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UNDERSTANDING THE MORPHOLOGY, NEUROPHYSIOLOGY AND BEHAVIOR OF THE INVASIVE *HALYOMORPHA HALYS* FOR ITS MANAGEMENT IN AGRICULTURAL ECOSYSTEMS

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ABSTRACT OF THE DISSERTATION

UNDERSTANDING THE MORPHOLOGY, NEUROPHYSIOLOGY AND BEHAVIOR OF THE INVASIVE *HALYOMORPHA HALYS* (HEMIPTERA: PENTATOMIDAE), FOR ITS MANAGEMENT IN AGRICULTURAL ECOSYSTEMS

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The rate of introduction of invasive alien species has been increasing dramatically over the past few decades, as a result of growing global trade and climate change. One successful invader in both agricultural and domestic settings is the brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae). Originating from East Asia, this polyphagous pest has been expanding its range since the early 2000's in North America and Europe, where it is known to feed on over 170 host plants, many of which are of economic importance. Currently, the control of *H. halys* relies on broad-spectrum insecticides that provide short term protection of crops and negatively impact the environment and non-target organisms. Despite the numerous studies conducted so far on this species, some gaps of knowledge related to its chemical ecology and vibrational communication still exist. In this light, the aim of this doctoral dissertation was to investigate the morphology, neurophysiology and behavior of *H. halys*, to enhance its sustainable management strategies.

We identified five types of sensilla on the antennae of *H. halys* adults and fifth instar nymphs, using scanning and transmission electron microscopy techniques. The examined sensilla were assigned putative functions based on their morphological characteristics. We found that the majority of these sensory structures are located on the flagellomeres and are thought to have an olfactory role. On another front, the morphological and physiological properties of some of the neurons involved in the vibrational communication of this pest were explored using intracellular recording and electrophoretic staining techniques. Four types of neurons were identified in the thoracic ganglion of *H. halys* adults, all of which are intersegmental with axons projecting anteriorly towards the prothoracic ganglion. The presumed roles of these neurons in processing the vibrational signals are discussed according to their sensitivity and location. Concerning the third and last objective of this dissertation, long-lasting insecticide nets were used under laboratory conditions to assess their effect on the behavior and survivorship of *H. halys* nymphs and adults. The nets showed promising potential for reducing the mobility and inflicting high mortality rates even after short exposure durations. Findings from these various studies are herein presented in the context of the chemical ecology, vibrational signaling and behavior of *H. halys*. Moreover, the future implications of this novel contribution and its relevance for integrated pest management strategies are highlighted.

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‘To know that we know what we know,

and that we don’t know what we don’t know,

that is true knowledge.’

Confucius

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Chapter 1

General introduction

1. Invasive alien species

When a species is introduced, deliberately or unintentionally, outside its native historic range and rapidly expands in this new area with negative effects on biodiversity, economy or human health, it is called an invasive species (Elton, 1958).

The rate of new introductions has been increasing drastically as a result of growing global trade (McGeoch et al., 2010), climate change (Hellmann et al., 2008) and assisted migration (McLachlan et al., 2007). Notable examples of invasive species include zebra mussel (*Dreissena polymorpha*), Argentine ant (*Linepithema humile*) and the spotted knapweed (*Centaurea maculosa*) (Pyšek and Richardson, 2010).

Blackburn et al. (2011) proposed a unified framework to further investigate and categorize the barriers that a species must overcome throughout the various stages in biological invasions, to become an invasive alien species (hereafter 'IAS'). Invasion stages include: transport, introduction, establishment and spread. Transport and introduction involve the intentional or accidental movement of species by humans, such as the introduction of natural enemies in agroecosystems and of marine species by ship ballast water, respectively. Introduced species succeed then in establishing in new areas as a viable population, capable of surviving and reproducing by itself, only after overcoming geographical and/or man-made barriers. Once established, the population will expand its geographical distribution and even disperse rapidly to new territories and become invasive. This chiefly depends on the dispersal abilities of the IAS and the presence of dispersal agents in the invaded site.

1.1. Factors influencing the invasion and establishment of insect pests

A Successful biological invasion is generally affected not only by the properties of the invading species and the frequency of the introduction events, but also by attributes of habitats that render them vulnerable to invasion (Simberloff, 2009; Lockwood et al., 2009).

Characteristics common to successful invaders have been described in many studies (Baker, 1974; Ehrlich, 1989; Simberloff, 1989; Williamson, 1996). These include: *r*-selected life histories (short generation time, high fecundity and high growth rates), have large native range, are rapid dispersers, have broad diet and behavioral flexibility, able to compete for resources and habitat with native species, have association with humans (aesthetic characteristics, domestication, agriculture, etc.…), have high genetic diversity and can adapt to environmental stress (phenotypic plasticity).

Traits associated with habitats and ecosystems vulnerable to invasions include features like high levels of natural and/or human-associated disturbances, low diversity of native species, absence of predators or pathogens and relevant abiotic conditions such as climatic match between host and source habitats (Lodge, 1993).

Recent studies have been focusing on two main hypotheses to explain the biological invasion mechanisms: ‘fluctuating resource availability’ and ‘enemy release’. The former hypothesis proposes that a plant community becomes more susceptible to invasion whenever there is an increase in the amount of unused resources, such as light, nutrients, and water (Davis et al., 2000). The fluctuation in the resources can occur under various circumstances, including periodic enrichments from external sources, release from the resident organisms, disturbances such as pest outbreaks and others (Davis and Pelsor, 2001).

The ‘enemy release hypothesis’ posits that the abundance or impact of some alien – often measured as individual size, population growth, or propensity to displace native species – is related to the scarcity of natural enemies in the introduced range compared with the native range (Keane and Crawley, 2002; Torchin et al., 2003; Mitchell and Power, 2003). Therefore, the biomass used for defense in the native range is reallocated into both reproduction and growth in the invaded region. This hypothesis assumes that (1) natural enemies are important in the regulation of populations, in the introduced community (2) native species are under stronger pressure from natural enemies than alien species and (3) exotic populations capitalize on this relatively lower enemy impact through increased abundance and distribution (Keane and Crawley, 2002).

1.2. Impacts of biological invasions

Invasive species have both ecological and socioeconomic impacts on the communities that they invade and understanding these impacts might aid in developing more efficient management strategies.

1.2.1. Ecological impacts

An ecological impact consists of any significant change in an ecological pattern or process (Pyšek et al., 2012). According to the ‘International Union for Conservation of Nature’, IAS are one of the top causes of biodiversity loss and are among the most significant factors associated with endangerment and extinction of other species, second only to habitat destruction (IUCN, 2019). Invasive species can outcompete native ones and reduce their richness and abundance, which can pose a threat to biodiversity. There are even events in which invasive species can alter the evolutionary pathways of native species through niche displacement and competitive exclusion (Mooney and Cleland, 2001). On the other hand, hybridization can cause loss of characteristics of resident species (Vilà et al., 2000) and can disrupt the existing mutualistic networks such as dispersal and pollination (Traveset and Richardson, 2006; Schweiger et al., 2010). In addition to predation and parasitism, IAS can also assist in the transmission of diseases in native species (Bax et al., 2003).

1.2.2. Socioeconomic impacts

The direct costs associated with the impacts of IAS are enormous and amount yearly to hundreds of billions of dollars worldwide. The total costs of invasive species in Europe is estimated to be well over €20 billion per year, and even that is an underestimate of the real impact (Pyšek and Richardson, 2010). In the United States, the damage and management of IAS cost around 120 billion dollars yearly (Pimentel et al., 2005). Furthermore, invasive alien insects alone, cost at least US$70 billion/year globally, due to their impacts on agriculture and forestry (IUCN, 2019).

IAS can also seriously affect human well-being by negatively impacting their safety, health, material and non-material assets as well as the social, spiritual and cultural relations (McNeely, 2005; Scalera et al., 2012; Bacher et al., 2018).

2. The invasion of *Halyomorpha halys*: a success story

2.1. Origin and distribution

Halyomorpha halys (Stål) (Hemiptera: Pentatomidae), commonly known as the brown marmorated stink bug (BMSB), is a successful invader in both agricultural and domestic settings (Leskey and Nielsen, 2018) in North America and Europe. The native range of *H. halys* is East Asia, it originates from China, Japan, Taiwan, and Korea (Lee et al., 2013a).

The first recorded *H. halys* sample in North America was retrieved from Allentown, Pennsylvania, in 1996, however the original introduction is believed to have occurred at least a several years earlier (Hoebeke and Carter, 2003). Since then, *H. halys* has rapidly expanded its range, and is currently established in over 42 U.S. states (Skillman et al., 2018). It has also reached Ontario, Canada (Gariépy et al., 2014) and has spread to other provinces including British Columbia (Abram et al., 2018). In 2017, *H. halys* was also recorded South America in Chile (Faúndez and Rider, 2017).

In Europe, *H. halys* was first detected in Switzerland in 2007 (Wermelinger et al., 2008) and subsequent detections have been reported in many countries: Germany (Heckmann, 2012), France (Callot and Brua, 2013), Italy (Pansa et al., 2013), Greece (Milonas and Partsinevelos, 2014), Hungary (Vetek et al., 2014), Liechtenstein (Haye et al., 2015a), 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, 2018), Croatia (Šapina and

Šerić Jelaska, 2018), Bosnia and Herzegovina (Zovko et al., 2019) and Turkey (Güncan and Gümüş, 2019) (Fig. 1.1).

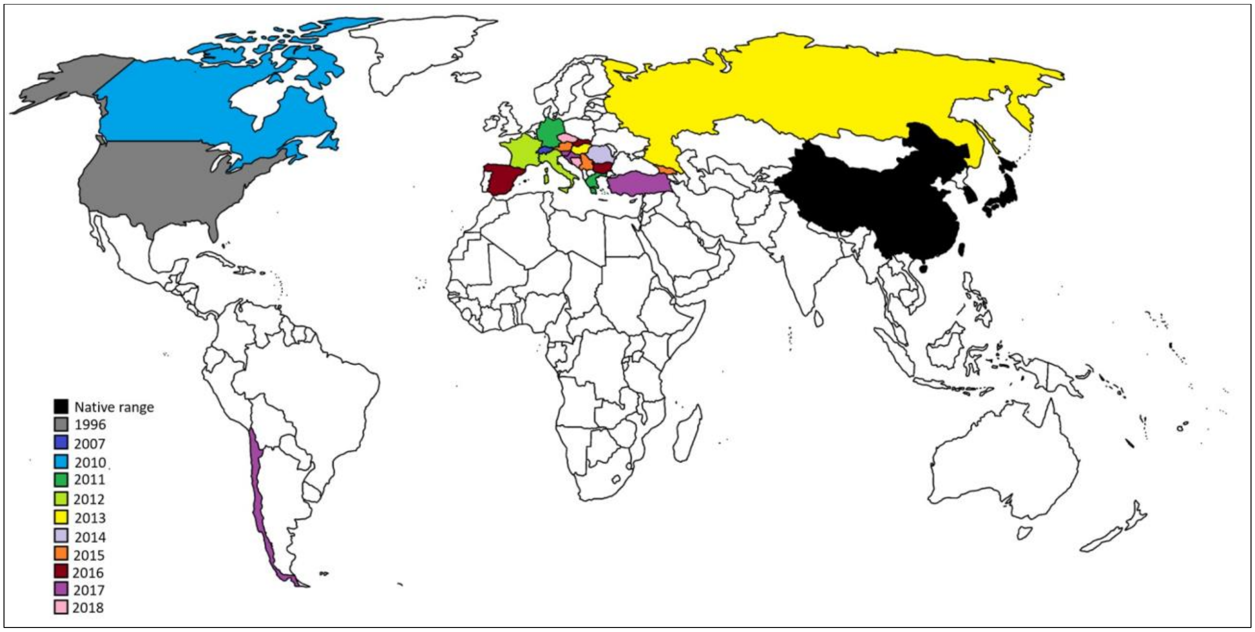

Figure 1.1. Current worldwide *H. halys* distribution map (as of October 2019) in the native and invaded range by year of detection (Leskey and Nielsen, 2018; edited and updated). Detailed information on the distribution of *H. halys* in the United States and Canada and Europe is available at www.stopbmsb.org and https://www.cabi.org/.

In addition, considering the ability of the bug to hitchhike in luggage, cars and shipping containers, it is probable that it will be found in additional European countries (Haye et al., 2015a). Moreover, it is likely that *H. halys* will spread and establish in temperate areas in both the Northern and Southern Hemisphere including parts of South America (Uruguay, southern Brazil and northern Argentina), Africa (northern Angola and adjacent areas of Congo and Zambia), New Zealand and southeast and southwest Australia, based on ecological and bioclimatic niche modelling studies (Zhu et al., 2016; Kriticos et al., 2017). A growing body of work is being generated on this pest even in areas where it hasn’t been recorded yet. In Australia and New

Zealand, a lot of effort is put into analyses to assess the biosecurity risks associated with the importation of goods from countries with established *H. halys* populations (Duthie et al., 2012; Horwood et al., 2019).

As noted earlier, many factors influence the successful establishment and spread of non-native species into a new region. The brown marmorated stink bug managed to proliferate and persist outside its native range thanks to a combination of favorable biological and physiological characteristics. These include primarily *r*-selected life history, association with human-modified landscapes, polyphagy and ability to compete with endemic species (Leskey and Nielsen, 2018). Moreover, in a laboratory flight mill study, *H. halys* males and females flew an average of 2442 and 2083 m, respectively, over a 22-h period, which confirms their strong dispersal capacity (Lee and Leskey, 2015).

2.2. Life cycle and description of *H. halys*

Like all stink bugs, *H. halys* is a hemimetabolous insect; it has three developmental stages: eggs, five nymphal instars and a final winged adult stage (Fig. 1.2). The life cycle of *H. halys* was studied by Funayama (2002), Niva and Takeda (2003), Nielsen et al. (2008), Medal et al. (2012) and Haye et al. (2014) and the stages were morphologically described by Hoebeke and Carter (2003). Adults exit their overwintering sites in the early spring, roughly April or May, to mate and reproduce. Females require between 117.65-147.65 degree-day (DD) accumulation until oviposition can begin, and they can lay an average of 240 eggs in their lifetime. Eggs are usually laid in clusters on the underside of leaves and are soft green in color, and they develop between 15° and 33 °C and can take between 22 and 26 days to hatch at 15° and 2-4 days at 30 °C (Haye et al., 2014). First instars have a tick-like appearance with an orange-red abdomen and stay close to the egg cluster to feed on it. As they grow, nymphs become darker in color and white markings appear on their legs and antennae. To develop from egg to adult, *H. halys* need 537.63-588.24 DD, whereas under controlled conditions, Haye et al. (2014) found that development was not possible at or below 15 and at or above 35 °C. Sexual dimorphism is observed in adults; females are often larger and heavier than their male counterparts, and the latter have a genital capsule on their ventral abdominal segment. *Halyomorpha halys* can be distinguished from other species by the banding of the antennae (white bands on the fourth antennal segment), rounded shoulders and alternating coloration on the margins of the abdomen.

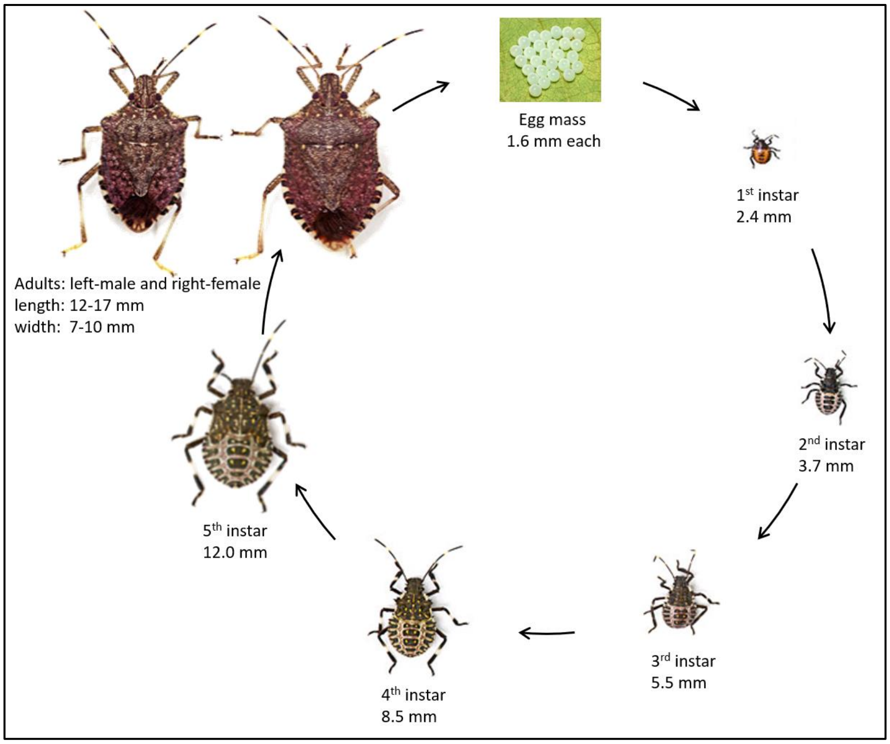

Figure 1.2. Life stages of *Halyomorpha halys*. The body length of each stage is included (Hoebeke and Carter, 2003). Image: http://www.stopbmsb.org/, edited).

The number *of H. halys* generations per year varies depending on location; the species is multivoltine with up to five generations reported in Southern China (Hoffman, 1931). Nielsen et al. (2016a; 2017) modeled voltinism and phenology of USA populations and predicted a bivoltine population throughout its range in the USA, with voltinism largely defined by regulation of photoperiod induction and termination. In Italy, two generations per year have been documented (Maistrello et al., 2017). Around mid-October, adults undergo facultative diapause and start clustering in dark locations in wild and man-made structures (Toyama et al., 2006). This behavior allows them to save resources in order to overcome unfavorable environmental conditions. Olfactory and tactile cues play an important role in maintaining aggregations over the winter compared to temperature and visual cues (Toyama et al., 2011). In late spring, adults emerge from overwintering sites and start a new cycle.

2.3. Sexual communication

The interaction of a series of chemical and vibratory signals is crucial during the intraspecific communication in the premating period of stink bugs (Miklas et al., 2003). Heteropteran insects are well-known for their defensive secretions released upon disturbance from the metathoracic scent glands (Aldrich, 1995). These secretions serve as an alarm pheromone to disperse conspecifics from predators, and their release in abundant amounts might be overshadowing the identification of possible sex pheromone compounds. McBrien and Millar (1999) also suggested that the alarm pheromone compounds themselves may be part of the sex pheromone, when emitted in much smaller quantities.

In Pentatomidae, the main sound producing mechanism is body vibration (Gogala, 2006). This mechanism was described in detail in *Nezara viridula* (Linnaeus), a model for the whole subfamily Pentatominae (Čokl, 2008). The thorax and abdomen are dorsally connected by a tergal plate, formed by fusion of the first and second abdominal tergites. Repeated longitudinal and lateral muscle contraction underlying the plate vibrate the abdomen in the dorso-ventral direction and these vibrations are transmitted over the thorax and legs to the substrate. The vibrations are then detected by special receptors present in the leg of *N. viridula*, namely subgenual and chordotonal organs.

Kawada and Kitamura (1983) outlined the mating behavior of *H. halys*, including several courtship phases, based on visual observations. Recently, communication via substrate-borne vibrational signals of this species was recorded under laboratory conditions, using a laser vibrometer (Polajnar et al., 2016). Males initiate the communication by spontaneously emitting long narrowband vibrational signals. Nearby active females respond thus triggering the male searching behavior. Afterwards, both sexes emit a combination of signals until they come into physical contact. It was noted as well that the male signals do not attract other individuals, whereas the female signals significantly attracted *H. halys* males (Mazzoni et al., 2017). Further investigations in this field are needed to better understand the characteristics of the processing system (such as vibratory interneurons) involved in emitting and receiving the vibratory signals. Moreover, exploring the potential of the female vibrational playback under field conditions will render the behavioral manipulation of *H. halys* feasible in the future.

2.4. Host plants

As a highly polyphagous pest, *H. halys* is known to feed on over 170 host plant species; many of these are of economic importance (Rice et al., 2014). Nymphs and adults can feed on all above-ground structures of a plant: leaves, stems, fruits, pods, seeds, and even through bark (Leskey et al., 2012a). They feed on field crops such as corn (*Zea mays* L.) and soybean (*Glycine max* L.) (Kuhar et al., 2012) and orchard fruit including apple (*Malus* spp.), pear (*Pyrus* spp.) and peach (*Prunus* spp.) (Nielsen and Hamilton, 2009a; Maistrello et al., 2014). Small fruits such as grapes (*Vitis* spp.), blueberry (*Vaccinium corymbosum* L.), raspberry (*Rubus* spp.) and blackberry (*Rubus* spp.) are also used as food sources for *H. halys* (Bergmann et al., 2014; Wiman et al., 2015). This pest has the potential to feed on vegetable crops as well, for instance on tomato (*Solanum lycopersicon* L.), eggplant (*Solanum melongena* L.), pepper (*Capsicum* spp.) and common bean (*Phaseolus vulgaris* L.) (Haye et al., 2015a).

In addition to agricultural crops, *H. halys* feed on a range of ornamental hosts, such as red maple (*Acer rubrum* L.), tree of heaven (*Ailanthus altissima* Miller), English holly (*Ilex aquifolium* L.) and cherry laurel (*Prunus laurocerasus* L.) (Bergmann et al., 2014). It has been reported that the host preference of *H. halys* appears to change throughout the season, depending on the survival and developmental needs of the pest (Nielsen and Hamilton, 2009b). The fitness of nymphal *H. halys* is also enhanced when they feed and develop on multiple plant species versus single-host diet (Acebes-Doria et al., 2016).

2.5. Feeding signs and symptoms

Pentatomids, including *H. halys*, suck sap from plants by inserting their sharp needle-like stylets into the tissues. This initially destroys cells upon penetration and the destruction of nearby cell walls by dissolving proteins follows, after the spread of the salivary enzymes (Depieri and Panizzi, 2011). This feeding behavior can cause deformities such as: discoloration, necrosis, dimpling, scars, wounds, shriveled and deformed seeds and overall decline in plant vigor (Leskey et al., 2012b). Young and ripening fruits are generally more susceptible than older tissues to feeding damage *by H. halys*.

Martinson et al. (2013) found that the bark of *Malus*, *Ulmus*, and *Acer* trees wounded by *H. halys* feeding attracted several species of native ants and wasps in the mid-Atlantic region of the United States. In its native range, *H. halys* is also a vector of a phytoplasma causing witches' broom on *Paulownia tomentosa* (Thunberg) trees in China (Sun et al., 1999) and Japan (Nakano et al., 1997). Moreover, heavy feeding by *H. halys* on some herbaceous perennials has been reported to cause wilting and death of the trees (Leskey et al., 2012a). Feeding injury by *H. halys* is usually concentrated on crop borders adjacent to wooded areas (Joseph et al., 2014; Venugopal et al., 2015), therefore exploiting this perimeter-driven behavior can improve the management of this pest by developing push-and-pull strategies (Blaauw et al., 2015).

2.6. Economic impact: *H. halys*- an agricultural pest

Many of the species used as host plants by the polyphagous *H. halys* are of significant economic importance, such as high-value nut and small fruit crops. In 2010, *H. halys* outbreaks in the mid-Atlantic region of the United States alone caused over 37 million US$ in losses to tree fruit producers (Leskey et al., 2012b). Over $21 billion worth of crops in the United States have been estimated to be threatened by *H. halys* feeding damage (ODA, 2016). In Italy, more than 50% of pears were damaged by *H. halys* feeding in the fruit-growing areas of Emilia-Romagna region (Bariselli et al., 2016).

Feeding injury by *H. halys* nymphs and adults, especially on fruiting structures, may severely affect agricultural crops and renders produce unmarketable. Maturity delay of crops, caused by *H. halys* feeding, can cause difficulties in harvesting soybeans for instance (Owens et al., 2013) which may result in significant yield loss. Cissel et al. (2015) reported significant quality reduction in corn kernels even at low *H. halys* population densities (one individual/year). Additional control methods also increase production costs in invaded areas due to the urgent need for frequent applications of insecticides to control *H. halys* and secondary pest outbreaks.

2.7. Urban impact: *H. halys*- a nuisance pest

Halyomorpha halys overwinters as adults and seeks shelters after being induced by shortened photoperiods in autumn (Niva and Takeda, 2003). Natural landscapes in the wild can act as an overwintering destination for *H. halys* such as beneath the bark, in rocky outcroppings and in crevices of dead and standing trees, particularly *Quercus* spp. and *Robinia* spp. (Lee et al., 2014a). However, this species is considered a nuisance pest for it also tends to aggregate in massive numbers in human-made structures such as on walls, windows, door frames, sheds, piles of firewood and even inside buildings and other anthropogenic settings (Inkley, 2012; Hancock et al., 2019). Infestations can exceed 20,000 individuals in houses invaded by *H. halys* (Watanabe et al., 1994). There is no record of *H. halys* causing direct damage to humans (stings or bites) or residential homes (structural damage), however the species can induce allergic sensitization during autumn dispersal period (Mertz et al., 2012). An investigation into the pattern of overwintering *H. halys* in college dormitories revealed that a higher proportion of adults overwinters towards the top of urban structures than towards the ground level (Cambridge et al., 2015). The seasonal dispersal pattern and numbers of *H. halys* varied by wall aspect, with higher densities in recessed doorways than on associated walls, in a multi-year assessment of adults' distribution on the walls of a building in Virginia (Bergh and Quinn, 2018). Understanding the overwintering ecology, behavior and dispersal patterns of *H. halys* will help refine our management techniques to mitigate nuisance to homeowners.

2.8. Management practices

2.8.1. Monitoring

Monitoring of pentatomids is generally difficult and time consuming because of their nocturnal activity and hiding behaviors, including dropping off plants or switching sides of a leaf or fruit when disturbed (Nielsen and Hamilton, 2009a). Field monitoring for *H. halys* is a key component of Integrated Pest Management (IPM) in agricultural systems to help growers in understanding the pest population dynamics and therefore reaching a management decision at precise timing (Fig. 1.3).

Methods for detection and monitoring of *H. halys* include: sweep-net sampling, beat sheet sampling, pheromone trapping, blacklight traps and timed visual counts (Fig. 1.3). Blacklight traps were attractive to *H. halys* early season populations as well as at low population densities and can give an indication of spread, abundance and adult flight activity during the night

(Nielsen et al., 2013). On the other hand, pheromone traps baited with the two-component aggregation pheromone (Khrimian et al., 2014) secreted by *H. halys* males: (3S,6S,7R,10S)-10,11-epoxy-1-bisabolen-3-ol and (3R,6S,7R,10S)-10,11-epoxy-1-bisabolen-3-ol in a 3.5:1 ratio, in addition to methyl (2E,4E,6Z)-2,4,6-decatrienoate (MDT) proved most efficient late in the season (Weber et al., 2014) (Fig. 1.3). MDT is the aggregation pheromone of the Oriental Stink Bug, *Plautia stali* Scott (Hemiptera: Pentatomidae) and has been shown to have a synergistic effect on the attraction of *H. halys* (Weber et al., 2014). Various trap designs of different colors and sizes to capture *H. halys* have been assessed, including: funnel traps (Aldrich et al., 2007), ground-deployed pyramid traps (Leskey and Hogmire, 2005), baffle traps (Aldrich et al., 2009) and bucket-style water traps (Leskey et al., 2012c) (Fig. 1.3). Black ground-deployed pyramid traps made of corrugated plastic has become the standard trap (Leskey et al., 2012c). Clear sticky traps baited with commercial lures baited with the aggregation pheromone and MDT in combination were also found to be reliable tools to monitor *H. halys* when deployed on borders of agricultural or urban locations, in a season-long study conducted throughout the United States (Acebes-Doria et al., 2019).

