UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Head Office: Università degli Studi di Padova

Department of Agronomy, Food, Natural resources, Animals and Environment (DAFNAE)

Ph.D. COURSE IN: CROP SCIENCE

SERIES XXXII

Seasonal population dynamics, thermal tolerance, and damage assessment of the invasive pest Brown Marmorated Stink Bug, *Halyomorpha halys*

Coordinator: Chiar.mo Prof. Sergio Casella

Supervisor: Chiar.mo Prof. Alberto Pozzebon

Ph.D. student: Davide Scaccini

Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the University or other institute of higher learning, except where due acknowledgment has been made in the text.

Padua, 29 September 2019 Davide Scaccini

A copy of the thesis will be available at *http://paduaresearch.cab.unipd.it/*

Dichiarazione

Con la presente affermo che questa tesi è frutto del mio lavoro e che, per quanto io ne sia a conoscenza, non contiene materiale precedentemente pubblicato o scritto da un'altra persona né materiale che è stato utilizzato per l'ottenimento di qualunque altro titolo o diploma dell'Università o altro istituto di apprendimento, a eccezione del caso in cui ciò venga riconosciuto nel testo.

Padova, 29 settembre 2019 Davide Scaccini

Una copia della tesi sarà disponibile presso *http://paduaresearch.cab.unipd.it/*

Table of contents

Riassunto

La cimice asiatica, *Halyomorpha halys*, è una specie invasiva nativa dell'Asia orientale, introdotta in USA, Europa e Sud America. Questo insetto dannoso si sviluppa a carico di più di 170 specie vegetali tra cui diverse colture frutticole, orticole, estensive e piante ornamentali. È in grado di causare danni alle colture e provocare fastidio alla popolazione umana quando si sposta nelle abitazioni per svernare.

Nella presente tesi sono stati considerati alcuni aspetti relativi ai processi di invasione, all'impatto del fitofago sulla vite e agli effetti di fattori abiotici (alte e basse temperature) e biotici (controllo biologico) che possono influenzare la dinamica di popolazione di *H. halys*.

Lo studio della struttura genetica dell'insetto associata a quella del suo simbionte primario, '*Candidatus* Pantoea carbekii', ha permesso di chiarire le vie di invasione della cimice asiatica. Alcune popolazioni del nord-est d'Italia hanno mostrato la più alta variabilità genetica, suggerendo eventi di colonizzazione multipli da aree diverse.

Nello studio delle dinamiche di popolazione stagionali di *H. halys* in vigneti multi-varietali è emersa una maggiore presenza dell'insetto su alcune cultivar a bacca rossa rispetto a quelle a bacca bianca, così come sono stati osservati livelli più elevati d'infestazione nelle zone di margine dei vigneti rispetto al centro. In studi specifici è emerso come le infestazioni di *H. halys* possano causare danni alle bacche, anche se è stata evidenziata una diversa suscettibilità varietale. Danni diretti sono rilevanti solo nel caso di elevati livelli d'infestazione, difficilmente osservabili in vigneto. Tuttavia è emersa una associazione tra l'incidenza di danni da *Botrytis cinerea* e la presenza di *H. halys*. Questo aspetto rappresenta la problematica principale dovuta alla cimice asiatica su vite e deve essere tenuta in considerazione nella definizione delle strategie di difesa.

Gli studi sulle esposizioni a basse e alte temperature hanno dimostrato che gli individui a fine svernamento sono più sensibili ai trattamenti termici rispetto a quelli che iniziano lo svernamento. Tra i primi, la mortalità degli insetti con livelli nutrizionali più bassi è stata maggiore in seguito alle esposizioni a basse temperature. Queste ultime hanno anche causato un aumento della longevità e una riduzione della fertilità nelle femmine svernanti. Lo studio evidenzia come l'esposizione a basse temperature possa influenzare il tasso di sopravvivenza e le dinamiche di popolazione di *H. halys*. Inoltre, le curve di mortalità derivate dallo studio sulle alte temperature possono essere impiegate per definire trattamenti termici per il controllo di *H. halys*.

Infine, tra i parassitoidi oofagi di *H. halys* è stato ritrovato in nord Italia la specie asiatica *Trissolcus mitsukurii*, che ha mostrato un importane impatto sulle uova dell'ospite. Grazie a dei marcatori molecolari è emerso come gli individui del parassitoide raccolti in Italia siano affini ai campioni raccolti in Giappone e Cina. Lo studio sul complesso di ooparassitoidi naturali di *H. halys* in nord Italia fornisce risultati promettenti sul controllo del fitofago, ma ulteriori indagini saranno necessarie per valutare l'insieme di possibili altri ospiti del parassitoide in Europa, così come i fattori che ne influenzano la distribuzione.

