterk polyfage schildwants ook grote economische schade kan aanrichten in de teelt van appel en peer.

Dit proefschrift onderzoekt de dieetvoorkeuren van deze twee schildwantsen van economisch belang in relatie tot hun aantrekking tot gemengde hagen en bloemenstroken in pitfruitboomgaarden. Daarnaast werd de verspreiding en genetische diversiteit van de invasieve *H. halys* in België onderzocht. Het potentieel van de groene gaasvlieg, *Chrysoperla carnea*, als een biologische bestrijder voor *H. halys* en *Nezara viridula* werd ook verder uitgediept. Ten slotte documenteert dit onderzoek eiparasieten van schadelijke schildwantsen die in België voorkomen.

Om de genetische structuur van de Belgische *H. halys* populaties te onderzoeken, werden moleculaire analyses uitgevoerd. De COI- en COII-barcodegebieden van verzamelde *H. halys* individuen werden gesequeneerd. De verspreiding van de Belgische populatie van *H. halys* werd onderzocht via data afkomstig van burgerwaarnemingen. Aan de hand van de genetische analyse en de gevonden haplotypes ontdekten we dat de populaties van de bruingemarmerde schildwants in België een relatief hoge genetische variatie hebben, vergelijkbaar met de variatie in Italië. De invasies van *H. halys* zijn dus recent en nog steeds aan de gang. De invasies zijn voornamelijk afkomstig uit andere Europese landen waar deze aziatische schildwants reeds gevestigd is. Dit gaat dan vooral om Italië, maar in mindere mate ook landen als China en Japan, waar de soort uit afkomstig is (Hoofdstuk 3).

Individuen van zowel *P. rufipes* als *H. halys* werden bemonsterd in boomgaarden, tuinen en bosgebieden in België. De daaruit volgende moleculaire analyse van maaginhoud met behulp van next-generation amplicon sequencing van de ITS2 barcode regio toonde aan dat *H. halys* een uitgebreidere dieetvoorkeur heeft dan *P. rufipes*. Beide schildwantsen hebben vooral een voorkeur voor loofbomen, waaronder genera als *Acer*, *Fagus*, *Prunus*, *Betula* en *Corylus*. Dit impliceert dat gemengde hagen en bosranden die dergelijke waar dergelijke genera abundant zijn in pitfruitboomgaarden een extra aantrekkingsfactor kunnen vormen of als een extra voedingsbron kunnen dienen voor populaties van schadelijke schildwantsen. Deze schildwantsen voeden zich in mindere mate met kruidachtige planten en daarom is het zaaien van bloemenstroken tussen boomrijen in boomgaarden waarschijnlijk geen extra aantrekkingsfactor voor deze schadelijke schildwantsen in pitfruitboomgaarden, terwijl het nog steeds voordelen met zich meebrengt, zoals het voorzien van nectar en pollen voor bestuivers en habitatcreatie voor natuurlijke vijaden van schadelijke plagen waaronder schildwantsen (Hoofdstuk 4).

Het potentieel van de groene gaasvlieg, *C. carnea* als biologische bestrijder van *H. halys* en *N viridula* werd geëvalueerd door het bepalen van de predatiecapaciteit van verschillende levensstadia van de gaasvlieglarven op de eerste twee levensstadia van deze schildwantsen bij verschillende temperaturen in een eenvoudige en een meer complexe arena. Zowel het tweede als derde larvale stadium consumeerde eerste-instar nimfen van beide schildwantsen. Het derde larvale stadium van *C. carnea* vertoonde een hogere predatiecapaciteit dan het tweede larvale stadium en was ook in mindere mate in staat om tweede-instar nimfen van beide schildwantsen te consumeren. Predatie door de gaasvlieglarven was over het algemeen hoger op nimfen van *N. viridula* in vergelijking met *H. halys*. Dit verschil is mogelijks te wijten aan interspecifieke verschillen in de eerste twee levensstadia; eerste-instar nymfen van *H. halys* zijn bijna twee keer zo zwaar als eerste-instars van *N. viridula*. Tweede-instars van *H. halys* zijn mobieler dan tweede-instar nimfen van *N. viridula* waardoor gaasvlieg larven een langere tijd nodig hebben om *H. halys* nimfen te consumeren. Bijkomstige veldexperimenten zijn nodig om het predatiepotentieel van *C. carnea* in open veldomstandigheden volledig te kunnen inschatten (Hoofdstuk 5).

Afgedode eipakketten van *H. halys* werden in boomgaarden en tuinen geplaatst om potentiële eiparasieten aan te trekken. Daarnaast werden in enkele boomgaarden opportunistische stalen genomen met een net en werden ook natuurlijk afgelegde eipakketten van schadelijke of relevante schildwantsen verzameld. Geen van de uitgezette eipakketten van *H. halys* werd geparasiteerd tijdens de studie. Echter, 31% van de natuurlijk gelegde eipakketten die tijdens deze studie werden verzameld, was geparasiteerd. Uit deze geparasiteerde eimassa's werden vijf verschillende soorten eiparasieten geïdentificeerd, daarnaast werden nog twee belangrijke eiparasieten geïdentificeerd uit de net stalen van boomgaarden. Alle eiparasieten werden morfologisch geïdentificeerd en behoorden tot de familie Scelionidae (Hymenoptera: Platygastridae). De gedocumenteerde eiparasiten van belangrijke schildwantsen waaronder *H. halys*, *P. rufipes* en andere schildwantsen in deze studie zijn: *Trissolcus basalis*, *Trissolcus belenus*, *Trissolcus colemani*, *Trissolcus cultratus*, *Trissolcus semistriatus*, *Telenomus truncatus* en *Telenomus turesis* (Hoofdstuk 6).

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Diet quality influences chronic toxicity of copper to the freshwater snail *Lymnaea stagnalis* chronic metal toxicity estimates. SETAC Europe 25th Annual Meeting. 3-7/05/2015. Barcelona, Spain. Flash talk poster presentation.

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Tutoring

Cornelis F, Verhaert L, Morobé L, Vanden Bulcke L. Wat is dat nog, “Inheems”? Hoe de herkomst van plagen en hun natuurlijke vijanden regelgevers kopzorgen geeft. Bachelor dissertation, Ghent University, 2022-2023

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