2.8.2. Chemical control

The most effective control tools to protect agricultural crops from *H. halys* feeding damage are insecticides. This option offers only short-term crop protection. The efficacy of insecticides is influenced by their formulation (dry residues/ liquid sprays) and by the bugs' life stage and phenology. Nymphs are more sensitive than adults (Bergmann and Raupp, 2014) and overwintering adults are more susceptible than F1 and F2 adults (Leskey et al., 2014). In conventional farming systems, synthetic chemicals belonging to neonicotinoid, pyrethroid, organophosphate and carbamate families are the most used and effective against *H. halys* (Leskey et al., 2012b) (Fig. 1.3). Recovery of *H. halys* from a range of pyrethroids (Lee et al., 2013b) and neonicotinoids (Leskey et al., 2012d) has been repeatedly reported. In addition, these broad-spectrum insecticides are highly toxic to many beneficial arthropods and their use is disrupting established IPM programs, where growers are often forced to increase the frequency of application, as much as fourfold in outbreak years (Leskey et al., 2012b), to reduce the economic losses caused by *H. halys*. Reliance on insecticides can also lead to increasing pesticide resistance and secondary pest outbreaks, especially spider mites, woolly apple aphids, and scale insect species (Rice et al., 2014).

Organic growers have limited options of chemicals effective against *H. halys*. Organically-approved insecticides with natural products including insecticidal soap, neem oil, essential oils, pyrethrins and capsaicin were only effective against nymphs with less than 30% mortality of adults (Bergmann and Raupp, 2014) (Fig. 1.3). Laboratory studies found that dry residues of potassium salts of fatty acids, spinosad, pyrethrins, and pyrethrins + kaolin significantly increased mortality of *H. halys* relative to water controls (Lee et al., 2014b), however none of these products were effective against adults in field trials on pepper (Dively et al., 2013).

Chemicals used to control *H. halys* in agriculture may also be effective for control in and around homes, especially during adults' fall dispersal to overwintering sites. Bergh and Quinn (2018) explored the potential of insecticide nets in mitigating homeowner issues from *H. halys*, by evaluating the use of framed panels of deltamethrin-incorporated netting, non-treated netting and an open frame with no netting placed on the wall of an invaded building. The presence of netting, independent of insecticides, increased the frequency of *H. halys* alightment and therefore could be further exploited to exclude adults from entering buildings (Bergh and Quinn, 2018).

2.8.3. Cultural and physical control

Cultural crop protection techniques against *H. halys* have received little attention. Changing the time of planting and introducing alternative hosts during fruit development of the main crops can for example reduce levels of damage by *H. halys* feeding (Fig. 1.3). Beans and sweet corn planted late in the season suffered less injury from *H. halys* when soybean was grown nearby (Kuhar et al., 2012). Moreover, the development of soybean cultivars with *H. halys* resistant genes is currently underway (Bansal et al., 2013). Growing a less-economically important and more attractive plant in the vicinity of the target crop can protect it from insect attack (Stary, 1969) (Fig. 1.3). A trap crop composed of sorghum and sunflower provided a 5-wk attraction period coinciding with peak *H. halys* activity in organic farms (Nielsen et al., 2016b). The same combination of trap crops grown close to organic peppers resulted in significantly less injury of *H. halys* relative to control peppers, however the feeding damage was insufficient to be economically viable (Mathews et al., 2017). Sunflowers alone also failed in reducing fruit damage in bell pepper fields when utilized as trap crops (Soergel et al., 2015).

Mechanical removal of nymphs and egg masses, removal of nearby alternative hosts, the use of exclusion nets and bagging of fruits have also been considered as physical measures to protect crops against *H. halys* (Lee et al., 2013a; Candian et al., 2018) (Fig. 1.3).

2.8.4. Biological control

Biocontrol agents of *H. halys* can be employed after determining *H. halys* population buildup in the field. Several entomopathogenic fungi, predators and parasitoids were assessed (Fig. 1.3). Three *Beauveria bassiana* and two *Metarhizium anisopliae* isolates were tested against *H. halys*, where one of the *B. bassiana* resulted in 85-100% mortality in 9-12 days post application, whereas the other isolates were less effective (Gouli et al., 2012). The formulations of commercially available products, containing a strain of *B. bassiana* as an active ingredient, were also explored against *H. halys* second instars. At a dose of 1×10^7 conidia/ml, both the wettable powder and the emulsifiable formulations were efficacious in causing 67% and 80% mortality 9 days post treatment and 95% and 100% after 12 days, respectively (Parker et al., 2015).

A two-year study in organic agriculture systems in the eastern U.S. revealed that chewing predators accounted for the majority of control of *H. halys* eggs (Ogburn et al., 2016). Another study showed that katydids and ground beetles in addition to earwigs, jumping spiders and crickets (to a lesser extent) were the most frequent and efficient predators of *H. halys* eggs (Morrison et al., 2016). Generalist predators such as lacewings and ants have also been reported (Leskey et al., 2012a).

Throughout its native range, several hymenopteran egg parasitoids in the genera *Trissolcus* Ashmead, *Telenomus* Haliday (Scelionidae), *Anastatus* Motschulsky (Eupelmidae) and *Ooencyrtus* Ashmead (Encyrtidae) are involved in limiting *H. halys* populations (Lee et al., 2013a). In the invaded areas in North America and Europe, *Trissolcus japonicus* (Ashmead) is the most promising candidate as a classical biological control agent, with parasitism rates often ranging from 50 to 90% in Asia (Yang et al., 2009). Since its first discovery in Maryland in 2014 (Talamas et al., 2015), *T. japonicus* has been reported in 10 states (Morrison et al., 2018) and very recently adventive populations were also recovered from sentinel *H. halys* egg masses in Switzerland (Stahl et al., 2019) and Italy (Moraglio et al., 2019; Pantezzi, 2019). Haye et al. (2015b) explored the potential of generalist egg parasitoids of European Pentatomidae for controlling *H. halys* and found that platygastrids in the genera *Trissolcus* and *Telenomus* developed poorly on fresh eggs, whereas the eupelmid *Anastatus bifasciatus* is the only potential candidate for augmentative biological control in Europe, as it succeeded in parasitizing and developing in *H. halys* eggs. *Trissolcus mitsukurii*, another egg parasitoid that play a key role in the population dynamics of *H. halys*, was recently recorded for the first time in Europe and is currently under evaluation (Sabbatini Peverieri et al., 2018).

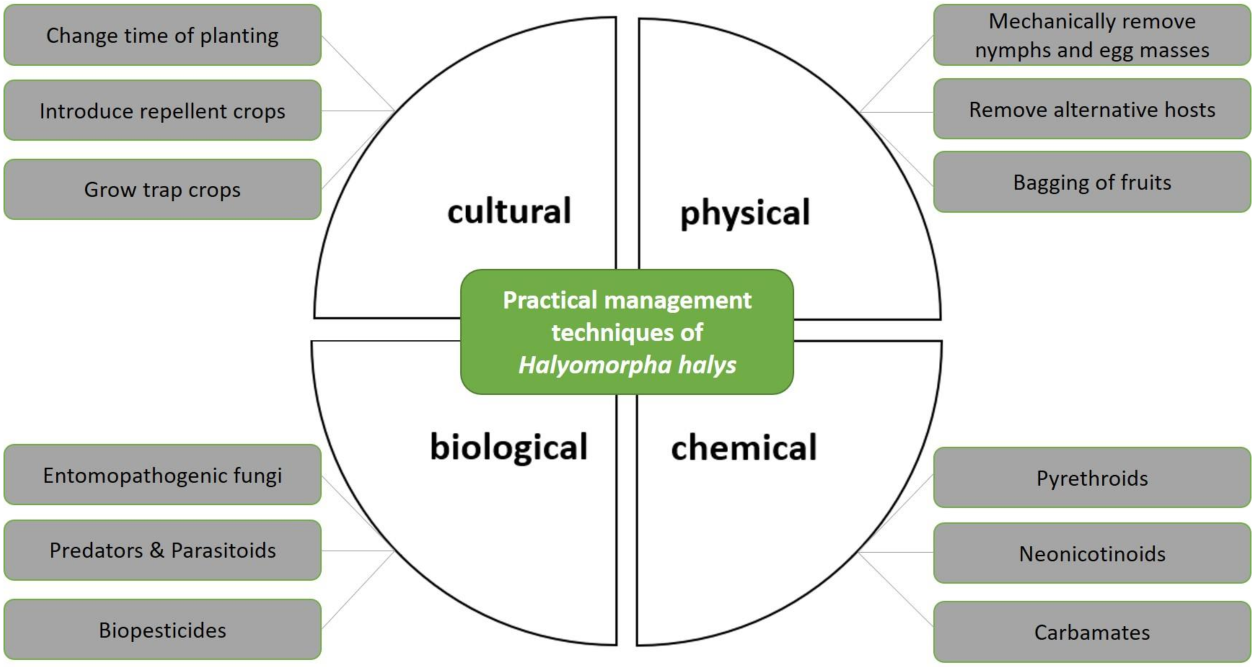

Figure 1.3. Scheme of practical control tactics against *Halyomorpha halys* in agricultural settings.

3. Aim and outline of the thesis

Chapter 1 provides a literature review on *Halyomorpha halys* (Hemiptera: Pentatomidae) to display the current state of knowledge and demonstrates how the studies included in this dissertation expand our understanding of this serious pest. **Chapter 2** investigates the morphology of different sensillum types on the antennae of *H. halys* adults and fifth instar nymphs, based on scanning and transmission electron microscopic observations. The results of this study provide valuable basis for future electrophysiological investigations, based on the putative functions of the investigated sensilla. **Chapter 3** elucidates the morphological and physiological properties of some of the neurons found in the thoracic ganglion of *H. halys* that are involved in the vibrational communication of this species. Using intracellular recording combined with electrophoretic staining techniques, we were able to characterize four neuron types; namely: *CG-AC-1*, *CG-AC-2*, CG-AB-1 and *CG-AI-1*. The findings of this study will provide a potential avenue of future control options of *H. halys* via exploiting the vibrational signals in novel behavioral manipulation techniques. **Chapter 4** explores the effect of direct contact on a deltamethrin-impregnated long lasting net (LLIN) on the behaviour and survivorship of *H. halys* nymphs and adults, following exposure at 3 different durations. Under laboratory conditions, the findings indicate a significant reduction in the vertical and horizontal mobility of adults. The data of nymphs also show a significant suppression in their vertical mobility with no differences in their horizontal movement. High levels of mortality (more than 70 %) of both adults and nymphs were recorded 7 days after initial exposure to the LLIN. The potential of using LLINs and their limitations for managing *H. halys* in agricultural settings are addressed in this study. **Chapter 5** briefly summarizes another four projects (novel marking methods, harmonic radar tag, BugMap and vibro-trap) that I was involved in during my PhD. **Chapter 6** reviews the key findings of all the studies mentioned in this dissertation and concludes with recommendations for future research and practical applications.

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Chapter 2

A closer look at the antennae of the invasive *Halyomorpha halys*: fine structure of the sensilla

Ibrahim, A., Giovannini, I., Anfora, G., Rossi Stacconi, M.V., Malek, R., Maistrello, L., Guidetti, R. and Romani, R., 2019. *Bulletin of Insectology, 72* (2), pp. 187-199. eISSN 2283-0332

Abstract

The brown marmorated stink bug, *Halyomorpha halys*, is an invasive agricultural and urban pest capable of feeding on over 100 species of host plants. The antennae of this bug play an important role not only in detecting food and mates but also in short-range location of conspecifics when aggregating for diapause. The morphology and distribution of antennal sensilla of *H. halys* were investigated at an ultrastructural level using scanning and transmission electron microscopy approaches. Adults have 5-segmented antennae, made up of a scape, a 2-segmented pedicel and two flagellomeres, while 5th instar nymphs have shorter, 4-segmented antennae, with only one pedicel segment. Five types of sensilla are distinguished, based on their shape, length and basal width and the presence of basal socket and pores: sensilla basiconica (types A, B, C, D and E), sensilla coeloconica, sensilla trichoidea and sensilla chaetica (types A and B). Sexual dimorphism was not observed in this species, with respect to the morphological features and abundance of sensilla. The most abundant sensory structures are sensilla trichoidea showing characteristics typical of olfactory sensilla, whereas the least abundant are sensilla coeloconica which may be involved in thermo- and hygro-reception. Basiconic sensilla type A are solely identified on the antennae of 5th instar nymphs, where they presumably have a dual mechanosensory-gustatory role. The putative function of the remaining sensilla are herein discussed.

Keywords: Pentatomidae, phytophagous, ultrastructure, sensory receptor, sexual dimorphism

Introduction

Antennae of insects are indispensable sensory organs that bear a wide range of sensilla, serving an array of sensory modalities, such as olfaction, gustation, mechanoreception and thermo- and hygro-reception (Altner and Prillinger, 1980; Zacharuk, 1985; Steinbrecht, 1984). The role of sensilla can be presumed based on their morphology, number and distribution. For instance, multipororous sensilla are usually associated with olfactory function, whereas mechano- thermo- and hygro-receptors are often attributed to aporous sensilla (Keil, 1999).

Numerous investigations handled the structure and function of sensilla in pentatomid bugs (Brézot et al., 1997; Sinitsina and Krutov, 1996; Rani and Madhavendra, 1995; 2005; Silva et al., 2010; Zhang et al., 2014a; Ahmad et al., 2016; Barsagade and Gathalkar, 2016). Among the studied species, similar sets of sensilla have been recorded, including sensilla basiconica, trichoidea, coeloconica and chaetica. Pentatomids heavily rely on olfaction in their intra- and interspecific communication, through pheromones (aggregation, sexual and alarm) and kairomones (plant volatiles) respectively. Porous basiconic sensilla described in these studies were the major receptors involved in volatile perception.

The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera Pentatomidae), is an invasive agricultural and domestic pest, native to eastern Asia, that was accidently and repeatedly introduced into North America and Europe (Hoebeke and Carter, 2003; Haye et al., 2015; Cesari et al., 2018). Currently, *H. halys* has been detected in almost all the states of USA and Canada, as well as 15 European countries (EPPO, 2018; Fogain and Graff, 2011). This highly polyphagous pest, capable of feeding on over 100 species of host plants, thrives on many economically important crops, some of which are apples and pears. Over $21 billion worth of crops in USA have been estimated to be threatened by *H. halys* feeding damage (ODA, 2013). In Italy, *H. halys* was first recorded in the province of Modena in 2012 and has ever since expanded its range to most of the Italian regions (Maistrello et al., 2018; Malek et al., 2018). Due to its overwintering behaviour, *H. halys* is also considered an urban nuisance pest as it often aggregates in anthropogenic structures during late fall (Lee, 2015).

A previous study focused on the role of *H. halys* antennae in short-range location of conspecifics and aggregation (Toyama et al., 2006), and later electrophysiological studies demonstrated the presence of olfactory receptors on its antennae capable of detecting various essential oils (Zhang et al., 2014b). Paula et al. (2016) recently identified 30 odorant binding proteins involved in the chemosensory perception in the antennae of *H. halys*. Therefore, given their significance in host and mate location mechanisms, it became essential to closely investigate the antennae of this invasive pest, to provide insight into possible behavioral manipulation techniques. The latter can be applied by amending already existing pheromone traps with host plant volatiles aimed at mass trapping, and/or vibrational signals for mating disruption of *H. halys* to mitigate its spread and damage (Mazzoni et al., 2017).

The aim of the present study was to describe the morphology, location, abundance and distribution of the sensilla present on the antennae of *H. halys* adults (females and males) and fifth instars nymphs by means of Scanning Electron Microscopy (SEM). Transmission Electron Microscope (TEM) techniques were employed to confirm the classification of the sensilla types based on SEM, while highlighting their possible functions. This work is a prerequisite for our ongoing research on host location mechanisms and other electrophysiological studies of the antennal sensory system involved in the chemical communication of *H. halys*.

Materials and Methods

Insect rearing

Wild *H. halys* were collected periodically from July through September 2016 from the field in the cities of Modena and Reggio Emilia (Italy). The bugs were kept on a diet of organic peanuts, fresh organic green beans, carrots and tomatoes under controlled conditions (24 ± 2 °C; 70 ± 5% RH; 16:8 L:D). Fifth instar nymphs and adults were used in this study.

Length of antennal segments

To measure the length of each antennal segment, five replicates of male, female and fifth instar's antennae were used. Each antenna was mounted on a slide with Faure's mounting medium, and the length of each segment was measured using a stereo microscope (MZ 12.5, Leica) equipped with an ocular micrometer (Leitz).

Scanning Electron Microscopy (SEM)

Six *H. halys* adults (three males and three females) and five 5th instar nymph individuals were used. The insects were anesthetized by freezing and their antennae were excised from the head capsule using fine forceps and placed in a lens cleaner liquid for 24 h. Dehydration in a graded series of ethanol (from 70% up to 99%) followed, prior to overnight drying at 30 °C. After drying, the specimens were mounted on aluminum stubs with double-sided sticky tape. The specimens were then sputter-coated with gold (Emitech k550, Quorum Technologies Ltd., UK) prior to their examination with Scanning Electron Microscope (Nova NanoSEM™ 450, FEI, USA) at 15 -20 kV.

Sensilla mapping

To determine the number of sensilla and their spatial distribution, antennae were oriented on the stubs in a way to display either their dorsal or ventral sides, to obtain a total sensillar count for each replicate. The observation and classification of sensilla present on the antennae was carried out by analyzing the details of high-resolution images taken by SEM. SEM pictures of three portions (proximal, medial and distal) of each antennal segment were taken with a resolution of 6144 × 4096 dpi and at a magnification of 730X. Because SEMs offer a view of only one side of the antenna, we only examined sensilla on the dorsal surface (abundant in sensilla) of each antennomere. In order to avoid counting any sensillum more than once, different types of sensilla were marked with color-coded dots using the Microsoft Paint Software (Redmond, Washington). Afterwards, the sensilla were counted manually.

Transmission Electron Microscopy (TEM)

For TEM observations, four males and four females were paralyzed by exposure to cold temperatures (−18 °C) for 60 seconds, then immediately immersed into a solution of glutaraldehyde and paraformaldehyde 2.5% in 0.1 M cacodylate buffer + 5% sucrose, pH 7.2-7.3 (as in Romani and Rossi Stacconi, 2009). Each antenna was detached from its base at room temperature, single antennomeres were isolated and reduced in size by cutting them into two parts to facilitate fixative penetration and left at 4 °C for 2 hours. The specimens were rinsed at 4 °C overnight in the cacodylate buffer, then they were post-fixed in 1% OsO_4 (osmium tetroxide) for 1 hour at 4 °C and rinsed in the same buffer. Dehydration, in a graded ethanol series from 60% to 99%, was followed by embedding in Epon-Araldite with propylene oxide as bridging solvent. Thin sections were taken with a diamond knife on an LKB "Nova" ultramicrotome and mounted on formvar coated 50 mesh grids. Then, sections on grids were stained with uranyl acetate (20 minutes, room temperature) and lead citrate (5 minutes, room temperature). Finally, the sections were investigated with a Philips® EM 208. Digital pictures (1376 × 1032 pixels, 8b, uncompressed greyscale TIFF files) were obtained using a high-resolution digital camera MegaViewIII (SIS®) connected to the TEM.

Statistical analysis

To explore the differences between the length of each antennal segment and to compare the numbers of sensilla among them and among males, females and 5th instar nymphs, the statistical analyses were performed using parametric ANOVA test with Tukey HSD post-hoc test. This test was used since data showed a normal distribution evaluated with the Levene's test. All statistical analyses were carried out using the IBM SPSS Statistics Software (25 version).

Results

General description of H. halys antennae

The filiform antennae of *H. halys* are located frontally on the head capsule, anterior to the eyes. In adults (both males and females), the antenna is composed of five distinct segments: a short basal scape, a two-segmented pedicel and two segments of the flagellum (flagellomeres Fl1 and Fl2) (Fig. 2.1 A, C). It has a dark brownish color with characteristic light-colored bands on the base and apex of the Fl1 segment and the base of the Fl2 segment (Fig. 2.1 C). The antennae are not sexually dimorphic in length (Table 2.1), even though the body size of females is generally bigger than that of males (Nielsen et al., 2008).

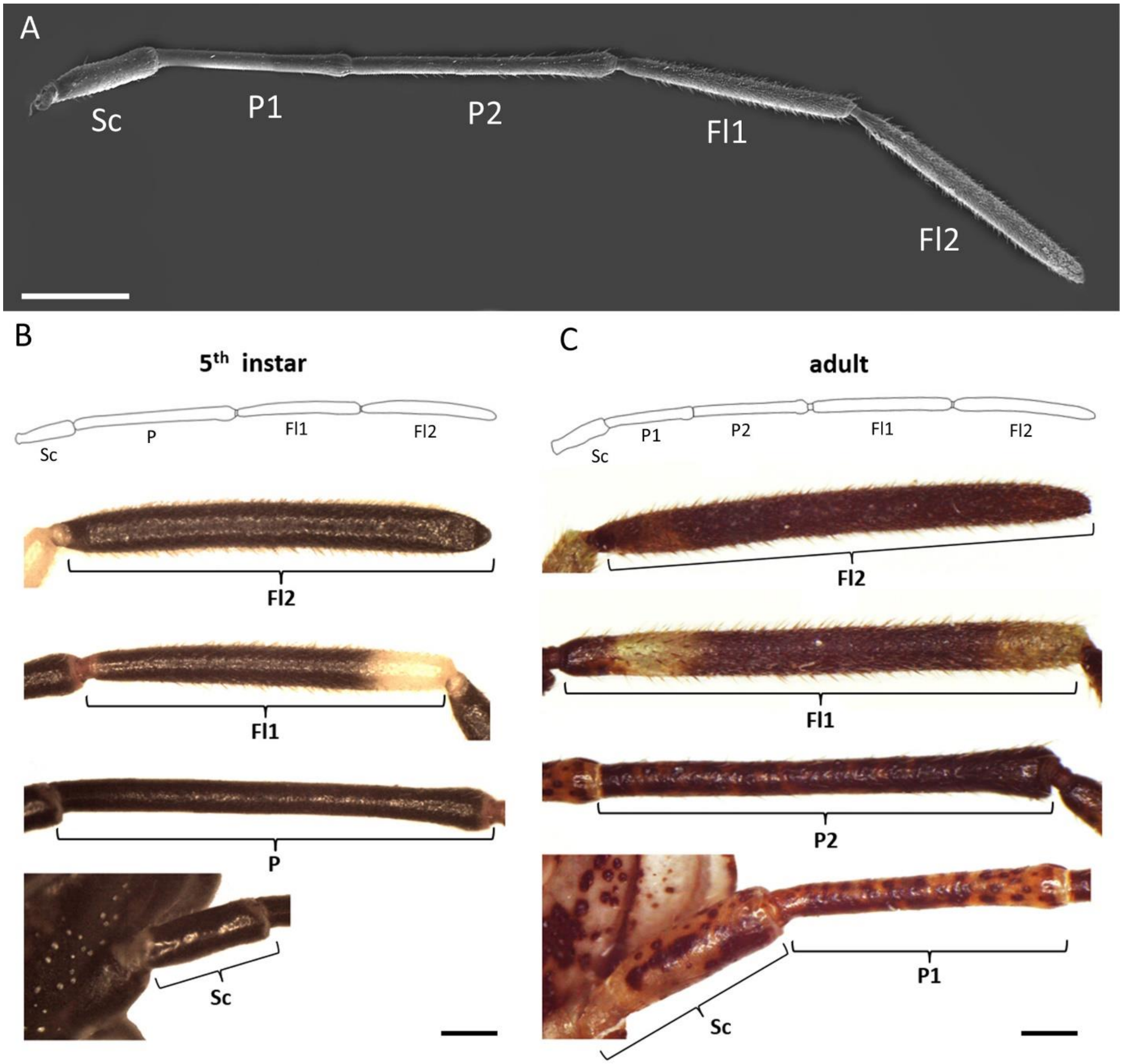

Figure 2.1. *H. halys* antennae. (A) adult female (SEM). (B) 5th instar nymph. (C) adult female. Sc, scape. P, pedicel of nymph. P1 and P2, pedicels of adult. Fl1 and Fl2, flagella. Scale bars = 1 mm.

The antenna of the 5th instar nymph consists of four segments, with only one pedicel segment, and thus is evidently shorter than the antennae of adults (Fig. 2.1 B, Table 2.1). The scape and flagellomere 1 of 5th instar nymphs are shorter with respect to those of the adult females ($p < 0.001$ and $p = 0.006$, respectively) and males ($p = 0.001$ and $p = 0.005$, respectively). In both males and females, the length of each segment of the antennae increases gradually from the scape to the first flagellomere (Table 2.1).

Table 2.1. Mean length ± SE (in mm) of antennal segments of *H. halys* females, males and 5th instar nymphs (n = 5).

Developmental stage	Sc	P1	P2	Fl1	Fl2	Whole antenna
Adult female	0.92 ± 0.03	1.58 ± 0.07	2.10 ± 0.08	2.52 ± 0.10	2.35 ± 0.16	9.47 ± 0.31 a
Adult male	0.95 ± 0.07	1.60 ± 0.11	2.15 ± 0.16	2.53 ± 0.12	2.47 ± 0.15	9.69 ± 0.42 a
5th instar nymph	0.90 ± 0.15	3.00 ± 0.15	-	2.25 ± 0.11	2.30 ± 0.06	8.44 ± 0.25 b

Sc: scape; P1: first pedicel segment; P2: second pedicel segment; Fl1: first flagellomere, Fl2: second flagellomere. Means with different letters in the same column are significantly different.

The total number of sensilla does not differ significantly between male and female antennae, while 5th instar nymphs have a lower number with respect to females ($p = 0.020$) and males ($p = 0.031$), in relation to the lower number of sensilla in Fl1 ($p = 0.004$ and $p = 0.005$, respectively) (Table 2.2 and supplemental material Table S1). The scape and pedicel are sparsely populated while the flagellar segments showed a higher density of sensilla for both adults and nymphs.

In both adults and 5th instar nymphs, the surface of the scape, pedicels and proximal part of the flagellomere Fl1 is characterized by a rhomboid pattern (Fig. 2.2 A), while the surface of the distal part of the flagellomere Fl1 and the flagellomere Fl2 is smooth. Small rounded pores (≈ 0.5 μm in diameter) are scattered on the rhomboid surface of the antennomers (Fig. 2.2 B-C). In addition, short filaments (1-2 μm in length, ≈ 0.5 μm in diameter) protrude from some scattered small pores (≈ 0.5 μm in diameter) present in the scapes, pedicels, and flagellomeres Fl1 of adults and 5th instar nymphs (Fig. 2.2 E). In addition to these short filaments, longer and flexible filaments (10-25 μm) are also observed in nymphs; with highest numbers on the pedicel (200-400) (Fig. 2.2 D, F), while they are less abundant on the scape (40-100) and flagellomere Fl1 (10-30). However, no filaments are recorded on flagellomere Fl2. The structure and function of the pores and filaments are still unknown.

Sensilla types

The identification of sensilla and the terminology used in the present study were based on the classifications made on different species of Hemiptera (Brézot et al., 1997; Romani and Rossi Stacconi, 2009; Silva et al., 2010; Gonzaga-Segura et al., 2013; Missbach et al., 2014; Olson et al., 2014; Zhang et al., 2014a; Kim et al., 2016).

Based on their shape, size, presence or absence of pores, grooved or smooth surface, and presence or absence of socket at the base of the sensilla, four main types of sensilla (subdivided in morphologically different types) were recorded on the antennae of *H. halys* adults and 5th instar nymphs. These include sensilla basiconica (SB) with five types (A, B, C, D and E), sensilla chaetica (SCh) with two types (A and B) and one type of sensilla coeloconica (SCo) and of sensilla trichoidea (ST). The distribution of the different sensilla types on each antennal segment is shown in Table 2.2 (and supplemental material Table S1).