Summary

Brown marmorated stink bug, *Halyomorpha halys*, is an invasive pest species native from East Asia and introduced into the USA, Europe, and South America. This pest can feed on more than 170 plant species among orchards, vegetables, row crops, and ornamentals, and it causes damage on crops and nuisance to the human population due to its overwintering behavior.

Here we studied the invasion pattern of the pest, its impact on grapevine and the effect of abiotic (low and high temperatures) and biotic (natural enemies) factors that can influence the population dynamics.

The genetic structure of *H. halys* and of its primary symbiont, '*Candidatus* Pantoea carbekii', were studied to elucidate the pathways of expansion of the pest across the invaded area. Some populations from north-eastern Italy showed the highest values of genetic diversity, and possible multiple introductions from different countries are suggested considering the affinity with symbiont haplotypes.

Seasonal dynamics of *H. halys* in multi-cultivar vineyards and the damage on grape clusters were also studied, showing a higher *H. halys* abundance on red-berry cultivars than on white ones, on varieties ripening late in the season and on the border of the vineyards. *Halyomorpha halys* infestations caused damage on berries, showing a different cultivar and plant phenological stages susceptibility, and that damage was relevant only with high infestations, not commonly found in vineyards. *Botrytis cinerea* occurrence was correlated to *H. halys* presence, and this represents the main issue related to *H. halys* effect on the grapevine and should be considered in the definition of integrated pest management strategies on this crop.

Lethal temperature exposure studies showed that exiting diapause adults were more sensitive to low- and high-temperature exposures than entering ones. Insects with a low nutritional status showed higher mortality levels after low-temperature exposures. Low-temperature exposures resulted in an increase in longevity and a reduction of fecundity of entering diapause *H. halys* females. The results showed that low temperatures in winter and spring can have an impact on the survival rate and population development of *H. halys*. Moreover, the mortality-curves obtained from high-temperature exposures can be used for the definition of cost-effective heat treatments aimed at the *H. halys* control.

Finally, the *H. halys* egg parasitoid complex was studied. Here we found that *Trissolcus mitsukurii*, a species previously found in Asia, was recorded in northern Italy. This parasitoid shows a high impact on host eggs. By studying the genetic variability of the parasitoid population, the relation

between Italian samples and Japanese and Chinese ones was detected. The study performed on the egg parasitoid complex of *H. halys* in northern Italy provides promising possible avenues for future control of the pest. Further studies are needed to assess the host range of *T. mitsukurii* in Europe and the factors influencing its distribution.

Chapter 1

Introduction and aims

Invasive insects play a crucial role in pest management in agriculture. In Europe, more than 2.500 species of invertebrates are reported as agriculturally important invasive species (Vilà et al. 2010). In these habitats, they have significant influences on populations, communities, and ecosystems, and are found to reduce plant fitness, animal diversity and abundance (Cameron et al. 2016). As pest species, predators/parasitoids of the native fauna, or vectors of diseases, non-native species cause both ecologic and economic impacts (Kenis and Branco 2010; Vaes-Petignat and Nentwig 2014). These impacts are strongly reflected by high costs associated with these species (e.g., Pimentel et al. 2001; 2005; Pimentel 2007; Nentwig 2008; Kenis et al. 2017), but these costs may be difficult to estimate (Vilà and Hulme 2017). Invasive species are present in large numbers in arable lands, where agricultural practices generate new and more homogenous habitats (Roques et al. 2009; Fried et al. 2017).

The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), is an invasive pest species native from East Asia and introduced into the US in the mid-1990s (Hoebeke and Carter 2003), into Europe in the 2000s (Wermelinger et al. 2008), detected in South America in 2017 (Faúndez and Rider 2017), and intercepted in points-of-entry of other countries as in New Zealand (Duthie 2012; Vandervoet et al. 2019). It is a polyphagous species that feeds on more than 170 plant species among crops as fruit species, vegetables, row crops, and ornamentals (Leskey and Nielsen 2018). Adults are capable to long-distance flight (Lee and Leskey 2015; Wiman et al. 2015), and nymphs are particularly mobile (Lee et al. 2014). These features evidence *H. halys* pest status and influence management practices against this pest (Leskey and Nielsen 2018). *Halyomorpha halys* is also a nuisance problem in residential areas during fall and winter when the adults invade the buildings searching for shelters for overwintering (Hoebeke and Carter 2003; Inkley 2012; Leskey et al. 2012; Cambridge et al. 2015).