Sensilla basiconica type A (SB-A)

SB-A arise from a flexible socket and are characterized by a long straight shaft, with a bulb-like structure at the tip (Fig. 2.3 A-B). Their length ranges from 7 to 35 μm (1.2-3.4 μm in basal diameter). When observed under high magnification, longitudinal grooves run along its cuticular surface and converge toward an apical area where numerous pores are likely to be located (Fig. 2.3 B).

Cross-sections at the base of the sensillum (just under the socket) show four dendrites (with neuro-tubules inside), (Fig. 2.3 C) that extend in the hair lumen. These sensilla have a grooved, single non-perforated thick wall (0.5-0.8 μm) and are inserted in the antennal wall through a socket, where a fifth sensory neuron becomes evident, ending in a tubular body (Fig. 2.3 C-D). SB-A are exclusive to 5th instar nymphs and are present abundantly on the pedicel and absent in the Fl2 (Table 2.2, Table S1).

Sensilla basiconica type B (SB-B)

SB-B are 13-30 μm long (1.4-3.5 μm in basal diameter) with a straight shaft and a rounded tip and are inserted in a flexible socket (Fig. 2.3 E-F). They are characterized by a smooth surface with only few longitudinal grooves on the apical part (Fig. 2.3 E-F). Apart from the second flagellomere, these sensilla are present on all the segments of the antennae in both adults and 5th instar nymphs, and their highest concentration is on the pedicel and lowest on Fl1 (Table 2.2, Table S1).

Sensilla basiconica type C (SB-C)

SB-C are short (9-12 μm in length; 1.7-2.1 μm in basal diameter) and straight sensilla ending with a blunt tip terminating with a molting pore (Fig. 2.4 A-B). Such pores were reported by Berg and Schmidt (1997) in their comparative studies on the basiconic sensilla of *Lepisma saccharina* L. and *Machilis* sp., whereby scars are left on the cuticular apparatus of the sensillum during the molting process. These sensilla are the second most numerous sensilla basiconica after SB-E (Table 2.2, Table S1), and they are shorter and thicker than SB-B. Evident longitudinal grooves are present on the central and distal parts of the sensilla, while the proximal part is smooth (Fig. 2.4 A, C). Many fine pores penetrate the bottom of the grooves.

The pores expand to form tubules, and the latter open inwards to the lumen of the sensillum. Cross-sections of SB-C show a double cuticular wall (outer and inner) in this region, with three dendrites housed in a narrow lumen (Fig. 2.4 C-D). SB-C are inserted in inflexible socket and are only present on the flagellomeres (predominantly on Fl2) of adults and 5th instar nymphs.

Sensilla basiconica type D (SB-D)

SB-D are 15-20 μm long, with a constant diameter of 1.5-2.0 μm, except for the distal part that tapers to terminate in a pore (Fig. 2.4 E-F). The base of the sensillum is smooth and bent towards the tip of the antennae, and the rest of the cuticular shaft is ribbed with longitudinal grooves. These sensilla are set into a tight inflexible socket, most of them are standing almost parallel to the antennal surface and they sometimes occur in groups of 4-7 (Fig. 2.4 G). SB-D are found exclusively on the flagellomeres of adults and 5th instar nymphs; predominantly on the Fl2 (Table 2.2, Table S1).

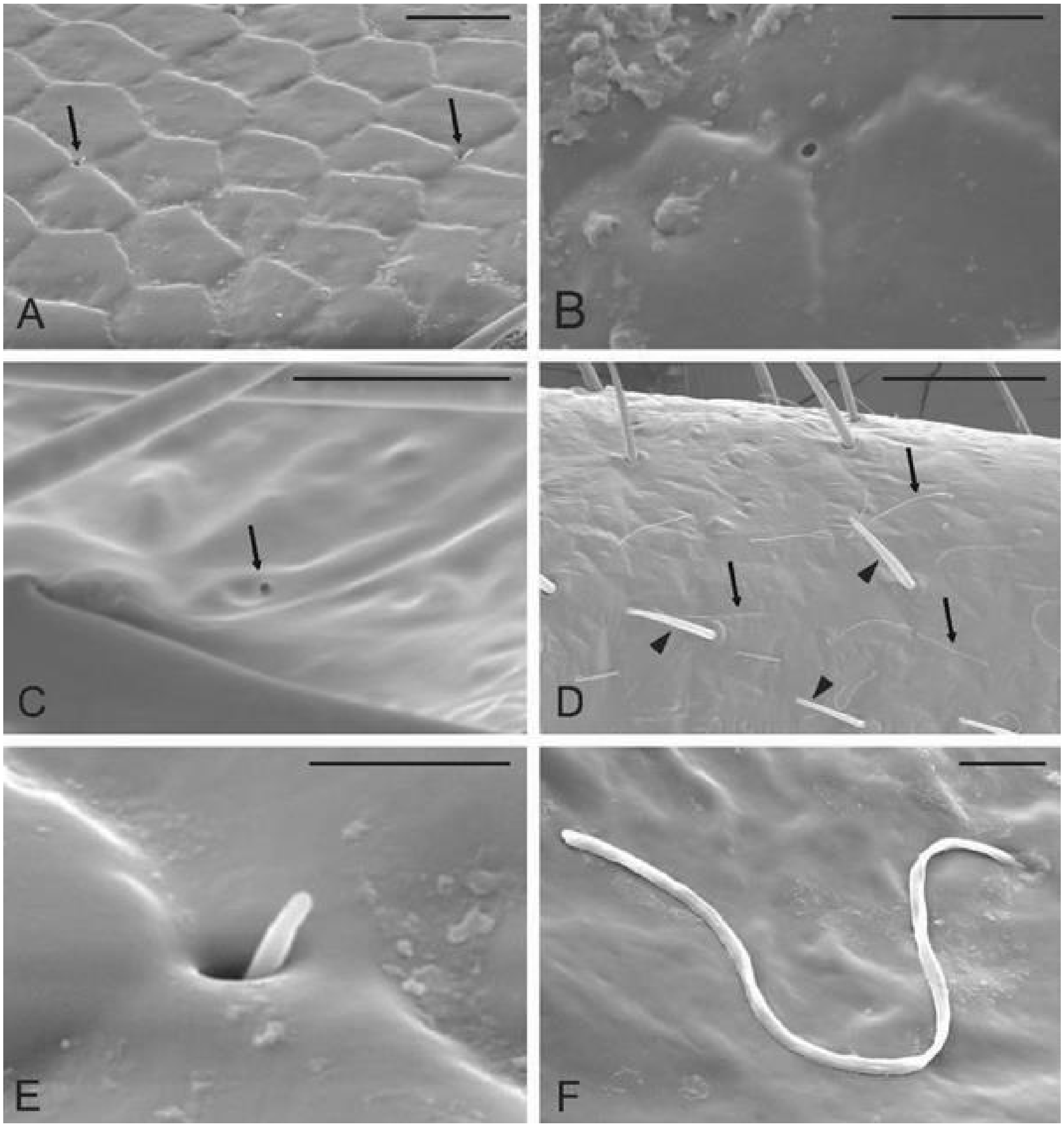

Figure 2.2. *H. halys* antennae. (A) rhomboid patterns on the surface of the scape of a female with short filament protruding from pores (arrows). (B) a pore on the surface of the scape of a female. (C) a pore (arrow) on the surface of the flagellum 1 (Fl1) of a female. (D) filaments (arrows) protruding from pores among sensilla SB-A (arrowheads) on the Fl1 of a 5th instar nymph. (E) short filament protruding from a pore of the scape of a female (enlargement of A). (F) long flexible filament present on the pedicel of 5th instar nymph. A-F (SEM). Scale bars: A = 10 μm; B, C = 5 μm; D = 50 μm; E, F = 2 μm.

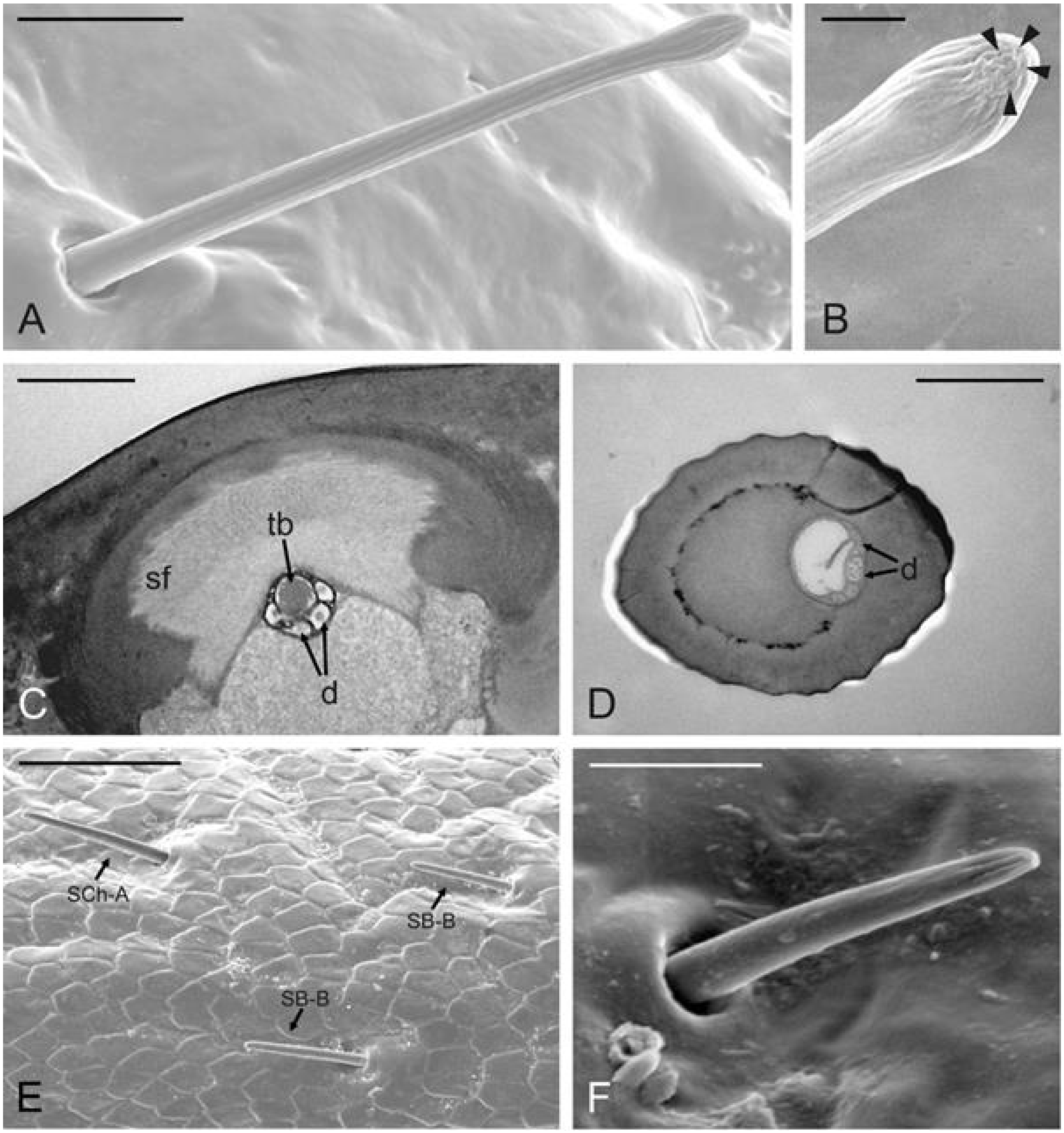

Figure 2.3. *H. halys* sensilla basiconica types A and B. (A-D) basiconic sensilla type A (SB-A). (A) sensillum, with a club shaped tip, on the pedicel of a 5th instar nymph. (B) close-up view of a SB-A showing longitudinal grooves along the shaft, depressions and numerous pores at the tip (black arrowheads). (C) cross section at the basal level part of SB-A, with the suspension fibers (sf), dendrites (d) and a tubular body (tb). (D) cross section of SB-A cuticular shaft showing four dendrites (d). (E-F) basiconic sensilla type B (SB-B). (E) two sensilla on the pedicel of a male (F) sensillum with longitudinal grooves on its apical part inserted in a well-defined socket. SCh-A, sensillum chaeticum. A, B, E, F (SEM); C, D (TEM). Scale bars: A= 10 μm; B, C = 2 μm; D = 1 μm; E = 40 μm, F = 5 μm.

Sensilla basiconica type E (SB-E)

SB-E are straight sensilla but curved near the apical region and composed of a shaft that tapers slightly from the base to a rounded tip (Fig. 2.4 H-I). Their surface appears to be smooth, lacking pores and longitudinal grooves. They are 30-40 μm in length (3.3-4.5 μm in basal diameter) and are inserted into inflexible sockets. The base of the sensilla is surrounded by 7-10 pores; the latter might either be the openings of exocrine antennal glands or molting pores (Fig. 2.4 I). SB-E outnumber the other types of sensilla basiconica present on the antennae.

None of them are recorded on the scape, very few on the pedicels, while the majority are present on the flagellomeres of adults and 5th instar nymphs (Table 2.2, Table S1). The 5th instar nymphs have a lower number of SB-E in the Fl1 with respect to females (p = 0.003) and males (p = 0.002).

Table 2.2. Abundance and distribution of different sensilla on the antennae of *H. halys* males, females and 5th instar nymphs (n = 3).

Sensilla	Developmental stage	Antennal segment				
		Sc	P1	P2	Fl1	Fl2
SB-A	Adult female	0	0	0	0	0
	Adult male	0	0	0	0	0
	5th instar nymph	3.7 (2.3)	36.3 (8.3)	-	17.3 (7.6)	0
SB-B	Adult female	29.7 (19.9)	45.3 (5.0)	33.0 (6.1)	10.0 (9.5)	0
	Adult male	16.7 (4.7)	41.3 (11.8)	34.3 (14.3)	5.3 (7.6)	0
	5th instar nymph	22.7 (13.7)	63.7 (16.3)	-	3.3 (2.9)	0
SB-C	Adult female	0	0	0	71.0 (6.2)	113.3 (9.9)
	Adult male	0	0	0	60.3 (11.0)	112.7 (23.5)
	5th instar nymph	0	0.3 (0.6)	-	41.0 (9.2)	85.0 (20.4)
SB-D	Adult female	0	0	1.0 (1.0)	13.3 (6.8)	46.7 (21.2)
	Adult male	0	0	0	10.7 (11.7)	48.0 (30.6)
	5th instar nymph	0.3 (0.6)	1.0 (1.0)	-	14.0 (8.5)	45.3 (10.7)
SB-E	Adult female	0	0	5.0 (5.0)	212.0 (30.06)	295.3 (37.0)
	Adult male	0	0.3 (0.6)	2.3 (4.0)	218.3 (27.8)	275.7 (29.1)
	5th instar nymph	0	0.3 (0.6)	-	92.3 (13.6)	200.7 (9.5)
SCo	Adult female	2.0 (1.7)	4.3 (3.5)	7.7 (4.9)	3.3 (3.1)	1.3 (1.5)
	Adult male	4.3 (3.2)	4.0 (4.4)	9.0 (5.2)	0.7 (1.2)	0
	5th instar nymph	1.3 (1.5)	2.0 (2.0)	-	7.3 (6.7)	3.7 (2.1)
ST	Adult female	0	0	0	542.0 (29.5)	793.7 (85.9)
	Adult male	0	0	0	528.0 (81.0)	754.0 (136.6)
	5th instar nymph	0	0.3 (0.6)	-	290.7 (44.3)	538.0 (87.0)
SCh-A	Adult female	12.3 (8.4)	5.0 (4.4)	42.9 (16.5)	140.3 (35.9)	112.7 (44.2)
	Adult male	13.0 (2.0)	6.0 (3.6)	38.7 (11.0)	151.0 (12.5)	146.0 (42.2)
	5th instar nymph	2.0 (2.0)	3.3 (4.2)	-	107.3 (13.7)	144.7 (18.0)
SCh-B	Adult female	4.0 (3.5)	5.3 (1.5)	15.0 (2.6)	7.6 (6.4)	31.0 (19.7)
	Adult male	4.3 (2.1)	8.3 (0.6)	18.3 (4.9)	12.0 (14.9)	2.3 (4.0)
	5th instar nymph	1.7 (1.5)	17.0 (7.2)	-	7.3 (7.5)	12.7 (14.2)

Values represent mean numbers and their respective standard deviations (in parentheses) of different types of sensilla on each antennomere. SB-A: sensilla basiconica type A; SB-B: sensilla basiconica type B; SB-C: sensilla basiconica type C; SB-D: sensilla basiconica type D; SB-E: sensilla basiconica type E; SCo: sensilla coeloconica; ST: sensilla trichoidea; SCh-A: sensilla chaetica type A; SCh-B: sensilla chaetica type B; Sc: scape; P1: first pedicel segment; P2: second pedicel segment; Fl1: first flagellomere, Fl2: second flagellomere.

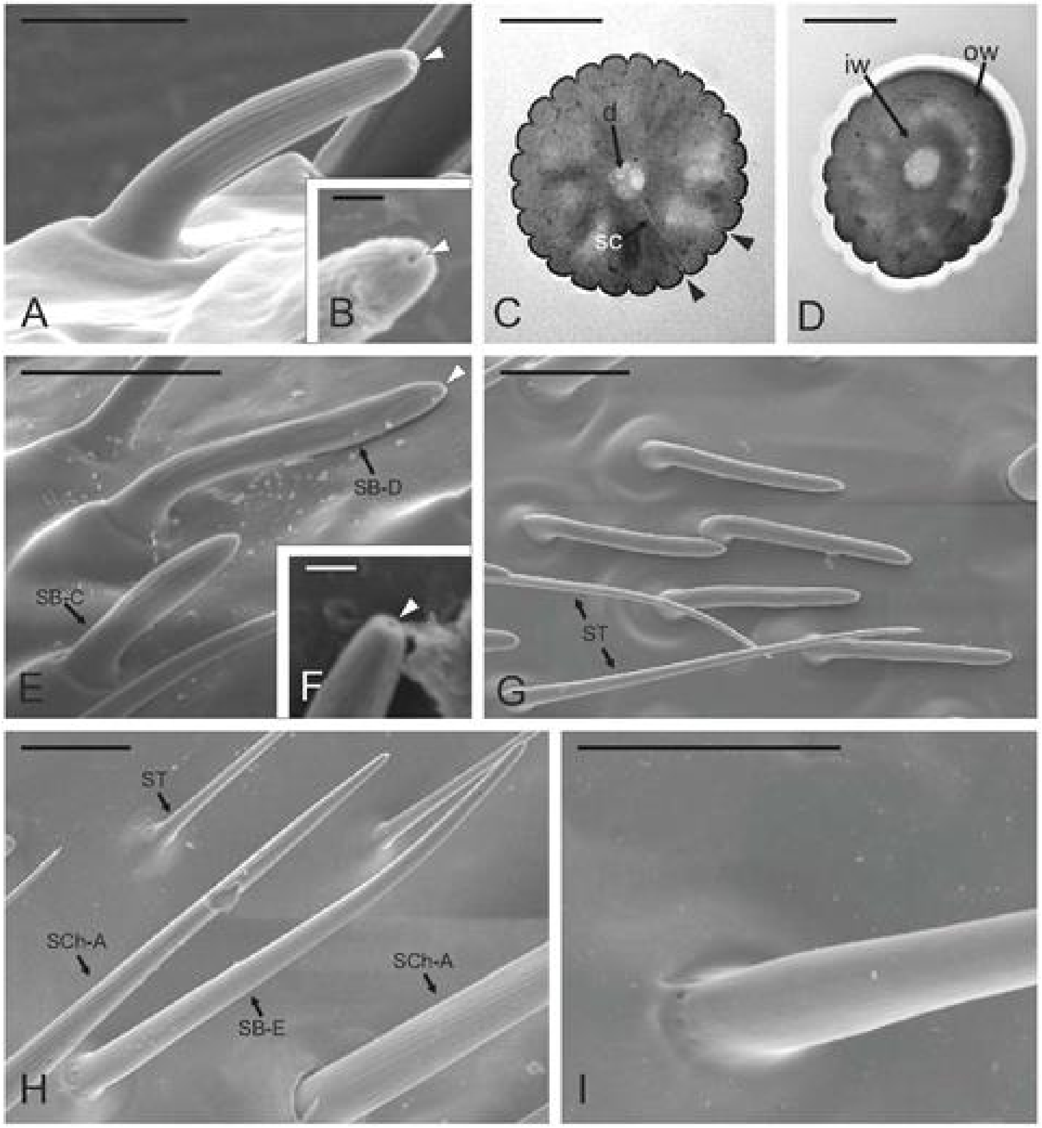

Figure 2.4. *H. halys* sensilla basiconica types C, D and E. (A-D) double-walled sensillum basiconica type C (SB-C). (A) sensillum with longitudinal grooves and an apical molting pore at the tip (white arrowhead). (B) tip of the sensillum with an apical molting pore (white arrowhead). (C-D) cross sections of the same sensillum SB-C taken at two different levels. (C) middle region of the shaft, with dendrites (d) located within the small lumen, and a few spoke channels (sc) connecting the external pores (black arrowheads) with the internal lumen. (D) more basal section, showing the typical double-walled organization with the outer wall (ow) and the inner wall (iw). At this level, part of the outer wall becomes smooth since longitudinal grooves are lacking at the basal region. (E-G) sensilla basiconica type D (SB-D). (E) single elongated sensillum with a pore at the tip (white arrowhead). (F) tip of the sensillum with a pore (white arrowhead). (G) cluster of five SB-D located on the flagellum 2 (Fl2) of a female. (H-I) sensilla basiconica type E (SB-E). (H) sensillum with smooth surface (I) base of the sensillum with pores. ST, sensilla trichoidea. SCh-A, sensilla chaetica type A. A-B, E-I (SEM); C, D (TEM). Scale bars: A = 5 μm; B-D, F = 1 μm; E, G-I = 10 μm.

Sensilla coeloconica (SCo)

SCo consist of short straight pegs with blunt tip protruding from a pit, however not projecting out of it (Fig. 2.5 A). They are inserted in a cuticular aperture having a diameter of 1.5-2.5 μm. SCo are the shortest and least abundant sensilla type recorded on the antennae of *H. halys* (Table 2.2, Table S1). A longitudinal section through the sensillum shows the absence of pores on the peg that is surrounded by finger-like projections and is concealed in the cavity of a cuticle inside a tight socket (Fig. 2.5 B). They are scattered on the whole antennae of both adults and 5th instar nymphs.

Sensilla trichoidea (ST)

ST are curved slender hair-like structures (1.5-2.0 μm in basal diameter), with a sharp tip and are 35-40 μm long (Fig. 2.5 C). Oblique sections through the inflexible socket reveal the presence of a single porous thick wall, whereby each pore opens through a tubule to reach the hair lumen (Fig. 2.5 D). The latter houses two dendrites (Fig. 2.5 D-E). ST are present solely on the flagellomeres (predominantly on the Fl2) and are the most abundant sensory structures on the antennae of *H. halys* (Table 2.2, Table S1).

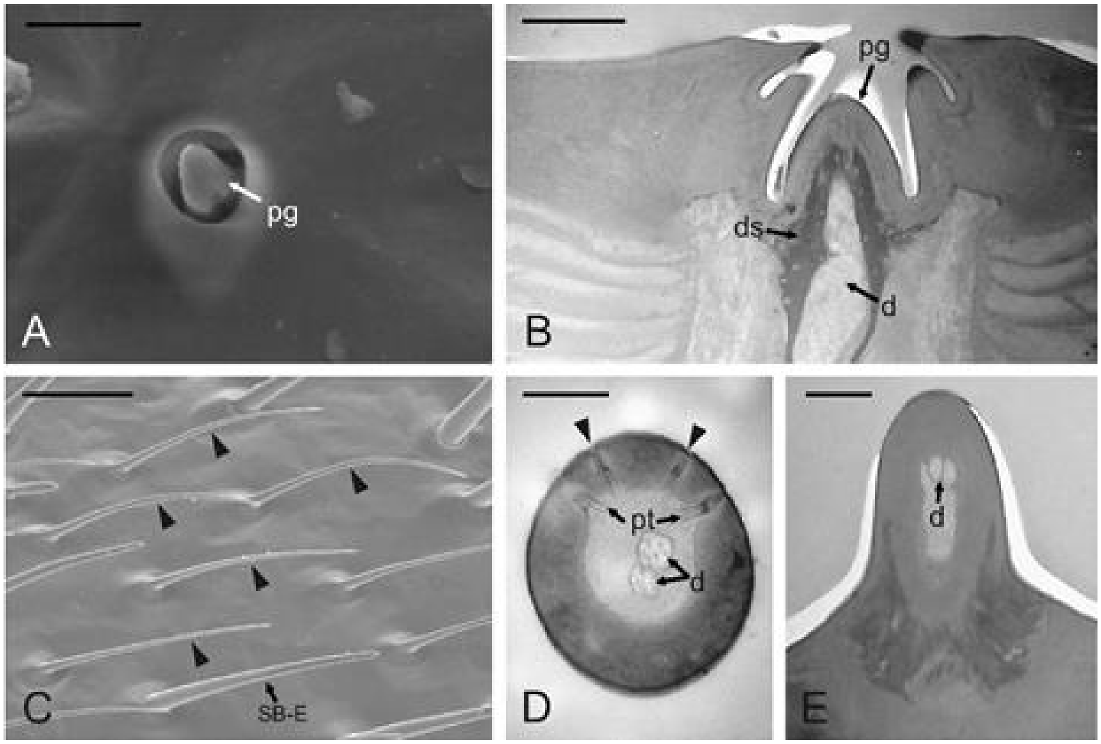

Figure 2.5. *H. halys* sensilla coeloconica and trichoidea (A-B) sensillum coeloconica (SCo). (A) the peg (pg) inserted into a pit. (B) longitudinal section of SCo near the base showing the peg (pg) located just below the cuticular opening. A thick dendrite sheath (ds) enveloping the distal part of the dendrites (d) is visible. (C-E) Sensilla trichoidea (ST). (C) ST (black arrowheads) on the Fl1 of a female. (D) a cross -section of the middle portion of the cuticular shaft with pores (black arrowheads), pore tubules (pt) and dendrites (d). (E) Oblique section through the base of the sensillum showing the dendrites (d). SB-E, sensillum basiconicum type E. A, C (SEM); B, D, E (TEM). Scale bars: A, B = 2 μm; C = 20 μm; D = 0.5 μm; E = 1 μm.

Sensilla chaetica type A (SCh-A)

SCh-A are characterized by a blunt conical apex and a grooved wall (Fig. 2.6 A-B). They are produced from a flexible socket at the base, and their length varies greatly between 30 μm and 120 μm (3.8-7.5 μm in basal diameter). They are the longest sensilla recorded on *H. halys* antennae, and their diameter decreases steadily from the base to the apex. In cross-section, they show a thin cuticular wall perforated by numerous pores irregularly distributed along its length. Each pore opens in-ward forming pore tubules that extend into the hair lumen, where around 50 dendritic branches are housed (Fig. 2.6 C-D). SCh-A are present on all antennomeres with the highest density on the flagellomeres in adults, but in the 5th instar nymphs, Fl2 has more SCh-A than Fl1 (Table 2.2, Table S1).

Sensilla chaetica type B (SCh-B)

These sensilla are 25-50 μm in length and 2.5-4.0 μm in diameter at the base tapering to 1.5-2.0 μm near the tip. They are characterized by a smooth surface, protruding between 50° and 70° from the antennae, and there is no evidence of the existence of pores neither grooves (Fig. 2.6 E). SCh-B are found on all the antennal segments; however, they are less abundant than SCh-A (Table 2.2, Table S1).

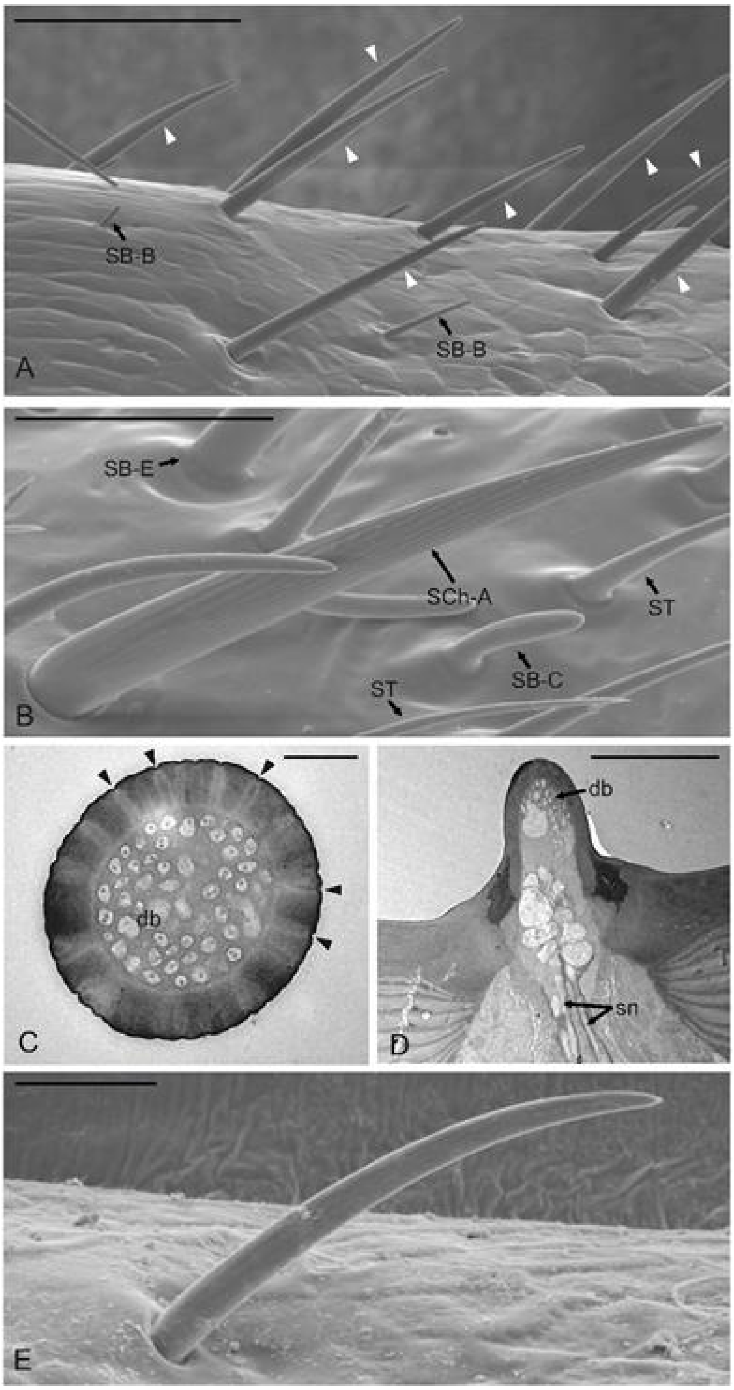

Figure 2.6. *H. halys* sensilla chaetica types A and B. (A-D) sensillum chaeticum (SCh-A). (A) SCh-A (white arrowheads) on the Fl1 of a female (B) sensillum with longitudinal grooves. (C) cross-section of the cuticular shaft with numerous pores (black arrowheads), and the sensillum lumen filled with dendritic branches (db). (D) longitudinal cross section through the base of the sensillum showing a thin cuticular wall where sensory neurons (sn) start to divide into sever-al dendritic branches (db). (E) sensillum chaeticum type B (SCh-B) with smooth surface. SB-B, basiconic sensilla type B. A, B, E (SEM); C, D (TEM). Scale bars: A = 50 μm; B = 20 μm; C = 0.5 μm; D = 5 μm; E = 10 μm.

Discussion

The antenna of *H. halys* adults is filiform and based on the nomenclature of Schuh and Slater (2007), it consists of five segments; scape, pedicel 1, pedicel 2, flagellum 1 and flagellum 2, whereas fifth instar nymphs have a single pedicel segment, and consequently a shorter antenna. The same authors highlighted that additional sclerites may occur during postembryonic development in many pentatomids causing the subdivision of the pedicel, which can explain the absence of pedicel 2 in nymphal stages. All the pentatomids studied so far were reported to have 5-segmented antennae like *H. halys*. These include both predatory: *Perillus bioculatus* (F.), *Eocanthecona furcellata* (Wolff), *Arma chinensis* (Fallou) and *Podisus maculiventris* (Say), and phytophagous species: *Dolycoris indicus* Stål, *Plautia crossota* (Dallas), *Nezara viridula* (L.), *Cyclopelta siccifolia* (Westwood), *Chrysocoris purpurea* (Westwood), *Euschistus heros* (F.), *Piezodorus guildinii* (Westwood), *Edessa meditabunda* (F.) (Rani and Madhavendra, 1995; 2005; Sinitsina and Krutov, 1996; Brézot et al., 1997; Silva et al., 2010; Ahmad et al., 2016; Barsagade and Gathalkar, 2016).

Both male and female of *H. halys* show no differences in antennal size, neither in types nor in numbers of antennal sensilla. Moreover, we report a lower density of sensilla on the antenna of *H. halys* nymphs when compared to adults. This difference in the number of sensilla is not a novel observation, for it was reported in other hemipterans (Rani and Madhavendra, 1995; Chinta et al*.,* 1997; Brézot et al., 1997). In her seminal study, Catalá (1997) concluded that insects have a general tendency to increase the number of receptors in the adult stage, especially chemoreceptors such as basiconic and trichoid sensilla. According to Chapman (1982), this phenomenon may be related to a differing chemical communication between adults and nymphs.

Given that in *H. halys* both life stages occupy the same habitat and feed on the same hosts, possessing more receptors might reflect the need for additional sensorial requirements in adults essential for the sexual maturity of adults and/or their dispersive capacity. For instance, the mating behaviour of *H. halys*, described by Mazzoni et al. (2017) and in *Halyomorpha mista* Uhler (synonym of *H. halys*) by Kawada and Kitamura (1983), involves antennation by males of the posterior end of sexually-responsive females; the step preceding copula formation. Other pentatomids exhibiting this behaviour include *E. meditabunda* (Silva et al., 2012) and *Murgantia histrionica* (Hahn) (Lanigan and Barrows, 1977). Likewise, many authors investigated the important role of hemipteran antennae in localizing suitable oviposition sites, such as the case in *Neomegalotomus parvus* (Westwood) (Alydidae) (Ventura and Panizzi, 2000), *Rhinocoris bicolor* (F.) and *Rhinocoris tropicus* (Herrich-Schaeffer) (Reduviidae) (Parker, 1969) and *Thyanta pallidovirens* (Stål) (Pentatomidae) (Wang and Miller, 1997). In addition, *H. halys* adults show greater capacity for active dispersal and colonization of new habitats when compared to the wingless nymphal stages (Lee et al., 2014; Lee and Leskey, 2015).

There are five main types of sensilla on the antennae of *H. halys* adults and fifth instar nymphs: basiconica, (types A, B, C, D and E), coeloconica, trichoidea and chaetica (types A and B). The density of sensilla increased from proximal to distal ends, and the dominant type was by far sensilla trichoidea, located especially on the flagellomeres.

Chemo- and mechanosensory sensilla

Olfaction

Olfaction is one of the most fundamental insect sensory modalities, mediating intra- and interspecific communication; including but not limited to foraging, host location, mate finding and aggregation. In *H. halys*, sensilla basiconica types C and D (SB-C and SB-D), trichoidea (ST) and chaetica type A (SCh-A) are presumed to possess olfactory roles, based on their morphological characteristics.

Both basiconic sensilla (types C and D) are inserted into inflexible sockets and have a single pore at the tip with longitudinal grooves running along their surface; features generally shared among olfactory sensilla. These sensilla are similar in appearance to other basiconic sensilla described in *D*. *indicus*, *P*. *crossota*, *E*. *furcellata* and *P*. *bioculatus* (termed “SBsh” by Ahmad et al., 2016), in *N*. *viridula* (termed “type 4 sensillum” by Brézot et al., 1997) and in *C. siccifolia* and *C. purpurea* (termed “s.b. II” by Rani and Madhavendra, 2005).

ST in *H. halys* are found only in the flagellomeres and are characterized by the presence of pores on the cuticular wall, with two sensory neurons innervating each sensillum; rendering their olfactory role plausible. Further validation of our assumption of ST sensilla as olfactory can be deduced from the study of Toyama et al. (2006), who observed that the ablation of the terminal flagellomere of *H. halys* reduced but did not completely inhibit aggregate formation. However, both antennectomy and the removal of the two flagellomeres totally inhibited this behaviour (Toyama et al., 2006), indicating that the sensory structures present on these segments are necessary for perceiving the aggregation pheromone. The authors referred to these structures as “hair-shaped sensilla” and suggested the interference of olfactory and/or tactile cues in their performance. Moreover, the morphology and presumable role of ST are in accordance with similar findings in *N*. *viridula* (termed “type 1 sensilla” by Brézot et al., 1997), in *E*. *heros*, *E*. *meditabunda* and *P*. *guildinii* (termed “ST2” by Silva et al., 2010) and in *A*. *chinensis* (termed “ST1” by Zhang et al., 2014a). Whereas, Ahmad et al. (2016) assumed a mechanosensory function of sensilla trichodea found on the flagellum of *D*. *indicus*, *P*. *crossota*, *P*. *bioculatus* and *E*. *furcellata* due to the presence of flexible sockets at the base of their aporous walls.

Electrophysiological studies by Zhang et al. (2014b) have shown that *H. halys* adults and nymphs can detect various essential oils thanks to the presence of olfactory receptors on their antennae. Furthermore, Zhong et al. (2017) discovered that (E)-2-decenal functions as an alarm pheromone for *H. halys*, repelling both males and females at higher doses. Also, green leaf volatiles (GLVs) were shown to be key foraging cues for *H. halys* adults (Morrison et al., 2018). Such results provide more evidence to the major role of the olfactory sensilla mentioned above in the chemical signaling, perception and behaviour of this pest. This knowledge can be exploited for establishing a more efficient management strategy based on the employment of improved lures, repellents and attract-and-kill techniques.

Mechano-olfaction

SB-B in *H. halys* share with the above discussed olfactory sensilla the presence of longitudinal grooves, however the fact that they are inserted in flexible sockets suggests a dual function: a mechano-olfactory role. This function might explain the observations of Toyama et al. (2006), who noticed that bugs encountering aggregations frequently contacted the settled bugs with their antennae prior to joining them. Justus and Mitchell (1996) suggested that the movement of the antennae might create a wind vortex around it, facilitating the contact of odor molecules with the sensilla. Nevertheless, the role of SB-B cannot be stated with definite certainty, given first their position on the pedicel, and second the lack of respective TEM micrographs. Additional morphological and electrophysiological investigation is needed to clarify their exact role.

Mechano-gustation

The presence of a tubular body and dendrites extending along the shaft in the cross sections of SB-A and its insertion in flexible sockets suggest a possible double gustatory-mechanosensory function. A similar type of sensillum was previously recorded on the flagellum of adults and fifth instar nymphs of two other pentatomid species: *E*. *heros* and *E*. *meditabunda* (Silva et al., 2010). Such sensilla possessed longitudinal grooves and two apical pores, which are indicative of chemoreceptive sensilla. The SB-A we observed in *H*. *halys* are probably responsible for providing gustatory cues for fifth instar nymphs from their surrounding food sources. SEM micrographs suggest that pores are present at the tip of the sensillum and provide easy passage for sub-stances from the exterior to the dendrites.

Thermo-hygrosensory sensilla

According to Altner and Prillinger (1980), hygroreceptors occur together with thermoreceptors in pegs that show no pore systems. The absence of pores in the sensillum wall of SCo precludes any olfactory function; these sensilla are thought to be involved in thermo- and hygro-reception, detecting wetness, dryness and changes in air temperature. Sensilla coeloconica were reported in other pentatomids: *N. viridula* (Brézot et al., 1997), *C. purpurea* (Rani and Madhavendra, 2005) and *P. guildinii* (Silva et al., 2010), where they were assigned to various presumed roles ranging from hygro-, thermo- to chemosensory reception.

The function of sensilla basiconica type E (SB-E) remains vague. Ventura and Panizzi (2005) described bristle sensilla with a curved extremity, bearing pores on the base and without flexible sockets on the last flagellomere of *N. parvus*. Similar sensilla were also observed on the last segment of the antennae of the milkweed bug *Lygaeus kalmii* Stål (Slifer and Sekhon, 1963). An olfactory function was assigned in both species. However, in *H. halys* the lack of pores on SB-E limits such an olfactory assumption; their function can be elucidated through further investigations.

Knowing the structure and function of the sensory system is necessary to characterize how the communication modalities interact in *H. halys* behaviour. The results of this study enhance our understanding of the sensory reception processes involved in the host plant selection and other behaviors of *H. halys*, which will indirectly help in developing more efficient tools to control this economically important pest.

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Supplementary Material

Table S1. Number of each sensilla type in the antennae of *H. halys* females, males and instars.

Animal	Flagellar segment	SB-A	SB-B	SB-C	SB-D	SB-E	SCo	ST	SCh-A	SCh-B
F1	Scape	0	44	0	0	0	1	0	7	6
	Ped. 1	0	50	0	0	0	1	0	8	4
	Ped. 2	0	36	0	1	10	2	0	61	17
	Flag. 1	0	19	66	8	199	6	551	163	3
	Flag. 2	0	0	118	66	296	3	819	159	37
F2	Scape	0	26	0	0	0	1	0	8	6
	Ped. 1	0	46	0	0	0	4	0	0	5
	Ped. 2	0	26	0	2	5	10	0	31	12
	Flag. 1	0	11	78	21	247	4	566	159	5
	Flag. 2	0	0	102	50	332	10	864	108	47
F3	Scape	0	19	0	0	0	4	0	22	0
	Ped. 1	0	40	0	0	0	8	0	7	7
	Ped. 2	0	37	0	0	0	11	0	34	16
	Flag. 1	0	0	69	11	190	0	509	99	15
	Flag. 2	0	0	120	24	258	0	698	71	9
M1	Scape	0	22	0	0	0	3	0	15	2
	Ped. 1	0	55	0	0	0	2	0	7	9
	Ped. 2	0	31	0	0	7	6	0	44	15
	Flag. 1	0	14	61	24	214	2	609	147	1
	Flag. 2	0	0	94	83	285	0	887	177	0
M2	Scape	0	15	0	0	0	2	0	13	6
	Ped. 1	0	35	0	0	1	1	0	2	8
	Ped. 2	0	50	0	0	0	6	0	26	24
	Flag. 1	0	2	49	2	248	0	528	165	29
	Flag. 2	0	0	105	35	299	0	761	163	7
M3	Scape	0	13	0	0	0	8	0	11	5
	Ped. 1	0	34	0	0	0	9	0	9	8
	Ped. 2	0	22	0	0	0	15	0	46	16
	Flag. 1	0	0	71	6	193	0	447	141	6
	Flag. 2	0	0	139	26	243	0	614	98	0
5th instar 1	Scape	5	32	0	1	0	3	0	2	2
	Pedicel	43	51	1	1	0	2	1	2	11
	Flag. 1	14	0	31	13	77	15	252	105	16
	Flag. 2	0	0	69	51	210	6	467	144	28
5th instar 2	Scape	1	29	0	0	0	1	0	0	3
	Pedicel	27	82	0	0	0	0	0	0	25
	Flag. 1	26	5	43	6	97	3	281	95	3
	Flag. 2	0	0	78	33	191	2	512	127	0
5th instar 3	Scape	5	7	0	0	0	0	0	4	0
	Pedicel	39	58	0	2	1	4	0	8	15
	Flag. 1	12	5	49	23	103	4	339	122	3
	Flag. 2	0	0	108	52	201	3	635	163	10

Chapter 3

Vibratory interneurons in the ventral nerve cord of *Halyomorpha halys* (Hemiptera: Pentatomidae): morphology and physiology

Ibrahim, A., Anfora, G., Mazzoni, V. and Stritih Peljhan, N., 2020. To be submitted once the project is concluded.

Abstract

The invasive brown marmorated stink bug, *Halyomorpha halys* (Stål), is a notorious agricultural and household pest. It relies on substrate-borne vibrations during courtship for mate finding and recognition. In this study, we tackled the neuronal mechanisms involved in vibratory communication of *H. halys* to increase our understanding of its mating process. The vibration-sensitive interneurons in the thoracic ganglion of adult *H. halys* were physiologically investigated using single-cell recordings and then stained and analyzed morphologically using fluorescent microscopy. Sine-wave pulses of frequencies ranging from 30-5000 Hz and intensities from 0.001-10 m/s^2 were used for stimulation. Four neuron types were described morphologically and physiologically, and two additional neurons were characterized only by their physiology. All morphologically identified neurons are intersegmental, with the axon(s) projecting anteriorly towards the prothoracic ganglion; two at the soma-contralateral side (*CG-AC-1* and *CG-AC-2*), one is a typical representative of DUM cells with a bilaterally symmetrical structure (*CG-AB-1*) and one shows a rare morphology with the soma-ipsilaterally positioned axon (*CG-AI-1*). The first two neurons morphologically and physiologically closely resemble and are thus putatively homologous to their namesakes in the green stink bug *Nezara viridula*. Physiologically, three neurons show a narrow low-frequency tuning with best responses at 50 Hz and weak or no responses above 100 Hz. Three neurons respond in the whole range of tested frequencies, two with the highest sensitivity around 200 Hz and one above 1000 Hz, being clearly shaped by a low-frequency inhibition. At 80 Hz and 160 Hz, the dominant and the first harmonic frequency of the female signal in *H. halys*, sensitivity is the highest for the broadband neurons, with thresholds at 0.018 and 0.032 m/s^2 and wide response dynamic ranges of at least 40-60 dB. The relevance of these findings for detection and processing of sexual signals and developing reliable behavioral manipulation techniques of *H. halys* is discussed.

Keywords: mating behavior, sexual communication, vibrational signal, intracellular recording, interneuron, sensory function

Introduction

Vibrational communication is utilized by a wide range of organisms and is essential for their survival and reproduction. In insects, substrate-borne vibrations remain the least investigated modality of communication, with studies focusing more on the visual, tactile and chemical channels to transfer information. Vibrational signals convey species-specific information that are vital in various behavioral contexts related to mating, defense and alarm (Virant-Doberlet and Čokl, 2004). These signals are perceived by specialized vibrosensory organs located in/on the body and appendages of insects (Čokl et al., 2005a).

Many hemipteran species communicate via substrate vibrations that are detected mainly using scolopidial (chordotonal) sense organs in insects (Lakes-Harlan and Strauß, 2014). In Pentatomidae, mechanisms involved in signal production include tremulation, percussion and dorsoventral abdomen vibrations (Hill, 2008). Extensive research on the sexual behavior and vibrational communication of the green stink bug, *Nezara viridula* L. has been conducted, considering the species a model for the whole family (Čokl and Virant-Doberlet, 2003; Čokl et al., 2005b; Čokl et al., 2007; Žunič et al., 2011; Prešern et al., 2018). Zorović et al. (2008) also described ten morphological types of interneurons involved in vibration detection in *N. viridula*, comprising low and medium frequency units depending on their sensitivity. Additionally, the sensitivity of these interneurons for temporal patterns of vibrations with varying pulse and pause durations was investigated (Zorović, 2011).

Another infamous pentatomid that has been gaining considerable attention lately is the invasive brown marmorated stink bug, *Halyomorpha halys* (Stål). It is an extremely polyphagous pest, feeding on more than 170 plant species in North America and Europe (Leskey and Nielsen, 2018). The mating behavior of *H. halys* involves decoding substrate-borne vibrational signals to find and attract potential mates (Polajnar et al., 2016). Past studies of vibrational communication in *H. halys* have been focusing predominantly on describing the vibrational signals during mating (Polajnar et al., 2016) and the use of vibrational playback for trapping techniques against this serious pest (Mazzoni et al., 2017; Polajnar et al., 2017).

Neuronal processing of vibrations that underlie signal detection in *H. halys* are still poorly understood. Exploring the fundamental characteristics of associated vibrational signals and understanding of which are crucial in their perception can help in developing reliable control methods using vibrations as an environment-friendly alternative to synthetic pesticides. Toward that end, the aim of the study was to investigate the morphology and physiology of vibration-sensitive interneurons of the ventral nerve cord of *H. halys* and to determine the extent to which they resemble the neuronal elements identified so far in *N. viridula*.

Materials and Methods

Study animals and preparation

Female and male *H. halys* were collected from host plants in Trento, Italy. The bugs were kept in plastic cages under controlled conditions at 24 ± 2 °C, 65 % R.H. and 16:8 (L:D). They were fed on green beans and peanuts and provided with water from cotton balls *ad libitum*.

Prior to recording, an animal was fixed with a mixture of beeswax and resin on a U-shaped holder with the dorsal side-up. The wings, scutellum, pronotum, underlying flight muscles and underlying adipose tissues were all removed to expose the central ganglion of the ventral nerve cord. The latter was continuously perfused with Ringer's saline solution (140 mM NaCl, 10 mM KCl, 4 mM $CaCl_2$, 4 mM $NaHCO_3$ and 6 mM NaH_2PO_4) to maintain its physiological activity during the recordings. The holder was then placed above a minishaker (Bruel & Kjær type 4810) which served in delivering the vibratory stimuli directly to the legs of the animal through a steel stud. The six legs of the animals were fixed using beeswax to the stud in such a way as to mimic the natural position of the insect on a stem (femur/tibia and tibia/stud angles approx. 60° and 85°, respectively). The central ganglion was then supported by a hooked steel spoon from below. A sliver wire inserted into the abdomen of the animal served as a reference electrode.

Intracellular recording and staining

Experiments were performed on an anti-vibration table in the Faraday cage (Fig. 3.1), on rearing temperature and humidity. The recordings were conducted in the central ganglion of *H. halys* adults using borosilicate glass capillaries with filament (Sutter Instrument, O.D: 1.2 mm, I.D: 0.69 mm and 7.5 cm in length). The latter were pulled using a horizontal microelectrode puller (Sutter Instrument, P-87). The resulting recording electrodes had a resistance of 90-130 MΩ after back-filling the tip with 5% Lucifer Yellow CH (Sigma-Aldrich, in 0.5 M LiCl) and the shaft with lithium chloride (0.5 M LiCl). The recording electrode was fixed on a mechanical micromanipulator (Leitz, Wetzlar, Germany) to allow for the fine penetration of the ganglion. Neuronal activity was amplified 50x using a microprobe system (Duo 773, WPI, Sarasota, USA) and digitally recorded and filtered (Power1401 mk2, Cambridge Electronic Design, Cambridge, UK) for storage and offline analysis with Spike2 software (Cambridge Electronic Design, Cambridge, UK). Following physiological characterization, the Lucifer Yellow dye was injected with 3-5 nA hyperpolarizing current to the neuron for at least 3-4 minutes.

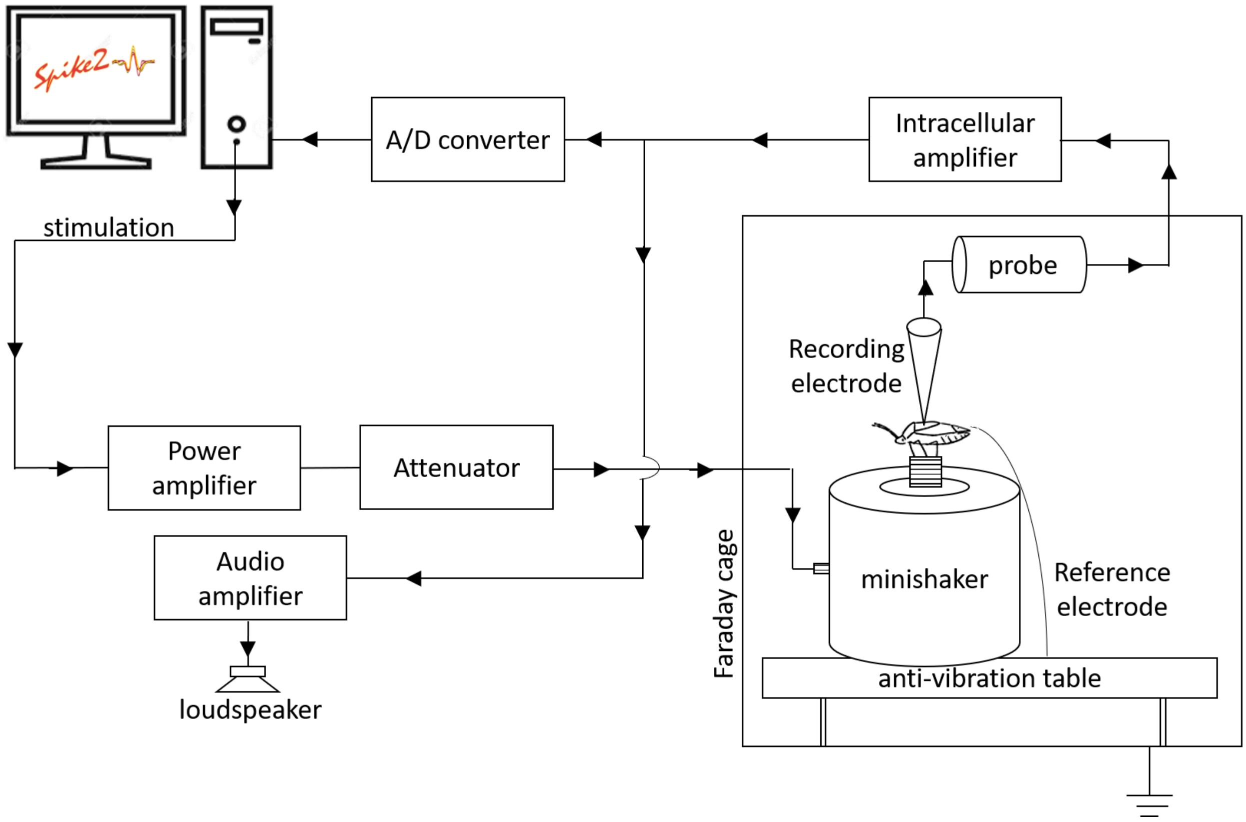

Figure 3.1. Scheme of the experimental setup used in the intercellular recordings in *H. halys*

Vibratory stimulation

All test vibratory stimuli were pure-tone sine-wave pulses of 100 ms duration, with 10 ms rise and fall time. The search sequence was composed of the exchanging 100 Hz and 400 Hz pulses delivered at an intensity of 1 m/s^2. Once a response to a search stimulus was detected, test pulses of frequencies ranging from 50−5000 Hz and intensities from 0.001−1 m/s^2 were fed to the minishaker. Neurons were not tested with frequencies below 50 Hz because of the limitations in the working range of the minishaker. The lowest frequency limit of 30-50 Hz, however, is typical for physiological characterizations of vibration-sensitive elements of insects (Kühne et al., 1984; Zorović et al., 2008; Stritih, 2009) as substrate vibrations of lower frequencies are usually not associated with insect communication signals but are induced in the environment by various sources of abiotic noise (Cocroft and Rodriguez, 2005; McNett et al., 2010).

The frequency and intensity tests were conducted in the following manner: we first tested the frequency preference of interneurons, using an equal-intensity test at 1 m/s^2 and 0.1 m/s^2, with 16-19 test stimuli increasing in frequency from 30-5000 Hz. Then, we did an intensity-response dependence test for 80 Hz and 160 Hz stimuli (the dominant[1] and first harmonic frequencies[2] in communication signals of *H. halys*). In cases where the recording potential was still stable, we tested for intensity dependence (in a search for threshold), at further test frequencies. Each test sequence was repeated at least three times.