After the first European record of *H. halys* in Switzerland (Wermelinger et al. 2008), the species have become widespread in multiple countries (Haye et al. 2015; Leskey and Nielsen 2018; Musolin et al. 2018; Vétek et al. 2018). In Italy, after an earlier detection in the Liguria region (north-western Italy) in 2007 (Cianferoni et al. 2018), *H. halys* was detected in Modena-Reggio provinces (northern Italy) in 2012, where it started to damage crops (Maistrello et al. 2013; 2016; Bariselli et al. 2016).

In Europe, *H. halys* distribution originated and continues from multiple introduction events (Cesari et al. 2018). The Italian haplotype diversity of *H. halys* was very high, and the invasion of the Italian peninsula may have occurred from different pathways, from Switzerland and from Asia and/or USA (Cesari et al. 2015; Morrison et al. 2017). Using genetic marker in *H. halys* genome, several studies investigated the origin and the pathways of *H. halys* invasion across the continents (Cesari et al. 2015;

2018; Xu et al. 2014; Zhu et al. 2016; Valentin et al. 2017; Lee et al. 2018; Kapantaidaki et al. 2019). However, additional information can be provided by studying the genetic diversity of the primary, vertically-transmitted, symbiont of *H. halys*, '*Candidatus* Pantoea carbekii'.

Furthermore, the impact of *H. halys* on crops is noteworthy, especially in orchards where the damage is mainly due to the feeding activity of the insect (Leskey and Nielsen 2018), even though possible correlations between *H. halys* presence and fungal, bacterial and phytoplasma infections on fruits were reported for some plants (Leskey et al. 2012; Kamminga et al. 2014; Rice et al. 2014; Dobson et al. 2016; Paltrinieri et al. 2016; Moore et al. 2019). However, little information is available on the impact of *H. halys* on *Vitis vinifera* L., particularly in Europe and despite its high economic importance. On this host plant, previously published results showed that direct damages on berries can be detected (Hori 2000; Bettiga et al. 2013), and also indirect damages may be referred to *H. halys* infestations, as the reduction of the quality of the wine (Mohekar et al. 2016; 2017a,b; 2018).

It is well known that the impact of an invasive pest can be influenced by biotic and abiotic factors. Among the latter, climatic conditions play a key role in shaping the world distribution of a pest. The predicted occurrence of *H. halys* covers most of the horticultural areas in Europe, the Americas and in the Southern Hemisphere (Kriticos et al. 2017). Temperature ranges influence the distribution of an organism, and previous studies evidenced that consistently low temperatures are required to kill *H. halys* adults in winter (Cira et al. 2016; 2018; Lowenstein and Walton 2018; Chapter 5), and maximum temperature thresholds for development were lower than 35.0 °C (Haye et al. 2014), implying possible restrictions in the distribution of the pest. Furthermore, the quantification of the effect determined by extreme temperature exposures is increasing in its importance in the context of climate change. Here we determined the effect of low and high temperatures taking into account different physiological statuses of the insect (i.e., before or after the winter diapause).

Among the biotic factors impacting *H. halys* populations, biological control represents the most important. Natural enemies may play a role in *H. halys* population dynamics and regulation. Classical or augmentative biological control may be an alternative to consider in the long-term management of *H. halys* populations. For example, in Asia the pest is exploited by several egg parasitoids belonging to Encyrtidae, Eupelmidae and Scelionidae (Hymenoptera) (Lee et al. 2013; Abram et al. 2017). The area of invasion of the stink bug however may have an impact on indigenous natural enemies of *H. halys*. In most of cases the complex of natural enemies have been observed to be low and variable across surveys (Abram et al. 2017). Furthermore, new records of *H. halys* egg parasitoids were recently reported in Europe, in particular two *Trissolcus* species, *T. mitsukurii* (Ashmead) and *T. japonicus* (Ashmead) (Hymenoptera: Scelionidae), previously known only from Asia and accidentally introduced in new areas (Stahl et al. 2018; Sabbatini Peverieri et al. 2018), which can potentially impact the population dynamics of the pest in invaded areas.