The stimulation sequences were created using Cool Edit Pro 2 software (Syntrillium, Phoenix, USA; sample rate 48 kHz, 16-bit resolution) and delivered to the minishaker using a sound card (Creative X-Fi Surrond 5.1. Pro, Singapore), via a power amplifier (Startech, A-50). Synthetized vibratory signals were high-amplitude stimuli giving the output between 1 and 10 m/s^2, that were attenuated for further 20 to 40 dB[3] using a custom-made voltage divider. The output intensity at the top of the stud attached to the minishaker was calibrated using a laser-Doppler vibrometer (PDV 100, Polytec, Waldbronn, Germany).

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[1] **Dominant frequency:** the frequency with the largest amplitude in a spectrum

[2] **First harmonic frequency:** the frequency that is a positive integer multiple of the fundamental frequency (the lowest component in a spectrum)

[3] **dB:** decibel, a unit of measurement used to express the ratio of one value of a power or field quantity to another on a logarithmic scale

Morphology

After completion of iontophoretic staining, the central ganglion was excised and fixed with few drops of 4% buffered formaldehyde solution for 1 hour at room temperature, or was left overnight at 4 °C. Dehydration in a series of ethanol (from 50% up to 100%) followed and the tissue was then cleared in methyl salicylate for at least 10 minutes prior to examination with fluorescent microscope (Leiz DMRB/E, Wetzlar, Germany). Neurons were photographed with a digital camera (AxioCam MR with AxioVision microscope software; Carl Zeiss, Oberkochen, Germany) attached to the microscope. Digital micrographs were adjusted for brightness and contrast using Photoshop (Adobe Systems, San Jose, USA) and neuronal morphology was reconstructed and drawn with a graphic tablet (Graphire 2, Wacom, Kazo, Japan) from a stack of digital images taken in different planes. In some cases, a representative whole-mount image was combined from these individual images using ImageJ (Abramoff et al., 2004).

Data evaluation

Spike 2 (CED, Cambridge, UK; version 7.0) software was used for data analysis. In spontaneously active neurons, spikes were counted, and spiking rates calculated for each 130-msec period prior to the stimulus. The response intensity refers to the mean number of spikes fired per stimulus; in case of spontaneously active neurons, this refers to the number of spikes fired above the level of spontaneous activity[4] present in the period of a specific test sequence. The lowest intensity eliciting at least one spike (above the level of spontaneous activity) per

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[4] **Spontaneous activity:** neural activity that is not driven by an external stimulus

stimulus was defined as threshold[5]. The response latency refers to the time between the stimulus onset and the peak of the first spike in response; in case of spontaneous activity it is the first spike fired above the rate of spontaneous spiking.

Terminology

We followed the terminology as used by Zorović et al. (2008) for labelling the neurons in *N. viridula*. Each neuron label is composed of the following parts that refer to:

1) soma location; which is *CG* for the central ganglion

2a) the course of the intersegmental axon; which is either ascending "*A*", descending "*D*", or both ascending and descending axons present (i.e. "T-shaped"; *T*), while the identifier "*L*" labels local neurons, without an intersegmental axon

2b) the position of the axon(s) with respect to the soma; which can be ipsilateral[6] "*I*", contralateral[7] "*C*" or bilateral "*B*", when two axons are present, each in one hemiganglion

2c) the characteristic number

To facilitate interspecific comparison, we labelled the putatively homologous neurons between *H. halys* and *N. viridula* with the same characteristic number. For the vibration-sensitive neurons that lack morphological identification, we used contemporary labels "V1" and "V2".

Results

The data presented here belong to six intracellular recording and/or staining experiments, each obtained from different *H. halys* adults. In four of them, the penetration was maintained

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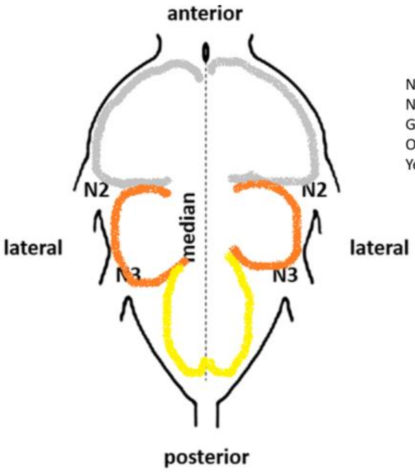
[5] **Threshold:** the intensity that must be exceeded for a response (spikes in neurons) to occur
[6] **Ipsilateral:** denoting the same side of the body in which a particular structure occurs
[7] **Contralateral:** denoting the side of the body opposite to that in which a particular structure occurs

long enough to complete the staining while the other two only provided information about the physiology of the detected neuron. The morphology is described based on the gross shape/appearance of the neuron and position of the axon with respect to the cell body (soma) and dendritic arborizations in the thoracic neuropils (Fig. 3.2).



N2: nerves of middle legs
N3: nerves of hind legs
Grey color indicates the mesothoracic neuropils
Orange color indicates the metathoracic neuropils
Yellow color indicates the abdominal neuropils

Figure 3.2. Scheme of the central ganglion with the different neuropils

CG-AC-1

Morphology

We stained a cluster of (at least) two morphologically homogenous, “L”-shaped neurons, with somata positioned laterally to intermedially in the cortex of the metathoracic neuropil, a central dendritic branch crossing the midline in the metathoracic neuropil, arborizations of “smooth” appearance (i.e. dendritic) in the ipsilateral side of the soma and ascending axons giving off two large collaterals at the soma-contralateral side of the ganglion (Fig. 3.3). Most dendritic branches are present in the metathoracic neuropil, an additional longer branch extends posteriorly[8] into the neuropil of abdominal neuromeres. The two long axonal collaterals bear short dense processes with beaded terminals along their length. One of them descends posteriorly into the abdominal neuropil, the other projects laterally into the metathoracic neuropil of the same hemiganglion. Some short, beaded processes branch off the axon also in the mesothoracic neuropil. Such morphology is also characteristics for the neuron labelled CG-AC-1 in *N. viridula* (Zorović et al., 2008) (Fig. 3.3).

Physiology

Recording from one of the co-clustered neurons showed some initial spontaneous activity and strongly phasic-tonic[9] bursts of spikes in response to vibratory stimuli (Fig. 3.4). The response was sharply tuned to low frequencies (Fig. 3.5), with suprathreshold[10] responses evoked in the range up to 200 Hz when tested with 1 m/s^2 and up to 80 Hz when tested with 0.1 m/s^2 stimuli (Fig. 3.6-A). Initially, still with the presence of some spontaneous activity, the threshold was 0.18 m/s^2 at 80 Hz and 0.32 m/s^2 at 160 Hz and in the course of recording decreased for 5 dB, to 0.32 and to 0.56 m/s^2, respectively (Fig. 3.6-B). With increasing stimulus intensity these responses increased throughout the tested range of 38 dB, reaching the mean of 15.75 and 12.80 spikes/stimulus at 80 and 160 Hz, respectively (Fig. 3.6-B), while their latency decreased to the minimal value of 11.4 and 12.4 ms, respectively (Fig. 3.6-C).

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[8] **Posterior:** nearer to the rear or hind end of the insect

[9] **Phasic-tonic response:** a response characterized by a short-lasting period of increased spiking rate at the beginning of the stimulus (phasic component) followed by a longer-lasting period of a lower spiking rate maintained to the end of the stimulus (tonic component)

[10] **Suprathreshold response:** refers to a stimulus that is large enough in magnitude to produce an action potential in excitable cells

CG-AC-2

Morphology

We recorded one example of the neuron with the soma located laterally in the cortex between the meso- and metathoracic neuromeres, an “U”-shaped dendritic segment supporting bilateral branches, which crosses the midline in the metathoracic neuropil and a soma-contralateral ascending axon (Fig. 3.7). Soma-ipsilaterally, the dendritic branches extend posteriorly into the metathoracic neuropil and anteriorly[11] into the mesothoracic neuropil, and soma-contralaterally one main dendritic branch extends posteriorly into the metathoracic neuropil. The ascending axon gives off some short collaterals, medially and laterally in the mesothoracic neuropil. Such morphology is typical for the neurone labelled CG-AC-2 in *N. viridula* (Fig. 3.7).

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[11] **Anterior:** nearer to the head end of the insect

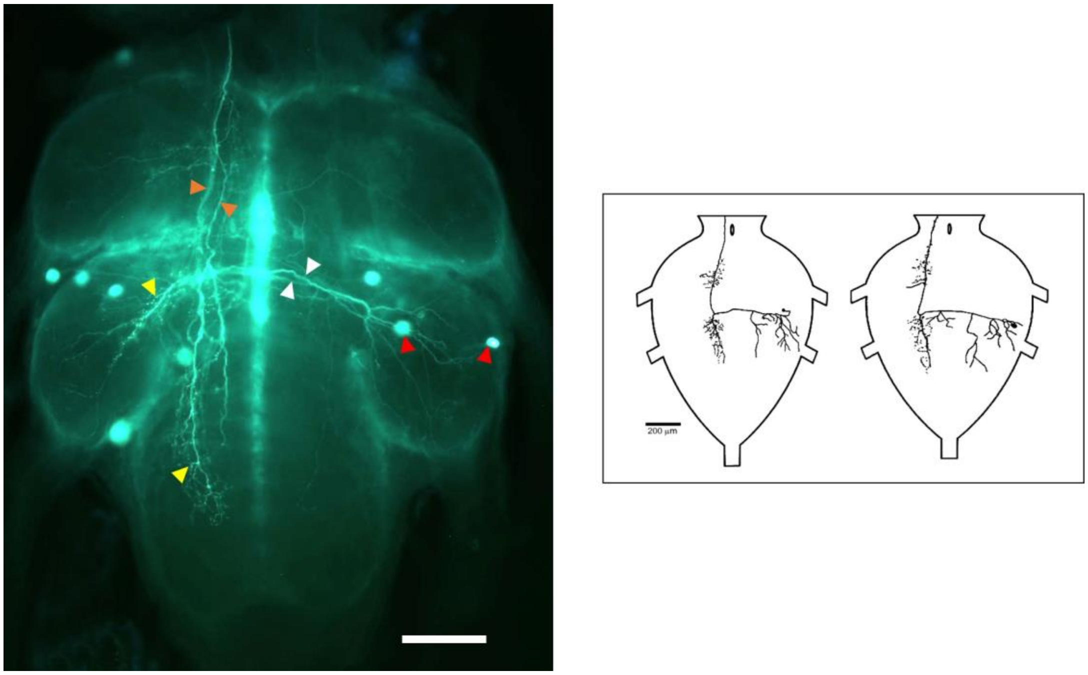

Figure 3.3. Photograph of Lucifer Yellow fills of clustered *CG-AC-1* type interneurons stained in a single preparation in the central ganglion of *H. halys* male (dorsal view) and whole mount drawings of two examples of morphologically (and physiologically; see below) similar neurons in *N. viridula* (Zorović, 2005). Red arrow heads mark the cell bodies located in the metathoracic neuropil. White arrow heads mark t central dendritic branches crossing the midline of CG. Yellow arrow heads mark axonal terminations in the metathoracic and abdominal neuromeres. Orange arrow heads mark ascending axons. Scale bar= 100 μm.

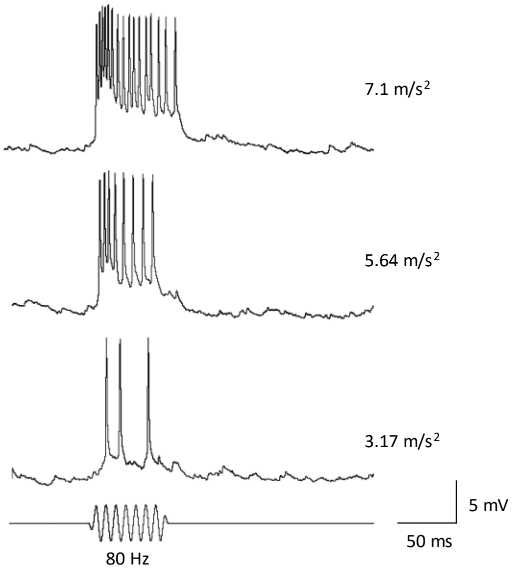

Figure 3.4. Phasic-tonic response of *CG-AC-1* neuron to vibrations of 80 Hz at increasing intensities.

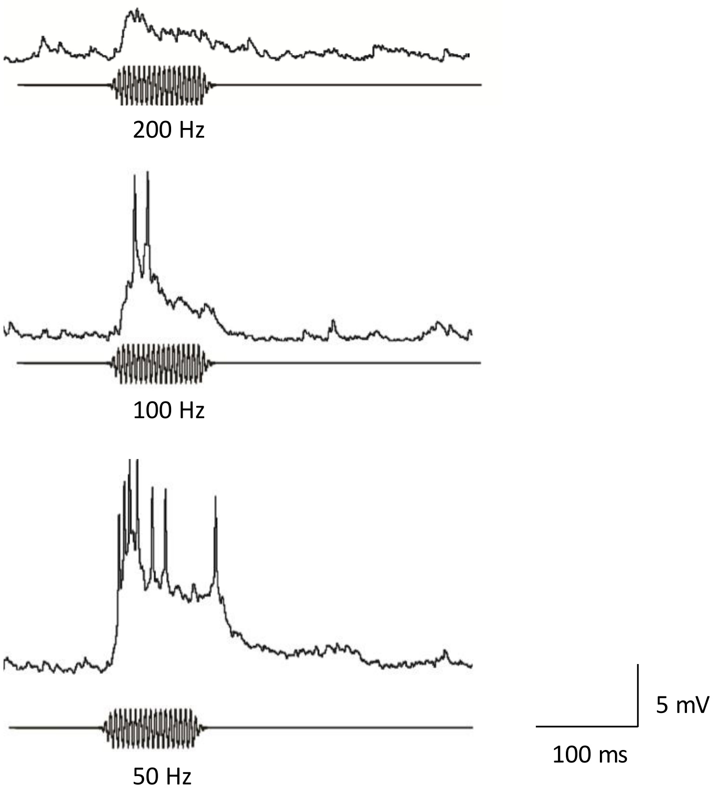

Figure 3.5. Response of *CG-AC-1* neuron to vibrations of increasing frequencies at 1 m/s^2.

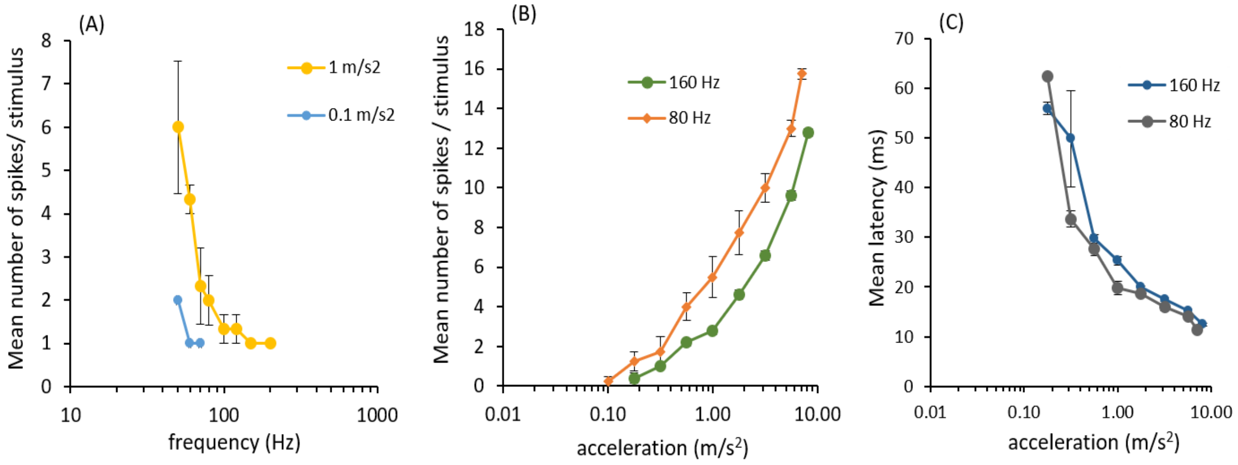

Figure 3.6. (A) Iso-intensity response functions of *CG-AC-1* neuron. (B) Intensity-response functions for 80 and 160 Hz stimuli. (C) Response latencies to stimulation at 80 and 160 Hz.

Physiology

In this neuron, full spike generation was prevented, probably due to the penetration damage, so its response intensity was evaluated as the area below the excitatory postsynaptic potential (EPSP[12]) in response to vibratory stimuli. This is a highly sensitive neuron that responded to stimuli in the whole range of tested frequencies up to 2000 Hz, both at 1 and 0.1 m/s^2, showing the strongest response at 200 Hz (Fig. 3.8-A, Fig. 3.9). The intensity-response tests at 80 and 160 Hz indicate the threshold between 0.018 and 0.032 m/s^2 and the increase of the response throughout the 60-dB range of tested intensities (Fig. 3.8-B, C). From the shape of the EPSP, the response appears to be phasic-tonic in most of the range and phasic[13] at high intensities of 50-100 Hz stimuli, where the shape of the synaptic potential clearly indicates a delayed inhibition.

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[12] **Excitatory postsynaptic potential (EPSP):** is the change in the membrane voltage of a postsynaptic cell following the influx of positively charged ions into the cell (that is detected as increase in voltage by intracellular recording).

[13] **Phasic response:** see footnote nb.9

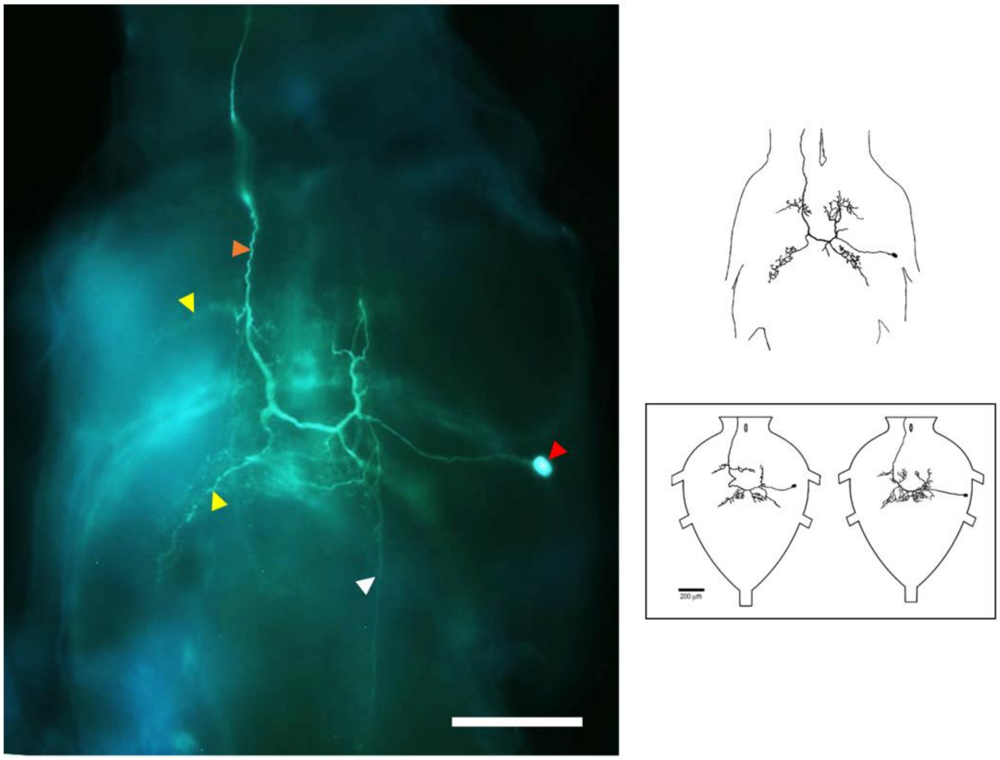

Figure 3.7. Photograph of Lucifer Yellow fills of *CG-AC-2* type interneuron in the central ganglion of *H. halys* male, taken from the dorsal view with the whole mount drawing of this neuron in *H. halys* (above) and of two examples of a putative homologue in *N. viridula* (below; Zorović, 2005). The red arrow head marks the cell body located in the cortex between the meso- and metathoracic neuromeres. Yellow arrow heads mark dendritic branches in the different neuromeres. The white arrow head marks the descending axon of another neuron that was stained less intensively in the same preparation. The orange arrow head marks an ascending axon. Scale bar= 100 μm.

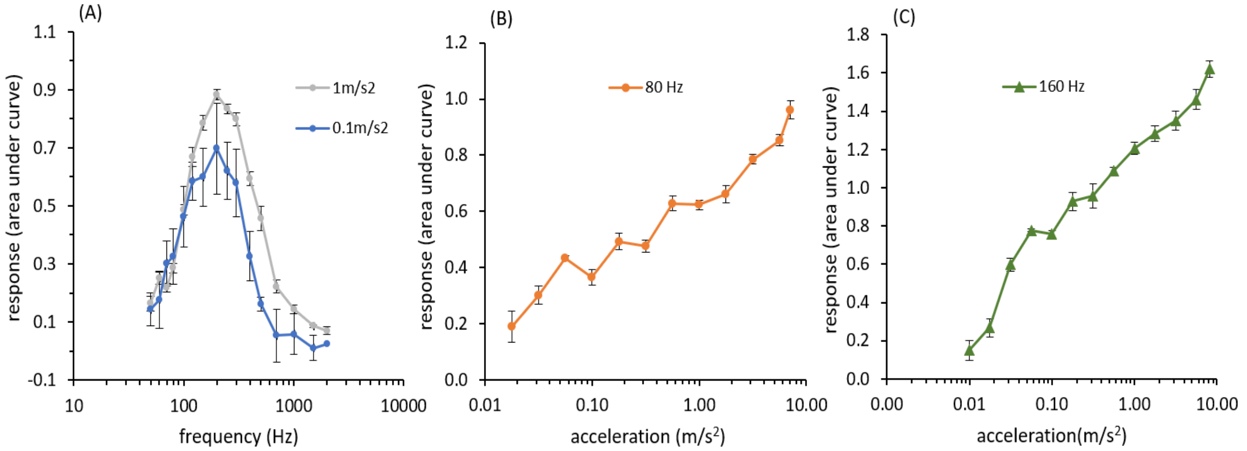

Figure 3.8. (A) Response of *CG-AC-2* neuron to vibratory stimuli of 50-2000 Hz when tested at 1 and 0.1 m/s^2. (B) *CG-AC-2* intensity-response at 80 Hz. (C) *CG-AC-2* intensity-response at 160 Hz.

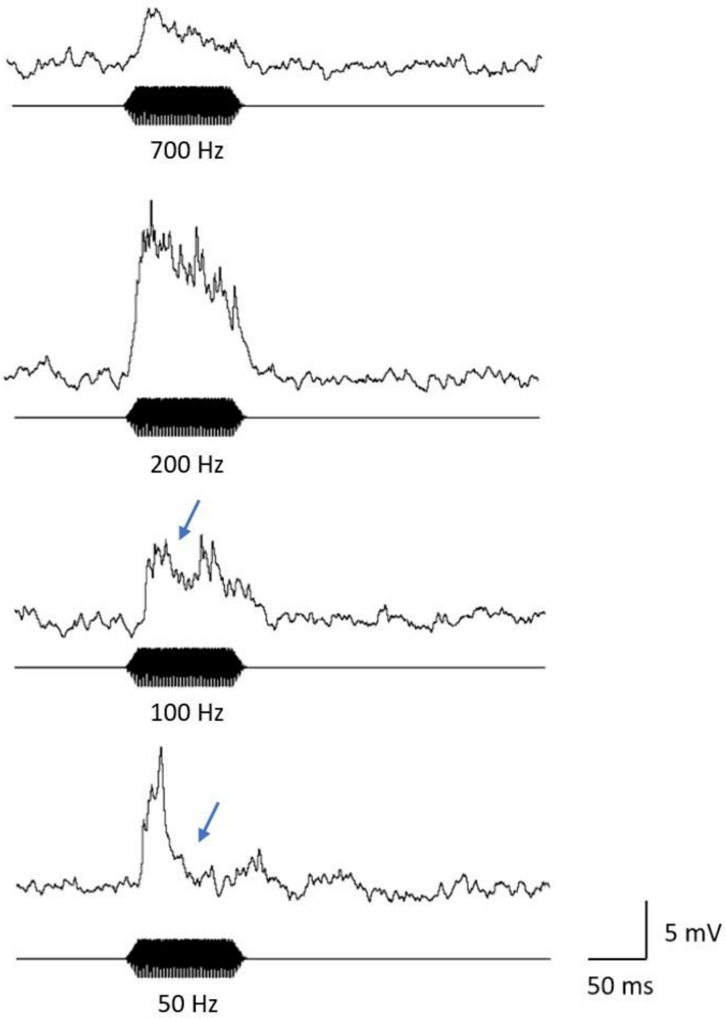

Figure 3.9. Response of *CG-AC-2* neuron to vibrations of increasing frequencies at 1 m/s^2. Blue arrows indicate the decrease of the membrane potential indicating inhibition.

CG-AB-1

Morphology

We stained one example of a neuron with a median soma and a bilaterally symmetrical structure (a DUM-type neurone) (Fig. 3.10). The soma is positined ventrally in the median cortex of the metathoracic segment. The dendritic segment is U-shaped, formed by two main branches projecting anteriorly and arborising medially in the mesothoracic neuropil. A short median branch originating at the midline of this segment gives rise to two bilatrally projecting axons that turn anteriorly as ascending projections into the prothoracic ganglion, and to two longer collaterals with short branhces that extend laterally along the metathoraric neuropil. Another branch originates from the midline-crossing segment and projects posteriorly into the anterior portion of the abdominal neuropil.

Physiology

The neuron *CG-AB-1* exhibited spontaneous activity, with the mean spiking rate increasing from around 30 Hz to 65 Hz and decreasing again to around 50 Hz in the course of recording. Responses to vibration were evoked by a broad frequency range of stimuli between 50 and 3000 Hz, the strongest between 150 and 300 Hz. Slight response peaks were expressed at 200 and 150 Hz with 0.1 and 1 m/s^2 stimulation, with an average number of peaks per stimulus reaching 7.5 and 17.2, respectively (Fig. 3.11-A). The neuron was tuned to 100-300 Hz, showing high sensitivity with thresholds at 0.018-0.032 m/s^2 in the 100-300 Hz range (Fig. 3.11-B). The response was tonic and, at high intensities of stimuli below 200 Hz, prolonged over the stimulus duration (Fig. 3.12). At higher frequencies, spiking activity was inhibited just after the stimulus offset, though no inhibitory postsynaptic potential (IPSP[14]) was visible (Fig. 3.13). At 80 and 160 Hz, the responses showed a dynamic range of at least 40 dB and 55 dB, increasing linearly from thresholds at 0.1 and 0.02 m/s^2 throughout the intensity ranges tested (Fig. 3.11-C). With the

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[14] **Inhibitory postsynaptic potential (IPSP):** is the change in the voltage of a postsynaptic cell following the influx of negatively charged ions into the cell (that is detected as decrease in voltage by intracellular recording)

increasing stimulus intensity, response latencies decreased to around 19 and 17 ms at 80 and 160 Hz (Fig. 3.11-D).

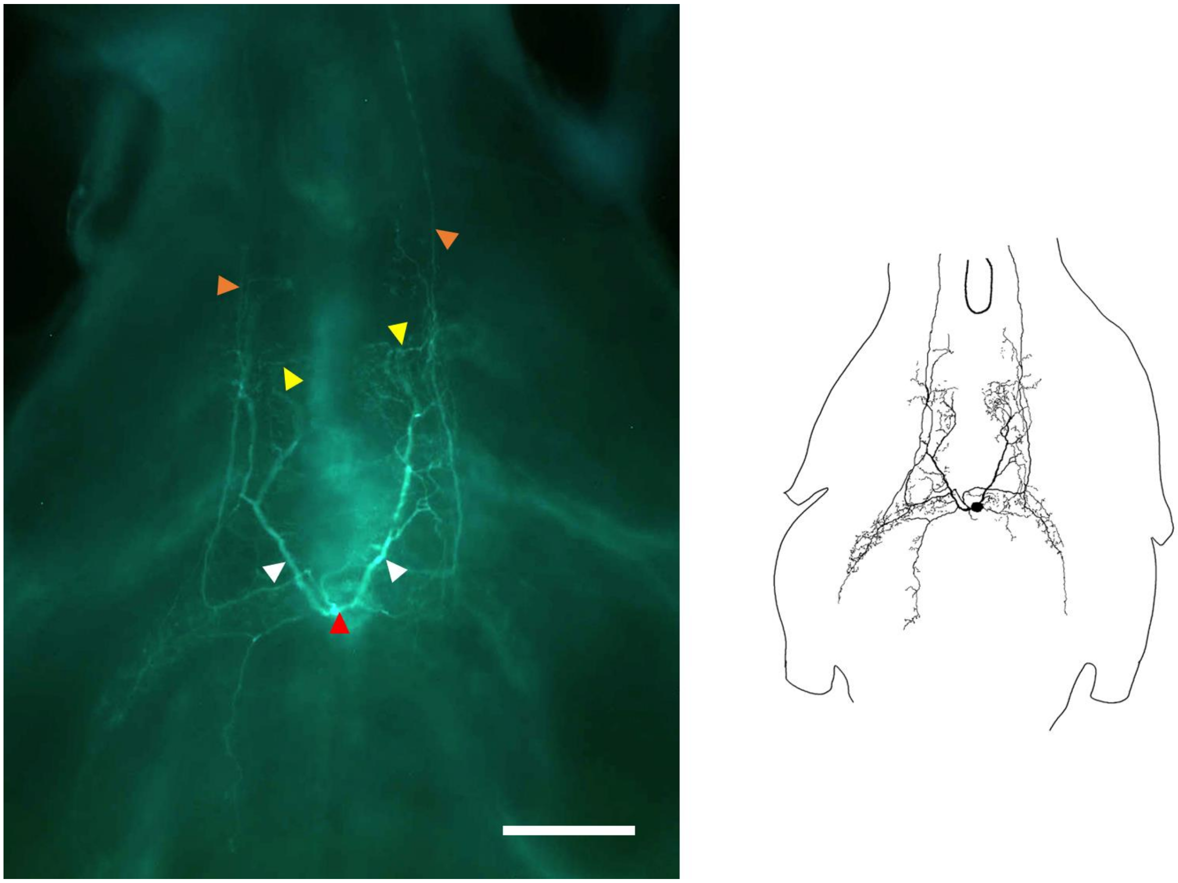

Figure 3.10. Photograph and whole mount drawing of *CG-AB-1* type interneuron filled with Lucifer Yellow in the central ganglion of *H. halys* female, taken from the dorsal view. The red arrow head marks location of the cell body in the median cortex of the metathoracic neuromere (outside the level of focus). White arrow heads mark the two main dendritic branches projecting anteriorly. Yellow arrow heads mark the dendritic arborizations in the mesothoracic neuropil. The orange arrow heads mark the two ascending axons. Scale bar= 100 μm.

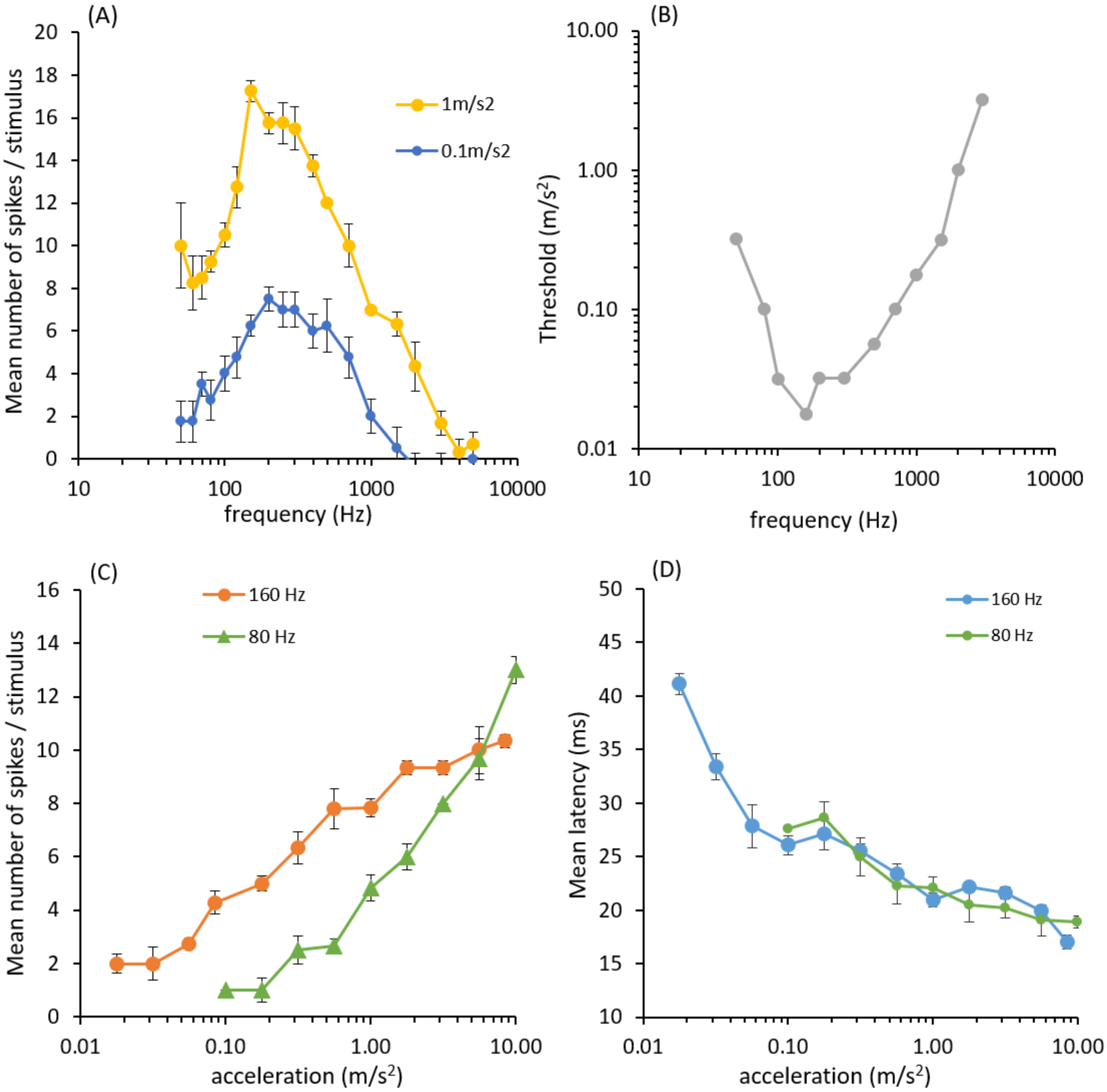

Figure 3.11. (A) Response of *CG-AB-1* neuron to vibratory stimuli of 50-3000 Hz when tested at 1 and 0.1 m/s^2. (B) *CG-AB-1* threshold curve over the tested frequency range. (C) *CG-AB-1* intensity-response curves at 80 and 160 Hz. (D) Response latencies to stimulation at 80 and 160 Hz.

CG-AI-1

Morphology

We stained a pair of mirror-imaged neurons of the same type, each originating in one lateral side of the central ganglion (Fig. 3.14). The soma is located in the dorso-lateral cortex between the meso-and metathoracic neuromeres. A long primary neurite projects medially to a dorsally positioned central branch, which crosses the midline but arborizes predominantly on the soma-ipsilateral side; in a sharp ventral loop, it gives rise to the main dendritic branch and an ascending axon, both forming diffuse arborizations over the meso- and metathoracic neuropils. Dendritic branches (of smooth appearance) arborize medially in the hemiganglion, with longer anterior and shorter posterior end projections, while axonal branches are located more laterally. A longer axonal collateral projects posterio-laterally into the metathoracic neuropil, and many medium-sized collaterals of the ascending projection end in the mesothoracic neuropil. Axonal terminations are clearly beaded in appearance.

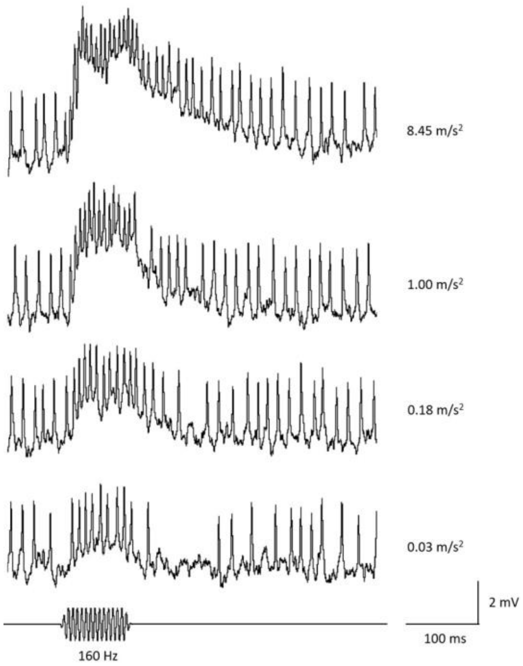

Figure 3.12. Tonic response of *CG-AB-1* neuron to vibrations of 160 Hz at increasing intensities. Spontaneous activity can be seen clearly. Note that at high intensities the response is highly prolonged over the stimulus duration.

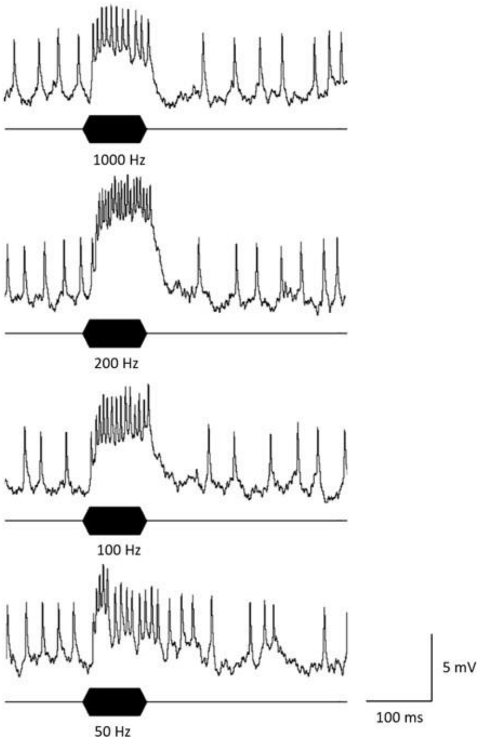

Figure 3.13. Response of *CG-AB-1* neuron to vibrations of increasing frequencies at 1 m/s^2. Spontaneous activity can be seen clearly.

Physiology

The neuron *CG-AI-1* was spontaneously active, with the spiking rate varying between 5 and 25 Hz in the course of recording. Low-frequency vibration up to 160 Hz evoked weak and often prolonged excitations with up to 5 additional spikes fired per stimulus at the maximal test intensities (10 m/s^2). The response latencies at 80 Hz were around 60.7 +/-6.7 ms. The lowest response threshold was at 3.2 m/s^2 for 50 Hz stimuli (Fig. 3.15).

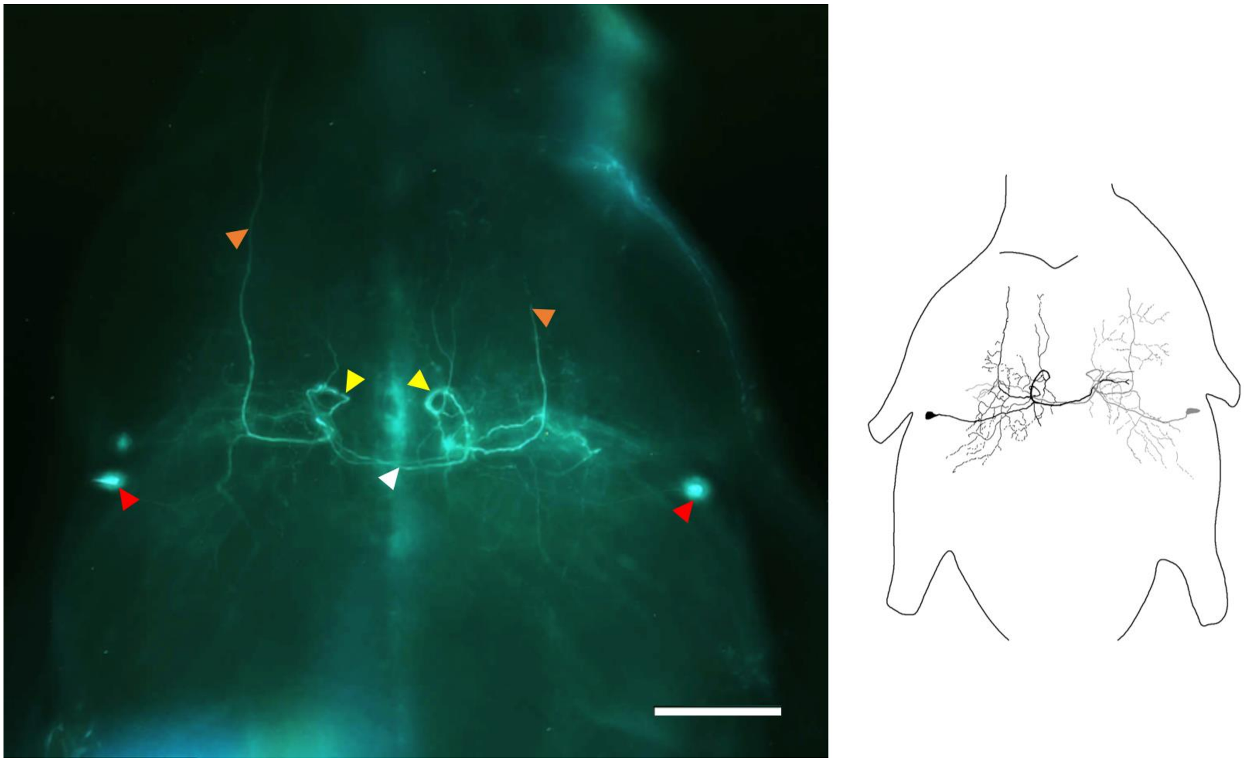

Figure 3.14. Photograph and whole mount drawing of *CG-AI-1* type interneuron filled with Lucifer Yellow in the central ganglion of *H. halys* female, taken from the ventral view. Red arrow heads mark the somata located in the dorso-lateral cortex between the meso- and metathoracic neuromeres. A white arrow head marks the central dendritic branches crossing the midline in a dorsal position. Yellow arrow heads mark the dendritic branch that originates from a "looped" projection. The orange arrow heads mark the two ascending axons. Scale bar= 100 μm.

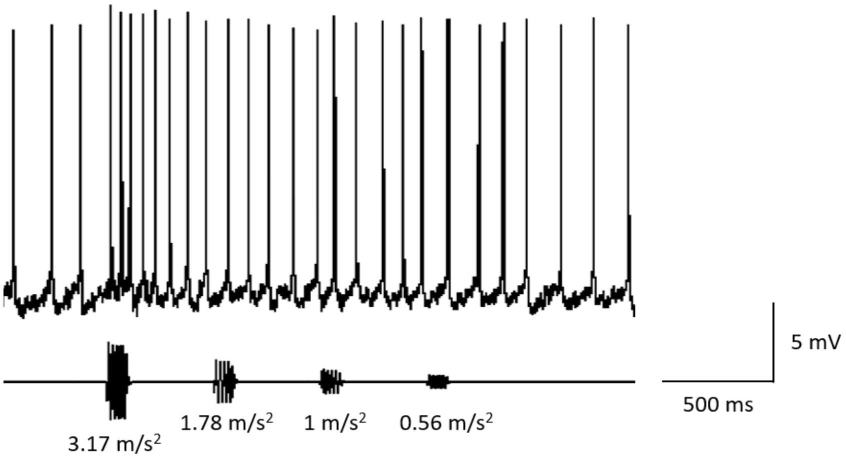

Figure 3.15. Response of *CG-AI-1* neuron to vibrations of 50 Hz at the indicated intensities; note that excitation only occurs at the highest intensity tested. Spontaneous activity can be seen clearly.

V1

Physiology

We recorded from an example of a low-frequency neuron in *H. halys* female, sharply tuned to 50 Hz stimuli with phasic-tonic response discharges (Fig. 3.16, 17). At 1 m/s^2, responses between 6 and 8 spikes per stimulus were fired at the best frequency[15] and already at 60 Hz the response dropped below threshold (Fig. 3.18-A). At 80 and 160 Hz tested, thresholds were at 1 and 3.2 m/s^2, respectively, and the response increased exponentially with the increasing stimulus intensity, reaching around 13 and 10 spikes/stimulus in the tested 20 and 10 dB, respectively (Fig. 3.18-B). Response latency strongly decreased to around 37 ms, not yet reaching its lowest values at the highest intensity tested (Fig. 3.18-C).

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[15] **Best frequency:** is the frequency showing the lowest threshold (or the highest response intensity) and therefore the highest sensitivity

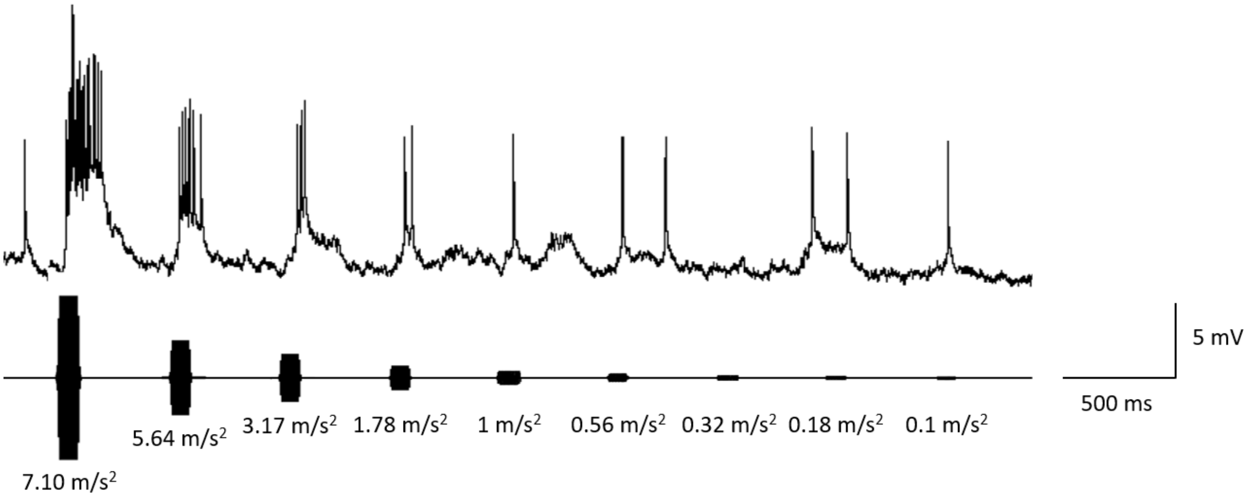

Figure 3.16. Response of *V1* neuron to vibrations of 80 Hz at decreasing intensities.

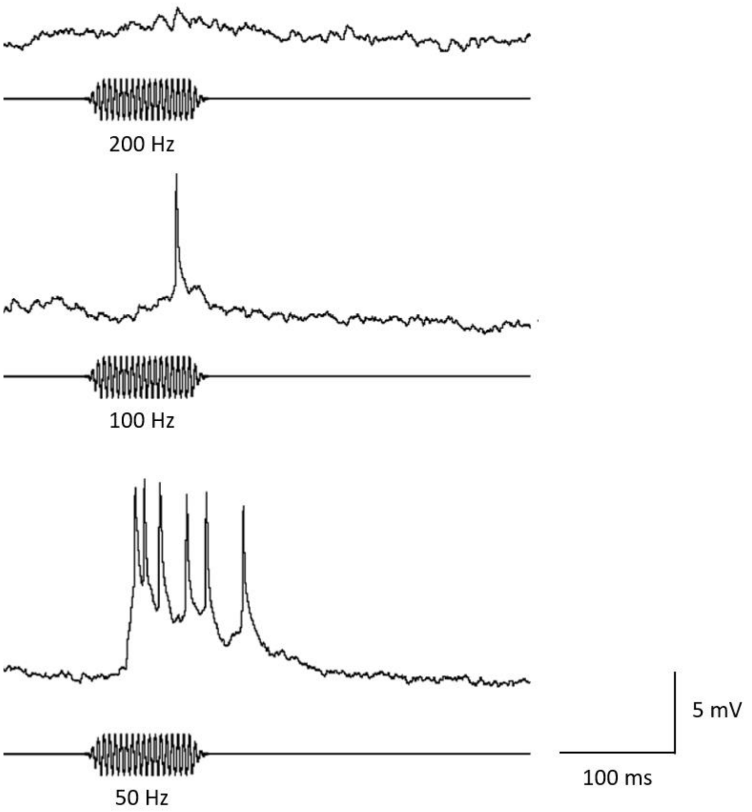

Figure 3.17. Response of *V1* neuron to vibrations of increasing frequencies at 1 m/s^2. Sharp tuning at 50 Hz is evident.

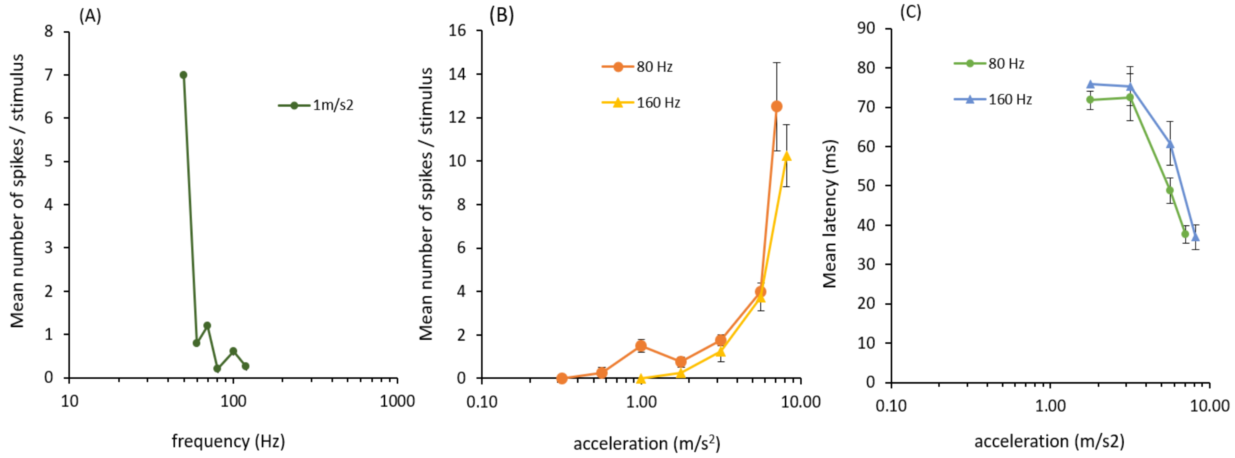

Figure 3.18. (A) Response of *V1* neuron to vibratory stimuli of 50-100 Hz when tested at 1 m/s^2. (B) *V1* intensity-response curves at 80 and 160 Hz. (C) Response latencies to stimulation at 80 and 160 Hz.

V2

Physiology

We recorded from an example of a high-frequency tuned neuron in *H. halys* male showing tonic discharges in response to vibratory stimuli, and some spontaneous activity with a rate of around 10 Hz. With 1 m/s^2 stimulation, the neuron responded in the whole range of tested frequencies between 50 and 2000 Hz, with just suprathreshold responses below 100 Hz and further strongly increasing responses with the increasing frequency (Fig. 3.19). The response was the strongest between 700 and 2000 Hz, where 30-33 spikes were fired per stimulus, with the latency between 11.5-12 ms (Fig. 3.20-A, B). The neuron received an inhibitory input at low frequencies, seen as a clear IPSP below 150 Hz, as well as a progressive reduction of the EPSP amplitude with decrease of frequencies in the excitatory responses at the high frequency side (Fig. 3.19).

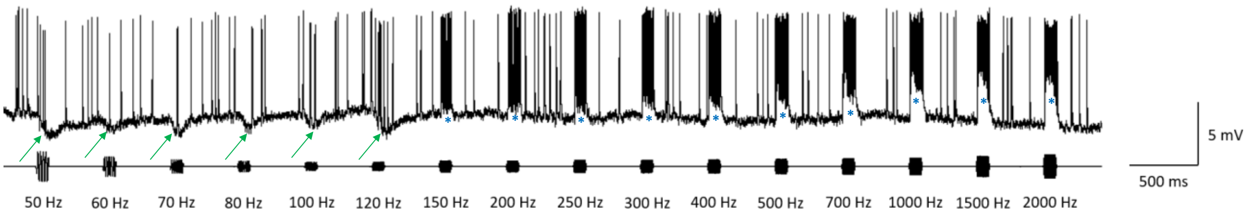

Figure 3.19. Response of *V2* neuron to vibrations of increasing frequencies at 1 m/s^2. Green arrows indicate the inhibitory postsynaptic potentials (IPSPs). Blue asterisks indicate the excitatory postsynaptic potentials (EPSPs).

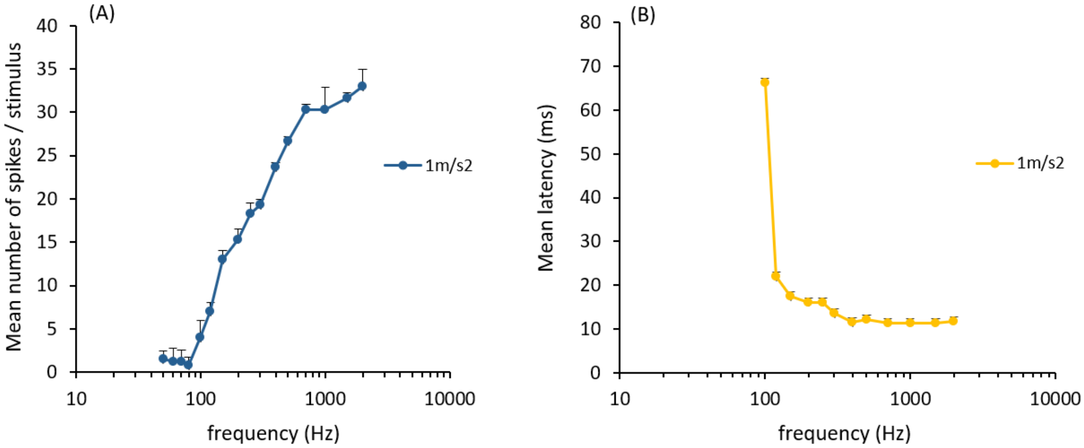

Figure 3.20. (A) Response of *V2* neuron to vibratory stimuli of 50-2000 Hz when tested at 1 m/s^2. (B) Response latencies to stimulation at 100-2000 Hz when tested at 1 m/s^2.

Discussion

In this study, we aimed to identify the morphology and physiology of part of the vibration sensitive-interneurons present in the central ganglion of the ventral nerve cord of *H. halys* adults, by means of the intracellular recording and staining technique. Our investigation has demonstrated so far, the existence of four intersegmental neuron types, two of which are thought to be putative homologues to the neurons identified previously in a species in the sister taxon, *N. viridula* (Zorović et al., 2008). Their homology can be claimed on the basis of morphological resemblance and is supported further by a close physiological similarity of the counterpart neurons. Comparative analysis in this regard, based on the structural and functional features of interneurons involved in the detection and recognition of substrate-borne vibrations, can add to the existing knowledge on this short-range communication mode in Pentatomidae and further our understanding on its underlying neuronal mechanism.