Aims

The research activities of this study investigated different aspects related to *H. halys*, a dramatic invasive pest in Italy. In particular, the research was aimed at investigating the pathways of introduction of this pest by using a molecular approach. In this research the genetic diversity of *H. halys* and its bacterial symbiont was studied in native and invaded areas elucidating host-symbiont relationships in these regions. Furthermore, a second activity was aimed at investigating the impact of *H. halys* on grapevine in northern Italy, which is one of the most crucial perennial crop in Europe. Finally, some biotic and abiotic factors that can influence *H. halys* population dynamics were considered: effects of extreme temperatures and biological control.

References

Abram P.K., Hoelmer K.A., Acebes-Doria A., Andrews H., Beers E.H., Bergh J.C., Bessin R., Biddinger D., Botch P., Buffington M.L., Cornelius M.L., Costi E., Delfosse E.S., Dieckhoff C., Dobson R., Donais Z., Grieshop M., Hamilton G., Haye T., Hedstrom C., Herlihy M.V., Hoddle M.S., Hooks C.R.R., Jentsch P., Joshi N.K., Kuhar T.P., Lara J., Lee J.C., Legrand A., Leskey T.C., Lowenstein D., Maistrello L., Mathews C.R., Milnes J.M., Morrison W.R., Nielsen A.L., Ogburn E.C., Pickett C.H., Poley K., Pote J., Radl J., Shrewsbury P.M., Talamas E., Tavella L., Walgenbach J.F., Waterworth R., Weber D.C., Welty C., Wiman N.G., 2017. Indigenous arthropod natural enemies of the invasive brown marmorated stink bug in North America and Europe. Journal of Pest Science 90:1009-1020.

Bariselli M., Bugiana R., Maistrello L., 2016. Distribution and damage caused by *Halyomorpha halys* in Italy. Bulletin OEPP/EPPO Bulletin 46: 332-334.

Bettiga L.J., Varela L.G., Bentley W.J., 2013. Brown marmorated stink bug. In Grape Pest Management – 3rd Edition, Bettiga L.J., Ed. University of California – Agriculture and Natural Resources, California, pp. 187-188.

Cambridge J., Payenski A., Hamilton G.C., 2015. The distribution of overwintering brown marmorated stink bugs (Hemiptera: Penatatomidae) in college dormitories. Florida Entomologist 98: 1257-1259.

Cameron E.K., Vilà M., Cabeza M., 2016. Global meta-analysis of the impacts of terrestrial invertebrate invaders on species, communities and ecosystems. Global Ecology and Biogeography, 25: 596-606.

Cesari M., Maistrello L., Ganzerli F., Dioli P., Rebecchi L., Guidetti R., 2015. A pest alien invasion in progress: potential pathways of origin of the brown marmorated stink bug *Halyomorpha halys* populations in Italy. Journal of Pest Science 88: 1-7, doi:10.1007/s10340-014-0634-y.

Cesari M., Maistrello L., Piemontese L., Bonini R., Dioli P., Lee W., Park C.-G., Partsinevelos G.K., Rebecchi L., Guidetti R., 2018. Genetic diversity of the brown marmorated stink bug *Halyomorpha halys* in the invaded territories of Europe and its patterns of diffusion in Italy. Biological Invasions 20: 1073-1092.

Cianferoni F., Graziani F., Dioli P., Ceccolini F., 2018. Review of the occurrence of *Halyomorpha halys* (Hemiptera: Heteroptera: Pentatomidae) in Italy, with an update of its European and World distribution. Biologia 73: 599-607.

Cira T.M., Venette R.C., Aigner J., Kuhar T., Mullins D.E., Gabbert S.E., Hutchison W.D., 2016. Cold tolerance of *Halyomorpha halys* (Hemiptera: Pentatomidae) across geographic and temporal scales. Environmental Entomology 45: 484-491.

Cira T.M., Koch R.L., Burkness E.C., Hutchison W.D., Venette R.C., 2018. Effects of diapause on *Halyomorpha halys* (Hemiptera: Pentatomidae) cold tolerance. Environmental Entomology 47: 997-1004.

Dobson R.C., Rogers M., Moore J.L.C., Bessin R.T., 2016. Exclusion of the brown marmorated stink bug from organically grown peppers using barrier screens. HortTechnology 26: 191-198.

Duthie C., 2012. Risk analysis of *Halyomorpha halys* (brown marmorated stink bug) on all pathways. (https://www.mpi.govt.nz/dmsdocument/2909-halyomorpha-halys-brown-marmorated-stink-bug-risk-analysis-november-2012). Accessed on 29 September 2019.