Neurons *CG-AC-1* and *CG-AC-2* have nearly the same morphology in both species, according to the location of the soma, midline-crossing segment, main dendritic branches, intersegmental axons and their main collaterals. Besides the morphological similarity, also the close resemblance in physiology justify considering *CG-AC-1* and *CG-AC-2* in *H. halys* putative homologues to those described earlier in *N. viridula* (Zorović et al., 2008). The term 'homologue' here is used to describe neurons that can be traced back to a common ancestral precursor (Bock, 1963), i.e. originating from the same neuroblast that divides producing ganglion mother cells to finally differentiate into neurons (Taghert et al., 1984). The implementation of CG-AC-1 and CG-AC-2 homologues in the receptor-interneuron network seems to be the same (not evolutionarily modified) in the two species. Differences, however, might occur in processing the temporal patterns of vibratory signals that play an important role in conspecific recognition during mating. Those were studied earlier in *N. viridula* (Zorović, 2011), but remain unexplored in *H. halys*.

The response of *CG-AC-1* neuron in *H. halys* is similar to that of its namesake in *N. viridula* (Zorović et al., 2008) and indicates they are both sharply tuned to low frequencies, with the highest sensitivity at 50 Hz. Slight differences occur in their response to a frequency range with 50-200 Hz in *H. halys* 50- 300 Hz in *N. viridula*. Moreover, both neurons had phasic-tonic responses to vibratory stimuli.

CG-AC-2 neuron in *H. halys* responded to the whole range of frequencies that has been tested (50-2000 Hz) and this response range was broader in comparison with that of its presumed homologue in *N. viridula* (50-1000 Hz), indicating this neuron was more sensitive in the former species (Zorović et al., 2008). However, neurons in both species were tuned to similar frequencies (200 Hz for *H. halys* and 150-200 Hz for *N. viridula*) and exhibited suppression of the responses at higher intensities of low-frequency stimuli, indicating inhibitory inputs (Zorović et al., 2008).

Neuron *CG-AB*-1 in *H. halys* responded to the whole range of frequencies that has been tested between 50 and 3000 Hz and was tuned to 150-300 Hz. This neuron with a bilaterally symmetrical distribution of the neurites and axons has the soma located ventrally at the midline, whereas the three *CG-AB* neurons found in *N. viridula*, also with a bilaterally symmetrical structure, had their somata located dorsally in the mesothoracic cortex, so they don't seem to be directly homologous. All unpaired median (called DUM) neurons of insects, however originate from the same unpaired median neuroblast and thus, as a common neuronal progeny, share many common morphological and physiological features. DUM neurons of the ventral nerve cord have received considerable attention because of their unique morphological and physiological characteristics. DUM neurons responding to acoustic or vibratory stimuli also occur in grasshoppers (Marquart, 1985; Thompson and Siegler, 1991), crickets (Gras et al., 1990), cave crickets (Stritih and Stumpner, 2009), bush crickets (Lefebvre et al., 2018) and fruit flies (neuron AV4 in Clemens et al., 2015). These neurons are believed to have a modulatory role and are important in shaping the frequency selectivity of neurons (Burrows, 1996; Bräunig and Pflüger, 2001; Stumpner, 2002; Lefebvre et al., 2018).

In *H. halys*, *CG-AB-1* itself was influenced by inhibition as well, most strongly above 160 Hz while the response was prolonged over the duration of the stimulus at higher intensities of lower frequencies. Lefebvre et al. (2018) also suggested an inhibitory function of DUM neurons in the auditory network of a bush cricket. Moreover, Stumpner (2002) concluded in his previous study on four species of bush crickets that the species-specific tuning of the neuron is not brought about by specific excitation, but by specific inhibition and suggested DUM neurons as likely candidates of these inputs.

Males and females of *H. halys* are known to emit low frequency signals for most signal types, with the dominant frequencies ranging between 50 and 80 Hz (Polajnar et al., 2016). Our data indicate that *CG-AC-1*, *CG-AI-1* and *V1* neurons are tuned to *H. halys* calling songs. *CG-AB-1* was tuned to higher frequencies and can be therefore assumed to play a role in sharpening the response of other neurons. Similarly, the dominant peak of *N. viridula* and other investigated stink bugs' communication signals lies between 85 and 120 Hz (Čokl et al., 2000; Čokl, 2008), and this further elucidates why neurons of stink bugs are mostly tuned to frequencies below 150 Hz.

As a polyphagous pest, *H. halys* has a wide range of host plants ranging from herbaceous annual vegetable crops to forest trees (Lee et al., 2013); all of which have different signal transmission properties due to differences in dimensions, architecture and tissue stiffness, which influence signal propagation velocity and tissue resonant properties (Michelsen et al., 1982). However, previous studies have shown that stink bugs are capable of actively modifying their output frequency according to the properties of the substrate they encounter (Polajnar et al., 2013).

The physiology of the neurons identified in this ongoing study provides valuable information about the sensitivity of the neurons involved in the sensory processing of the vibrational signals in *H. halys*. Knowing their best frequency, sharpness of tuning, occurrence of frequency-specific inhibitions and response patterns can help in developing a more efficient behavioral manipulation technique of *H. halys*. A field study conducted in a pear orchard in Northern Italy highlighted the potential of using *H. halys* female FS2 signal in attracting males (Polajnar et al., 2019) via a bimodal trap that employs dual stimuli, the aggregation pheromone and the vibrational signal. Therefore, the findings of this investigation can be integrated to create more attractive FS2 signals that might help in capturing more *H. halys* males and ultimately reduce the reproduction and damage of this polyphagous pest.

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Chapter 4

Effect of deltamethrin-incorporated nets on mobility and survivorship of *Halyomorpha halys* (Hemiptera: Pentatomidae) adults and nymphs in the laboratory

Ibrahim, A., Kirkpatrick, D., Nixon, L., Ludwick, D., Anfora, G. and Leskey, T.C., 2020. *Journal of Applied Entomology* (in review).

Abstract

The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), is an invasive pest that attacks specialty and row crops in North America and Europe. There has been a concerted effort to reduce frequent broad-spectrum insecticide applications made on vulnerable crops. One tool that has emerged recently is the use of long-lasting insecticide treated nets (LLINs) as a killing agent. Here, we conducted bioassays to evaluate the effect of direct contact on deltamethrin-impregnated LLINs on the behavior and survivorship of *H. halys* nymphs and adults in the laboratory. Following exposure at three different durations (1.25, 4.25 or 7.25 min), vertical and horizontal mobility of adults and nymphs and the flight capacity of adults were recorded and compared with individuals that were not exposed (control). Exposure to LLINs reduced the horizontal distance and velocity and increased the angular velocity of adults only but reduced vertical mobility of adults and nymphs. Adult flights were not significantly affected by LLIN exposure. Mortality of adults and nymphs at 7-d post exposure ranged from 73-77%

regardless of exposure time. We discuss our findings within the context of the potential for and limitations of deploying LLINs in vulnerable crops to manage *H. halys* populations.

Keywords: brown marmorated stink bug, long-lasting insecticide net, pyrethroid, behavior, flight capacity, mortality

Introduction

The brown marmorated stink bug, *Halyomorpha halys* (Stål), is an invasive pentatomid native to East Asia that has been expanding in North America and Europe since its accidental introduction (Hoebeke and Carter, 2003; Arnold, 2009). *Halyomorpha halys* is highly polyphagous and attacks tree fruits, vegetables and row crops (Leskey and Nielsen, 2018). Overwintering adults are also considered nuisance pests, as they often alight onto the exterior (Hancock et al., 2019) and seek shelter within homes and other anthropogenic structures (Inkley, 2012). Both adults and nymphal stages of *H. halys* have the ability to colonize and disperse among different host plants throughout the growing season (Rice et al., 2014; Acebes-Doria et al., 2016). Feeding injury caused by *H. halys* includes deformities, scars, discolorations, pitting and depressions of plant tissues that render produce unmarketable (Maistrello et al., 2017). Moreover, while injury to crops can be mitigated through application of broad-spectrum synthetic insecticides, these are very disruptive to beneficial arthropods, often causing secondary pest outbreaks (Leskey et al., 2012a; Leskey and Nielsen, 2018; Kuhar and Kamminga, 2017).

Toward that end, progress to limit insecticide applications against *H. halys* has included trap-based decision support tools (Short et al., 2017), attract and kill technology (Morrison et al., 2018) and trap cropping (Mathews et al., 2017). Recently, insecticide-treated nets, long used as bed nets for management of vector-borne mosquitoes in human dwellings (Lengeler, 1998) have received considerable attention for use in agricultural and forestry agroecosystems. Long-lasting insecticide-treated nets (LLINs) have been used in Poland (Skrzecz et al., 2015) and Germany (John and Zeilhofer, 2013) as a method to manage bark beetles attacking spruce. In the USA, LLINs were deployed on citrus trees to combat psyllid infestation (Trujillo, 2014). For *H. halys*, Kuhar et al. (2017) reported that deltamethrin LLINs are toxic to adults and nymphs under laboratory conditions. Sabbatini Peverieri et al. (2017) investigated the lethal and sub-lethal effects of the use of an α-cypermethrin LLINs on *H. halys* adults in the lab and observed higher mortality and lower recovery rates with longer exposures. Moreover, Sabbatini Peverieri et al. (2018) showed that the number of *H. halys* adults and nymphs killed by α-cypermethrin or deltamethrin LLINs vertically hung on pear trees, was significantly greater than the control in two pear orchards in Northern Italy. Bergh and Quinn (2018) also explored the potential of deltamethrin LLINs against *H. halys* when used in experimental panels on building walls during the period when adults move toward potential overwintering sites.

However, none of these studies quantified the potential impact of exposure on *H. halys* mobility. We know from previous studies that exposure to particular insecticides can have a profound impact on adult vertical and horizontal walking capacity (Lee et al., 2013) as well as flight capacity (Morrison et al., 2017). Even organically approved materials affected horizontal and vertical mobility of adults (Lee et al., 2014). These changes in mobility can have significant implications for management. Therefore, we quantified the impact of exposure to deltamethrin-incorporated LLINs on adult and nymphal horizontal and vertical mobility as well as adult flight capacity and measured mortality of exposed individuals in a series of laboratory studies.

Materials and Methods

Sources of Insects for Bioassays

For horizontal and vertical mobility bioassays, actively foraging *H. halys* adults and nymphs (3rd -5th instars) were collected from *Paulownia tomentosa* (Thunberg), in Kearneysville, West Virginia, USA from mid- August until October 2018. Additionally, a second *H. halys* colony from material provided by the New Jersey Department of Agriculture (USA) was maintained in the laboratory and used to supplement experiments with individuals as needed. Both sources of *H. halys* were held in a climate-controlled room in screen cages (30 cm^3; DP1000, BugDorm, BioQuip, Rancho Dominguez, CA) at 25℃, 60% R.H. and 16:8 (L:D) and provisioned with food (carrot, peanuts, sunflower seeds, green beans, and sundried tomato) and moistened cotton dental wicks. For all trials with nymphs, we combined 4th and 5th instars as their baseline dispersal capacity is statistically identical (Lee et al., 2014). For effects of flight capacity, wild *H. halys* adults, naturally settled within wooden overwintering shelters (Bergh et al., 2017) and held in a dark, unheated shed, were used. Prior to the start of the exposure period and flight bioassay, adults were removed from shelters and held for ~1h at 25℃ as per methods of Lee and Leskey (2015).

LLIN exposure

Black ZeroFly® (Vestergaard-Frandsen, Switzerland) LLINs, impregnated with deltamethrin at a dose of 3.85 mg a.i./g fiber and purchased in 2018 was used in this study. The untreated control net, Clear Advantage Charcoal Fiberglass Insect Screen (Saint-Gobain Adfors) had similar descriptions as the insecticidal net (black color and mesh size of 33 holes/cm^2). Exposure arenas consisted of plastic Petri dishes (100×15mm), with their interior base covered by a 100-mm disc of ZeroFly® or untreated net. Petri dish lids were perforated with 0.5 cm holes to allow ventilation.

Exposure durations used in this study were derived from those developed by Bergh and Quinn (2018), which were 1.25, 4.25 and 7.25 mins. For the control treatment, *H. halys* were exposed to untreated net for 1 min. For all treatments, *H. halys* adults or nymphs were introduced in an inverted position beneath the LLINs or untreated net disk with their abdomen facing up to facilitate constant contact during the exposure interval. For each replicate of nymphs and adults, all exposure treatments and the control were run simultaneously and then insects were moved directly to a mobility trial. All bioassays were conducted between August 2018 and April 2019.

Horizontal mobility

To assess the impact of exposure intervals to the LLINs or to an untreated control, horizontal mobility was measured using the EthoVision system (version 3.1, Noldus Information Technology Inc., Leesburg, VA) following the methods described by Lee et al. (2013). Replicates were collected over time for the bioassays of *H. halys* adults and nymphs exposed to LLINs for different time intervals for adults: control (n = 20), 1.25 mins (n = 27), 4.25 mins (n = 26) and 7.25 mins (n = 27) and for nymphs: control (n = 13), 1.25 mins (n = 21), 4.25 mins (n = 21) and 7.25 mins (n = 19). *Halyomorpha halys* nymphs or adults were tracked simultaneously in five glass Petri dish arenas (10 cm diam; 3 cm tall) using a camera (Re-350, Canon, Inc., Tokyo, Japan) suspended above the arenas. To aid in detection and tracking in EthoVision, we conducted tests in a darkened room with the arenas backlit using fluorescent lights and at 25 °C and 60% R.H. Following each trial, glass arenas were washed with soap and water, rinsed with 90% ethanol and dried.

The following variables were quantified during the 60-min recording period: total distance moved, mean velocity, and mean angular velocity. For data acquisition, an input filter for the movement distance was applied, whereby data were accumulated only if the position of test individual changed by >10% of the mean body length. This procedure accounted for variation in the position of the tracked individual due to recalculation of the center of the test subject; i.e. cursor bounce.

Vertical mobility

To record the climbing capacity of *H. halys* following different exposure periods to LLINs or to untreated net, adults and nymphs were placed separately into clear polycarbonate cylinders (7 cm diameter; 30 cm tall) following the methods of Lee et al. (2013). Adults or nymphs were visually observed for 15-minutes and their total vertical climbing distance on the interior surface of the cylinders was recorded. During each trial, once an individual reached the top of the cylinder, the cylinder was inverted to enable continuous vertical movement. Replicates were collected over time for the bioassays of *H. halys* adults and nymphs exposed to LLINs for different time intervals for adults: control (n = 20), 1.25 mins (n = 26), 4.25 mins (n = 28) and 7.25 mins (n = 26) and for nymphs: control (n = 20), 1.25 mins (n = 26), 4.25 mins (n = 27) and 7.25 mins (n = 27).

Flight capacity

Following the setup used previously by Lee and Leskey (2015) and Morrison et al. (2017), six computer-monitored flight mills were used to record the flight performance of overwintering *H. halys* adults. Replicates were collected over time for the bioassays of adults exposed to LLINs for different time intervals with control (n = 20), 1.25 mins (n = 26), 4.25 mins (n = 26) and 7.25

mins (n = 28). All trials were conducted in a bioassay room at ~24 °C, 60% R.H., 16:8 (L:D) and 1150 lux.

To tether exposed *H. halys* adults, a bead of glue from a low temperature glue gun (3M Scotch-Weld Hot Melt Applicator LT, 3M, St. Paul, MN) was applied to the head of an insect pin and affixed to its pronotum. The tip of the pin was then inserted into the rotation arm of the flight mill, in a way that maintained a perpendicular positioning of the adult. A counterweight was added at the opposite end of the mill to balance the weight of the tethered individual. All trials began between 0800-1000AM and lasted 22 h. The software system DASYLab (Measuring Computing, Norton, MA) recorded the time of flight initiation and cessation, as well as the number of mill revolutions. Based on the number of mill revolutions over a given time, the total flight distance (in meters) was computed for each individual.

Survival assessment and lethality index

The effects of LLINs exposure on the survivorship of *H. halys* nymphs and adults were assessed under laboratory conditions. The survivorship of the overwintering adults previously used in flight mill trials (control (n = 20), 1.25 min (n = 26), 4.25 min (n = 26) and 7.25 min (n = 28)) and laboratory-reared nymphs previously used in vertical and horizontal mobility trials (control (n = 25), 1.25 min (n = 33), 4.25 min (n = 34) and 7.25 min (n = 32)) was assessed. After trials were completed, *H. halys* specimens were held individually in transparent plastic containers (Fabri-Kal Clear KC32 Cups 32 oz) with food and water in a room at 25 °C, 60% R.H. and a photoperiod of 16:8 (L:D). Individuals were monitored daily for survivorship and assigned a physical condition (alive, moribund or dead), following the classification of Leskey et al. (2012b).

The lethality index (LI) was calculated to study the correlation between the exposure duration and mortality rates of *H. halys* adults and nymphs, using the formula by Leskey et al. (2012b), whereby the results range from 0 to 100; with 100 indicating complete mortality and 0 complete survival. This index allocates a specific weight not only to alive and dead specimens, but also to bugs in a moribund state that might recover from immediate knockdown.

Statistical analyses

All data were analyzed using R software (v.3.4.3). All data sets were subjected to Shapiro-Wilk test for normality. Data for the horizontal mobility of adults, vertical mobility of nymphs, and flight capacity of adults were not normally distributed, therefore a Kruskal-Wallis rank sum test was applied. Where there was significance, a Dunn's multiple comparison test was performed for pairwise comparisons between treatment groups. For the horizontal mobility of nymphs, angular velocity data were normally distributed, whereas, the distance moved, and velocity were square-root transformed (√x) to meet the assumptions of normality, and then analyzed using one-way ANOVA. For the vertical mobility of adults, the data were square-root transformed (√x) to meet assumptions of normality and analyzed using a one-way ANOVA with Tukey's HSD for pairwise comparisons. For the survival assessment of nymphs and adults, a generalized linear model with binomial distribution was used to compare the proportion of individuals classified as alive or dead 7 days after exposure. A chi-square test was conducted to compare the survival in each treatment with that in the control.

Results

Horizontal mobility

Exposure to the LLINs reduced the walking distance and velocity of *H. halys* adults, whereas no measurable differences were observed in nymphs. Exposure to LLINs significantly affected walking distance, velocity and angular velocity of adults ($X^2 = 16.836$, df = 3, $P = 0.00076$; $X^2 = 16.801$, df = 3, $P = 0.00077$; $X^2 = 20.729$, df = 3, $P = 0.0001198$, respectively).

Adults moved significantly shorter distances following exposure to the LLINs for 4.25 min (Dunn's $z = -2.829$, $P = 0.01398$) or 7.25 min (Dunn's $z = -3.910$, $P = 0.00055$) when compared with the control (Fig.4.1 A). Horizontal walking distance of adults exposed for 1.25 min was significantly greater when compared to those exposed for 7.25 min (Dunn's $z = 2.401$, $P = 0.0326$), whereas no significant differences were observed between adults exposed to 1.25 and 4.25 min or 4.25 and 7.25 min ($P > 0.05$).

As for the velocity, there was a significant decrease among adults exposed to the LLINs for 4.25 min (Dunn's $z = -2.823$, $P = 0.0142$) or 7.25 min (Dunn's $z = -3.904$, $P = 0.000567$) relative to the control (Fig.4.1 B). Adults that spent 7.25 min on the LLINs walked significantly slower than those that spent 1.25 min (Dunn's $z = 2.406$, $P = 0.0322$). In contrast, no significant differences in velocity were observed among the other treatments ($P > 0.05$).

Regardless of the exposure duration, significant increases in the angular velocity of adults exposed to the LLINs compared with the control were recorded: 1.25 min (Dunn's $z = 3.413$, $P = 0.001283$), 4.25 min (Dunn's $z = 4.205$, $P = 0.00015$) and 7.25 min (Dunn's $z = 3.711$, $P = 0.00061$), indicating irregular walking paths by exposed adults (Fig.4.1 C). No differences among the treatments themselves were recorded ($P > 0.05$).

There were no significant differences in the walking distance, velocity and angular velocity of nymphs among exposed and unexposed individuals (one-way ANOVA: $F = 1.226$; df = 3; $P = 0.307$; $F = 1.179$; df = 3; $P = 0.324$; $F = 1.483$; df = 3; $P = 0.227$, respectively) (Fig.4.1 D, E, and F).

Vertical mobility

Contact with the LLINs inhibited the vertical mobility of *H. halys* adults and nymphs. The distances climbed by *H. halys* adults and nymphs after exposure to LLINs or control nets at the three exposure durations are summarized in Table 4.1.

Among *H. halys* adults exposed to the LLINs and those exposed to the control net, significant differences were recorded (one-way ANOVA: $F = 10.06$; df = 3; $P < 0.0001$). No significant differences were observed in the climbing distance of *H. halys* adults exposed to the LLINs for 1.25 min or 4.25 min compared with the control. Adults exposed for 7.25 min to the LLINs climbed significantly shorter distances when compared with the control ($P < 0.0001$), 1.25 min ($P = 0.0026$) and 4.25 min ($P = 0.0034$).

LLINs exposure had a significant effect on vertical mobility of nymphs ($X^2 = 19.653$, df = 3, $P < 0.001$). The distance climbed by nymphs exposed to the LLINs was significantly shorter with longer exposure duration, when compared with the control (for 1.25 min: $P = 0.0018$; for 4.25 min: $P = 0.0026$; and for 7.25 min: $P = 0.0001$). No differences between treatments themselves was recorded ($P > 0.05$).

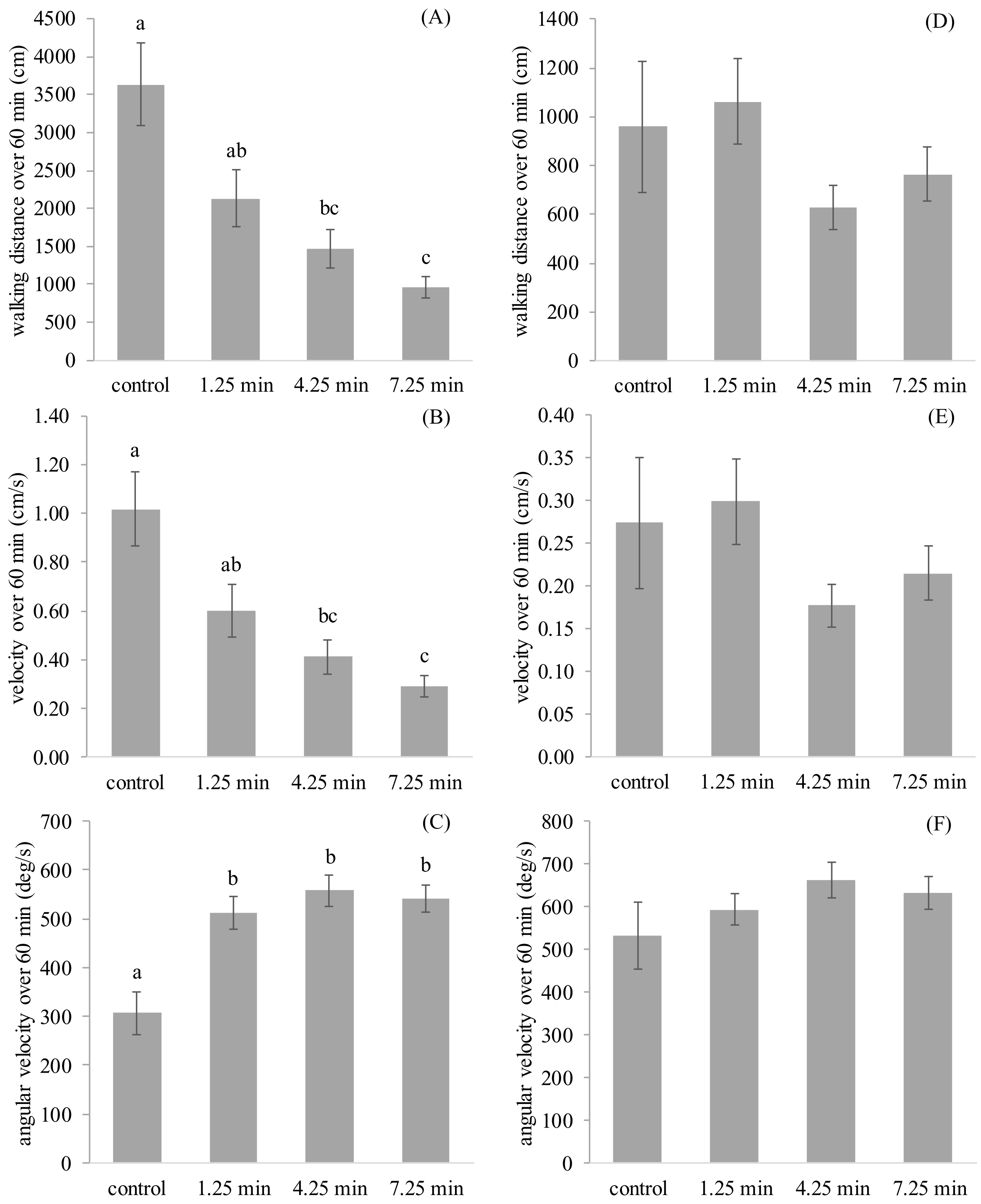

Figure 4.1. Bar graphs showing the mean ± SEM of (A) horizontal walking distance (cm), (B) velocity (cm/s) and (C) angular velocity (deg/s) of *Halyomorpha halys* adults and nymphs (D), (E) and (F) respectively, measured for 60 min using Ethovision software. Bars with different letters are significantly different between treatments (adults: Kruskal-Wallis followed by Dunn's test $P < 0.001$; nymphs: one-way ANOVA $P < 0.05$).

Table 4.1. Vertical climbing distance (mean ± SEM) of *Halyomorpha halys* adults and nymphs over a 15-min period following exposure to the LLIN for 1.25, 4.25 and 7.25 min.

	Vertical climbing distance (cm)/ developmental stage	
Treatment	Adults [1]	Nymphs [2]
control	622 ± 58.21 a	222.90 ± 39.57 a
1.25 min	494 ± 40.55 a	72.23 ± 24.74 b
4.25 min	454 ± 31.41 a	64.96 ± 22.30 b
7.25 min	313.23 ± 26.39 b	41.59 ± 15.02 b

[1] Data was square-root transformed and analyzed using one-way ANOVA followed by Tukey's HSD.
[2] Data analyzed using Kruskal-Wallis rank sum test followed by Dunn's test.
Values in the same column followed by different letters are significantly different at $P < 0.05$.

Flight capacity

The distance flown by overwintering adults for either the first 15-min on the flight mill or the entire 22-hours following LLINs exposure was not significantly different ($X^2 = 1.0493$, df = 3, $P = 0.7893$; $X^2 = 2.3827$, df = 3, $P = 0.4969$, respectively) regardless of the exposure duration (Table 4.2).

Table 4.2. Flight distance (mean ± SEM) of *Halyomorpha halys* adults 15 minutes and 22 hours following exposure to LLIN for 1.25, 4.25 and 7.25 min.

	Flight distance (m)	
Treatment	15 min after exposure	22 hours after exposure
Control	705.67 ± 99.76	2288.04 ± 575.16
1.25 min	571.83 ± 110.46	1417.92 ± 433.25
4.25 min	586.75 ± 116.14	2360.54 ± 549.32
7.25 min	547.45 ± 89.23	2053.92 ± 887.04

Data was analyzed using Kruskal-Wallis rank sum test, no significant differences were recorded ($P > 0.05$).

Survival assessment and lethality index

Survivorship of *H. halys* exposed to the LLINs was significantly different from that of unexposed control individuals on day 7 regardless of developmental stage (adults/nymphs) and exposure duration (for adults: $X^2 = 15.675$, df = 1, $P < 0.0001$, $X^2 = 17.696$, df = 1, $P < 0.0001$ and $X^2 = 17.232$, df = 1, $P < 0.0001$ for 1.25 ,4.25 and 7.25 min respectively) (for nymphs: $X^2 = 20.013$, df = 1, $P < 0.0001$, $X^2 = 18.728$, df = 1, $P < 0.0001$ and $X^2 = 19.26$, df = 1, $P < 0.0001$ for 1.25 ,4.25 and 7.25 min respectively) (Fig. 4.2-4.3).

Based on the lethality index, the LLINs was most effective at killing *H. halys* adults and nymphs when they were in contact with the net for the longest exposure duration (7.25 min) (Table 4.3). Approximately 73-77% of adults were classified as dead 7 days after being exposed to the LLINs for any of the exposure durations compared with only 10% in the control (Fig. 4.2). For nymphs, this percentage ranged from 74-76% for exposed nymphs in comparison with 16% in the control (Fig. 4.3).

Discussion

In this study, we performed laboratory bioassays to evaluate the effect of a deltamethrin-incorporated net on the mobility and survivorship of *H. halys* adults and nymphs. Our results indicate that exposure to the LLINs reduced the walking and climbing capacity of adults, with no measurable effects on the flight capacity. In addition, contact with the LLINs inhibited the climbing capacity of nymphs, whereas no significant differences were observed in their horizontal walking behavior. High mortality rates were observed in both adults and nymphs even following a brief 1.25 min exposure duration to the LLINs.

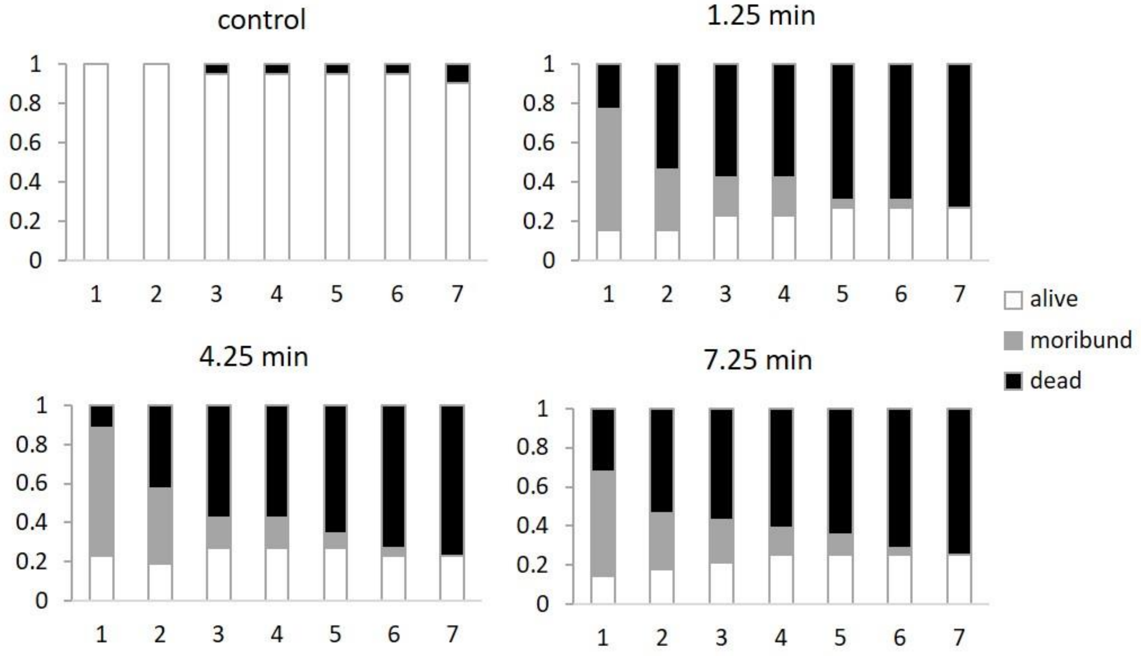

Figure 4.2. Bar graph representing the survival assessment of *Halyomorpha halys* adults over 7 days. The horizontal axis represents the days following exposure and the vertical axis represents the proportion of adults in the different treatments.

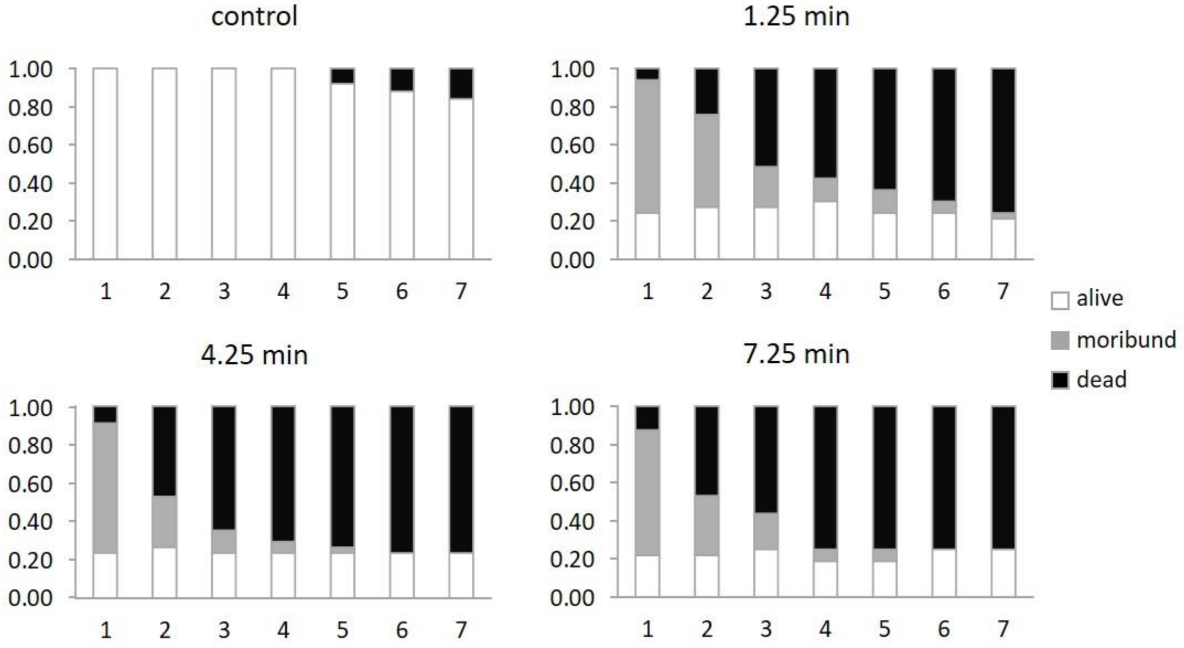

Figure 4.3. Bar graph representing the survival assessment of *Halyomorpha halys* nymphs over 7 days. The horizontal axis represents the days following exposure and the vertical axis represents the proportion of nymphs in the different treatments.

Lee et al. (2013) tested the effects of several organophosphates and pyrethroids on the mobility of *H. halys* after exposure to dry insecticide residues for 4.5 hours. Eight out of the nine tested pyrethroids significantly increased the walking distance of adults 10 minutes after exposure, and then decreased it one and a half hours later (Lee et al., 2013). In our study, LLINs were found to be more efficient in inducing uncoordinated and irregular movements in *H. halys* adults, for they resulted in comparable intoxication symptoms in much shorter exposure durations. To our knowledge, this study is the first to report the effect of LLINs on the mobility of *H. halys* nymphs. We found that the walking distance and velocity numerically decreased, although no significant differences were recorded between exposed nymphs and the control.

Table 4.3. Lethality index of the LLIN on *Halyomorpha halys* adults and nymphs following exposure to 1.25, 4.25 or 7.25 min over the 7-days trial.

	Treatment			
Development stage	control	1.25 min	4.25 min	7.25 min
Adults	4.29	67.43	65.38	69.46
Nymphs	5.14	62.34	67.86	68.53

Morrison et al. (2017) exposed *H. halys* adults to dry residues of another pyrethroid, bifenthrin, for a 5-min period. The authors noted a significant decrease in the vertical mobility, where adults only climbed 8% of the distance reached by the control. In our study, adults exposed for 4.25 or 7.25 min showed approximately 30% and 50% reduction in climbing distances respectively (Table 4.1). This suggests that dry residues of bifenthrin can induce faster and more intense changes in the climbing behavior of *H. halys* adults in comparison with deltamethrin-impregnated LLINs. The significant reduction in the climbing capacity of nymphs after contact with LLINs offers hope for limiting their mobility and dispersal in fruit trees and possibly reducing feeding damage.

The flight distances of exposed adults were not significantly affected 15-min and 22-hours after tethering on the flight mill, irrespective of the LLINs exposure duration. Similarly, Morrison et al. (2017) showed that 5-min exposure to pyrethroids (bifenthrin and dinotefuran) had no significant effects on the flight capacity of exposed adults versus the control in the first 15-min after exposure, whereas other insecticides including methomyl, thiamethoxam and thiamethoxam + λ-cyhalothrin significantly decreased the distance flown by adults. On the other hand, Zhao et al. (2011) reported a significant enhancement in the flight speed and distance of the brown planthopper *Nilaparvata lugens* Stål (Hemiptera: Delphacidae) following exposure to high concentrations of imidacloprid, triazophos and deltamethrin. Based on our results, it is likely that longer exposure intervals (>7.25m) to LLINs would be necessary to affect flight capacity of *H. halys* and is something that should be explored further in future studies.

Our survival assessment showed that mortality rates of *H. halys* adults and nymphs, following contact with the LLINs, ranged from 73-77% at day 7 for all exposure durations (Figs. 4.2 and 4.3). In other investigations using LLINs, different mortality rates were recorded, reflecting possible variability in the exposure method, duration, and/or the physiological status of assayed individuals (Kuhar et al., 2017; Sabbatini Peverieri et al., 2017, 2018; Bergh and Quinn, 2018). In particular, we selected exposure intervals following Bergh and Quinn (2018), and in our study, adult mortality was generally higher than observed in that study. However, Bergh and Quinn (2018) evaluated adults that were dispersing to overwintering sites, and during late season, adults are harder to kill with conventional insecticides than those earlier in the season

(Leskey et al., 2014). Thus, adults in the Bergh and Quinn (2018) study (those about to overwinter) were quite different than ours (primarily adults that were actively foraging on host plants). This difference in mortality is striking and one that could be worth following up with adults at different points in the growing season as well as during the overwintering period to determine if susceptibility to LLINs changes depending on physiological state. Moreover, while recovery from the knockdown effects of insecticides was reported in field and laboratory studies for *H. halys* (Nielsen et al., 2008; Leskey, 2011; Leskey et al., 2012b), in our case little recovery was observed (< 10%). These results suggest that brief contact to LLINs induce higher *H. halys* mortality rates compared with longer exposure to dry insecticide residues, but again, it would be good to confirm these results with adults throughout the growing season in future studies.

Since the identification of the male aggregation pheromone of *H. halys* by Khrimian et al. (2014) and the pheromone synergist (Weber et al., 2014), pheromone traps have been developed and validated season-long for detecting and monitoring of *H. halys* in the USA and Italy (Acebes-Doria et al., 2018, 2019; Leskey et al., 2015; Malek et al., 2018; Morrison et al., 2015, Rice et al., 2017). A recent small-scale pilot study also points to cues released by live *H. halys* (Suckling et al., 2019) as being attractive to adults, perhaps due to additional vibrational cues at short range (Polajnar et al., 2016; Mazzoni et al., 2017) or yet-to-be identified conspecific odors. Certainly, these advancements offer prospects for behavioral manipulation of *H. halys*, via the combination of its aggregation pheromone with LLINs for a potentially effective 'attract and kill' approach. This will lengthen *H. halys* arrestment on LLINs, thus ensuring a lethal dose uptake, providing farmers with an alternative to recurrent insecticide applications (Morrison et al., 2016), that while successful at managing *H. halys*, require additional labor due to weekly spray applications

(Morrison et al., 2019); LLINs can alleviate the need for weekly sprays. Considering *H. halys* perimeter-driven behavior (Leskey and Nielsen, 2018) and the fact that attract and kill has already proven successful in apple (Morrison et al., 2019) investigations on the placement of LLINs on baited apple trees in orchard borders is underway (T.C. Leskey, personal communication). Further inquiries are warranted, regarding the installation method and net size. Currently, limitations remain on the use of LLINs due to factors related to the registration of these products for pest management in agricultural settings, effects on non-target arthropods and natural enemies, and the acquisition of pyrethroid resistance.

In conclusion, exposing *H. halys* nymphs and adults to LLINs reduced their survivorship and mobility. Further research is required to elucidate the possible effects of sublethal doses of LLINs on physiological parameters of *H. halys* such as development and fecundity, feeding behavior as well as seasonal differences in mortality.

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Chapter 5

Satellite projects on *Halyomorpha halys*

The influence of marking methods on mobility, survivorship and field recovery of adult and nymphal *Halyomorpha halys* (Hemiptera: Pentatomidae)

Kirkpatrick, D.M., Rice, K.B., Ibrahim, A., Fleischer, S.J., Tooker, J.F., Tabb, A., Medeiros, H., Moshe Gish, M., Morrison, W.R. and Leskey, T.C., 2020. *Entomologia Experimentalis et Applicata* (in press).

Summary

Tracking insect movement is crucial to understand their behaviour, biology and population dynamics. Mark-capture and mark-release-recapture (MRR) techniques can be used to estimate feeding behaviour, dispersal, ecology and population densities in the field and therefore assist in providing framers with accurate monitoring tools and precise timing for interventions to mitigate crop losses. An ideal marker should not only be cost-effective, user-friendly, visible and non-toxic but also should not affect the behaviour, development, longevity or reproduction of the target insect. Furthermore, it should be retained on the insect for a sufficiently long period of time to conduct relevant studies. A variety of mass-marking techniques have been used with several insects including fruit flies, moths, beetles, mosquitoes and wasps. These involved the use of tags, paints, dyes, dusts, trace elements, radioactive isotopes and more recently, the use of protein and genetic marking.

In this study, we evaluated the influence of four fluorescent dusts on the mobility and survivorship of externally-marked *Halyomorpha halys* adults and nymphs under laboratory conditions. We also assessed the persistence of neon markings on the pronotum of *H. halys* adults recovered from soybean fields and woods 48 hours, 2 weeks and 3 weeks after release.

None of the four different colors of fluorescent dust used to mark *H. halys* affected the survival or development time of both adults and nymphs when compared with unmarked control over the 10-days study period. In addition, there was no significant difference in the horizontal mobility of *H. halys* adults and nymphs for all the studied parameters; distance moved, angular velocity and walking velocity. Similarly, the vertical mobility of *H. halys* adults and nymphs was not significantly influenced by the tested fluorescent dusts. For instance, the average vertical distance climbed ranged from 356 cm for pink fluorescent dust marked adults to 592 cm for green fluorescent dust treatment during the 15-min trials, compared with the unmarked control at 558 cm. As for nymphs, the averages reached 179 and 349 cm for orange and green fluorescent dust marked-individuals respectively, with 261 cm climbed by unmarked control. Marking adults with fluorescent dusts had no significant impact on their flight capacity, regardless of the color tested. The neon mark persisted for long durations (up to 3 weeks) on the pronotum of marked adults recovered from the field, however this marking method required more time to apply since each adult should be marked individually, whereas marking with fluorescent dust was applied to large groups (≤ 50 individuals) of adults at once. Our results prove that fluorescent dusts are a cost-effective marking tool that can be used reliably to better understand the dispersal behavior of *H. halys* adults and nymphs, without interfering in the insect's behavior and development.

Chapter 5

Influence of harmonic radar tag attachment on nymphal *Halyomorpha halys* (Hemiptera: Pentatomidae): mobility, survivorship and detectability

Kirkpatrick, D.M., Rice, K.B., Ibrahim, A., Morrison, W.R. and Leskey, T.C., 2019. *Entomologia Experimentalis et Applicata*, *167*(12), pp. 1020-1029. DOI: 10.1111/eea.12861

Summary

Dispersal of insects is one of the key features that contributed to their evolutionary success. Likewise, the movement of insect pests is a determining factor of their capacity to forage in complex environments and search for suitable hosts and mates, consequently impacting crop production. Therefore, understanding the movement of target pests, particularly invasive ones, can be instrumental for developing and enhancing monitoring and management programs. The tracking of individual insects mostly relied on the use of mark-release-recapture methods. The latter approach faces certain limitations related to low recapture rates, affecting the quality of the collected data. The development of refined harmonic radar techniques for entomological research offered scientists the possibility to continuously track insect movement, shedding new light on this behavior that can be exploited for enhancing integrated pest management efforts.

The harmonic radar tool operates by illuminating the tag attached on individual insects by a high-power microwave signal and recaptures the second harmonic of the transmitted signal radiating from the tag. This method has been adopted in the past decades and has proven to be

reliable in tracking the adult stages of several insect orders, including Coleoptera, Hymenoptera, Hemiptera, Lepidoptera and Diptera in their natural habitat.

A prerequisite of a successful tracking is the effective attachment of radar tags on individuals, without hampering their survival or normal behavior. Previous studies examined the effects of harmonic radar tag attachment on adult *Halyomorpha halys* by using harmonic radar to track their movements through landscapes. Since nymphal stages of *H. halys* are also known to feed on leaves, stems and fruits of host plants, their dispersal ecology thus deserves attention. In this study, we evaluated the impact of harmonic radar tag attachment on *H. halys* 4th and 5th instar nymphs and determined whether any negative effects on survivorship, movement and behavior would arise. Specifically, we evaluated the 1) retention of attached tags using four commercially available glues and their adhesive bond strength, 2) effect of tags on nymphal survivorship for 9 days following attachment with three glue types, 3) influence of tag attachment on horizontal and vertical walking behavior and 4) ability to track tagged *H. halys* nymphs using harmonic radar in the field in host (apple) and non-host plant (mowed grass) plots baited with pheromonal stimuli or left un-baited.

Our laboratory trials indicated that the Loctite glass glue treatment provided strong attachment of radar tags on nymphal instars, had no effect on movement after drop test and needed >65 g of force to be detached, making it suitable for use under natural conditions. Moreover, molting was not inhibited by the tag and nymphs could shed it along with their exuvia. We did not find any negative effects imposed by the minimal weight of the tag on the horizontal walking capacity of nymphs. In addition, tagged nymphs climbed on average 142.72 cm in 15 minutes, which was not statistically different than the distance climbed by untagged control.

In the field, our findings validated that *H. halys* nymphs can be successfully tracked and relocated on host and non-host plants, with or even without pheromonal stimuli 48 hours after release. Specifically, 83 % of nymphs were retained and tracked on baited and non-baited host plants, compared with 50 % in baited mowed grass plots. Our results demonstrated that portable harmonic radar system would enable tracking *H. halys* nymphs at various spatial scales and help understand their host use patterns, providing crucial information for improving pest management tactics and reducing crop damage.

Coupling traditional monitoring and citizen science to disentangle the invasion of *Halyomorpha halys*

Malek, R., Tattoni, C., Ciolli, M., Corradini, S., Andreis, D., Ibrahim, A., Mazzoni, V., Eriksson, A. and Anfora, G., 2018. *ISPRS International Journal of Geo-Information*, *7*(5), p.171. DOI: 10.3390/ijgi7050171

Summary

Invasive insect monitoring is a laborious process for a small group of scientists, who due to several limitations, cannot cover sufficient ground in space and in time to fully grasp the invasion. Citizen science is a powerful tool that may help circumvent these difficulties by enlisting volunteers who would work in close collaboration with expert scientists. This crowdsourcing approach enables students, farmers and everyday citizens to participate in scientific inquiries of local and regional significance, which also helps in raising awareness on issues of local and global concern.

To uncover the invasion of the brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), in the newly invaded North Italian region of Trentino, we adopted an approach that couples traditional monitoring and citizen science. BugMap, a freely available mobile application on both IOS and android was designed to allow citizens to report opportunistic sightings of the bug. Combining these BugMap reports with the systematic sampling carried out by technicians and scientists resulted in a large amount of data which we employed to better understand *H. halys* invasion.

We found that citizens provided coverage of areas that are usually out of reach for scientists and technicians. For instance, the majority of reports from buildings (99 %), private gardens (83 %), agricultural settings (78 %) and wild areas (62 %) were recorded by citizens. This highlights the significant contribution of BugMap users from highly diversified geographical areas. A seasonal classification of reports based on the locality of the sighting offered a better grasp of the stink bug invasion dynamics in Trentino. Our findings were analogous with what was already known about *H. halys*, it is a landscape level pest that aggregates and overwinters in dead or standing trees and anthropogenic structures. Rising temperatures in spring and longer days elicit an exit from man-made structures into adjacent fields in search for nutritive hosts and mates, a behavior that last until late summer. Afterwards, declining temperatures and shorter photoperiods in Autumn trigger the shelter-seeking behavior of the insects.

For modeling *H. halys* distribution we used MaxEnt, a machine learning algorithm that applies the principle of maximum entropy to predict the potential distribution of species from presence only data and a set of environmental parameters (digital elevation model, land use and hydrography). The jackknife test for assessing variable contribution revealed that digital terrain model, urban land use and distance from houses were factors that most affected *H. halys* dispersal. The model also revealed areas of high suitability for *H. halys* establishment in Trentino, including Val d’Adige, Val di Non, Valle dei Laghi and Valsugana. Urban and agricultural interfaces in these regions along with the availability of suitable hosts, might be facilitating the establishment of a polyphagous and public nuisance invader such as *H. halys*.

Our initiative of combining traditional monitoring and citizen science, termed monitoring 2.0, not only disentangled *H. halys* invasion in Trentino, but also helped contribute to elevating scientific citizenship across Trentino and other Italian regions.

Chapter 5

Can vibrational playback improve control of an invasive stink bug?

Polajnar, J., Maistrello, L., Ibrahim, A. and Mazzoni M., 2019. In Hill, P.S.M., Lakes-Harlan, R., Mazzoni, V., Narins, P.M., Virant-Doberlet, M. and Wessel, A. eds., *Biotremology: Studying Vibrational Behavior II*. Berlin: Springer, pp. 375-398. DOI: 10.1007/978-3-030-22293-2_19

Summary

To reduce the dependence of agricultural systems on pesticides, efforts are focusing on finding sustainable alternatives to protect crops against insect damage. In this regard, methods using stimuli to manipulate the behavior of target pests have been developed, such as the attract-and-kill and push-pull strategies. These methods exploit insects' sensory biases over a long distance, such as with the use of olfactory and visual stimuli or over a short distance via antifeedants and oviposition inhibitors. To a lesser extent, sounds and vibrational stimuli have been used to disrupt reproduction of leafhopper and planthopper pests as well as psyllids and beetles. Combining two or more stimuli could even lead to increased efficacy of the system via modifying pest distribution or inhibiting feeding, aggregation, reproduction and other insect behaviors.

The use of vibrations for pest management have been developing towards two options. The first is based on detecting pests in their natural habitat, yet this is faced by obstacles especially when dealing with highly mobile and cryptic insects such as stink bugs and the need for sophisticated and costly recording methods to identify them. The second option, however, seems more practical and involves methods to behaviorally manipulate target pests mainly by using artificial vibratory stimuli (playback).

In this book chapter, we reviewed the sexual behavior of *H. halys* described in an earlier study and described how the localization abilities of this bug were tested in another study. Moreover, we explored the potential of using vibrational playback in improving *H. halys* control.

The brown marmorated stink bug communicate via substrate-borne vibrational signals to recognize and find potential mates. Males are the more active partners and they initiate the vibrational communication by emitting MS1 signal that elicit receptive females to respond by emitting a similar FS1 signal and therefore initiating a duet. FS2 signals are later produced by the female triggering the male searching behavior to locate the calling female while repeatedly emitting MS2 signals. When the male encounters the female, the last male signal is emitted (MCrS) and abdomen vibration with rapid sideways shaking of the body can be seen until the insects come into physical contact.

In another study, the attractiveness of the female vibrational signal (FS2) to *H. halys* males was tested in different settings including natural substrates, arenas and a cage representing an acoustic trap, using video-tracking analysis. More than 50% of males were attracted to the source point and elicited a searching behavior. The ability of males to locate the vibrational sources varied depending on the type of substrate used.

To move this knowledge to the field, we explored the potential of using the FS2 female signal in manipulating the males by means of a prototype bimodal trap tested in a pear orchard in North Italy. We hypothesized that the male-female capture ratio in the bimodal trap (olfactory and vibrational stimuli together) would be higher in comparison with two commercially available pheromone traps (olfactory stimuli alone). The prototype trap was pyramid-shaped and had a square cardboard base and an electric shock was delivered to the internal parts of its cavities to kill the trapped bugs. Preliminary results showed a male-female capture ratio of 11.5:1 in the bimodal trap, which was substantially higher than that of the Dead-Inn pyramid (1:1) and clear sticky traps (0.6:1). Moreover, only a few non-target insect species were captured using the bimodal trap.

Optimization of the bimodal trap in terms of design, color and properties of the materials used as well as furthering our knowledge on the spectral and temporal characteristics of the vibrational playback are essential for maximizing its trapping capacity and refining this pest population reduction tactic.

Chapter 6

Conclusions and implications

This dissertation explores various aspects of the brown marmorated stink bug, *Halyomorpha halys*. After invading and successfully establishing its populations in North America and Europe, *H. halys* continues to create major agricultural and nuisance problems worldwide. Despite the increased disciplinary and interdisciplinary research efforts in recent years to mitigate the damage caused by this polyphagous pest, critical knowledge gaps still exist. The purpose of the studies covered in this dissertation was to address some of the missing links which will ultimately contribute to the development of *H. halys* management strategies.

It has been proved earlier that the antennae of *H. halys* play an important role in finding host plants, locating mates and in the reproductive and aggregation behaviors. A detailed characterization of the antennal sensilla of *H. halys* was reported for the first time, using scanning and transmission electron microscopy (Chapter 2). Five types of sensilla were distinguished on the antennae of adults and 5th instar nymphs with the vast majority showing characteristics typical of olfactory sensilla and were present on the flagellomeres. The morphological description and mapping data of these sensilla lay the foundation for future trials using the single sensillum recording technique to advance our understanding of the underlying mechanisms of *H. halys* sensory physiology and chemical ecology. Moreover, this would push forward the development of potent attractant (such as host-derived volatiles) and repellent formulations that increase the trapping efficacy of *H. halys* in agricultural and urban settings.

Another important line of research that remains understudied is the vibrational communication of *H. halys* and its underlying neuronal mechanisms. Substrate-borne signals carry both location and identity information that are crucial for the mating success of this species. Knowledge on the processing properties of the nervous system mediating this communication modality in *H. halys* is lacking. The structure and function of some of the vibration-sensitive interneurons found in the ventral nerve cord of adult *H. halys* were elucidated by combining intracellular recording and electrophoretic staining techniques (Chapter 3). Four neuron types were described morphologically and physiologically, and two additional neurons were characterized solely based on their physiology. Identifying the basic response characteristics of these neurons, i.e. frequency tuning, intensity response and response latency, as well as understanding their role in neuronal processing will bring us one step closer towards successfully manipulating the sexual behavior of *H. halys*. Without further research into the remaining neurons involved in processing vibrational signals, it will not be possible to achieve a more comprehensive view of this vital signaling system and, therefore, to successfully interfere with it. Another avenue for further study should address the sensory receptors responsible for detecting vibratory signals in *H. halys*.

The last research line in this dissertation focuses on the use of more sustainable management options against *H. halys* as alternatives to recurrent insecticide applications. Under laboratory conditions, the impact of exposure to a long-lasting insecticide net (LLIN) on the behavior and survivorship of adult and nymphal *H. halys* was quantified (Chapter 4). Our findings indicate reduced horizontal and vertical movement of adults and high nymphal and adult mortality rates. This highlights the promising potential of LLINs especially if combined with the aggregation pheromone of *H. halys* for a substantially effective ‘attract and kill’ approach. Further inquiries in the field are warranted to set the optimal installation method and net size as well as to investigate the effect of LLINs on non-target arthropods and natural enemies.

In conclusion, researchers have been trying to combat this aggressive pest for the past two decades. This dissertation sheds new light on the morphology, neurophysiology and behavior of *H. halys* and will hopefully contribute to the collective effort of the international scientific community.