Faúndez E.I., Rider D.A., 2017. The brown marmorated stink bug *Halyomorpha halys* (Stål, 1855) (Het.: Pentatomidae) in Chile. Arquivos Entomolóxicos 17: 305-307.

Fried G., Chauvel B., Reynaud P., Sache I., 2017. Decreases in crop production by non-native weeds, pests, and pathogens (pp. 83-101). In Impact of biological invasions on ecosystem services, Vilà M., Hulme P.E., Eds. Invading Nature – Springer Series in Invasion Ecology, vol. 12. Springer, Cham, Switzerland, 354 pp.

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

Haye T., Gariepy T., Hoelmer K., Rossi J.-P., Streito J.-C., Tassus X., Desneux N., 2015. Range expansion of the invasive brown marmorated stinkbug, *Halyomorpha halys*: an increasing threat to field, fruit and vegetable crops worldwide. Journal of Pest Science 88: 665-673.

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

Hori K., 2000. Possible causes of disease symptoms resulting from the feeding of phytophagous Heteroptera. In: Heteroptera of economic importance, Schaefer C.W., Panizzi A.R. (eds.). CRC Press, Boca Raton, Florida, pp. 11-35.

Inkley D.B., 2012. Characteristics of home invasion by the brown marmorated stink bug (Hemiptera: Pentatomidae). Journal of Entomological Science 47: 125-130.

Kamminga K., Herbert D.A., Toews M. D., Malone S., Kuhar T., 2014. Halyomorpha halys (Hemiptera: Pentatomidae) feeding injury on cotton bolls. Journal of Cotton Science 18: 68-74.

Kapantaidaki D.E., Evangelou V.I., Morrison W.R. III, Leskey T.C., Brodeur J., Milonas P., 2019. *Halyomorpha halys* (Hemiptera: Pentatomidae) genetic diversity in North America and Europe. Insects 10: 174, doi:10.3390/insects10060174.

Kenis M., Branco M., 2010. Chapter 5: Impact of alien terrestrial arthropods in Europe. In: Alien terrestrial arthropods of Europe, Roques A., Kenis M., Lees D., Lopez-Vaamonde C., Rabitsch W., Raspulus J.-Y., Roy D. (eds.). BioRisk 4(1): 51-71.

Kenis M., Roques A., Santini A., Liebhold A.M., 2017. Impact of Non-native Invertebrates and Pathogens on Market Forest Tree Resources (pp. 103-117). In: Impact of biological invasions on ecosystem services, Vilà M., Hulme P.E. (eds.). Invading Nature – Springer Series in Invasion Ecology, vol. 12. Springer, Cham, Switzerland, 354 pp.

Kriticos D.J., Kean J.M., Phillips C.B., Senay S.D., Acosta H., Haye T., 2017. The potential global distribution of the brown marmorated stink bug, *Halyomorpha halys*, a critical threat to plant biosecurity. Journal of Pest Science 90: 1033-1043.

Lee D.-H., Leskey T.C., 2015. Flight behavior of foraging and overwintering brown marmorated stink bug, *Halyomorpha halys* (Hemiptera: Pentatomidae). Bulletin of the Entomological Research 105: 566-573.

Lee D.-H., Short B.D., Joseph S.V., Bergh J.C., Leskey T.C., 2013. Review of the biology, ecology, and management of *Halyomorpha halys* (Hemiptera: Pentatomidae) in China, Japan, and the Republic of Korea. Environmental Entomology 42: 627-641.

Lee D.-H., Nielsen A.L., Leskey T.C., 2014. Dispersal capacity and behavior of nymphal stages of *Halyomorpha halys* (Hemiptera: Pentatomidae) evaluated under laboratory and field conditions. Journal of Insect Behavior 27:639-651.

Lee W., Guidetti R., Cesari M., Gariepy T.D., Park Y.L., Park C.G., 2018. Genetic diversity of *Halyomorpha halys* (Hemiptera, Pentatomidae) in Korea and comparison with COI sequence datasets from East Asia, Europe, and North America. Florida Entomologist 101(1): 49-55.

Leskey T.C., Nielsen A.L., 2018. Impact of the invasive brown marmorated stink bug in North America and Europe: history, biology, ecology, and management. Annual Review of Entomology 63: 599-618.

Leskey T.C., Hamilton G.C., Nielsen A.L., Polk D.F., Rodriguez-Saona C., Bergh J.C. et al., 2012. Pest status of the brown marmorated stink bug, *Halyomorpha halys* in the USA. Outlooks on Pest Management 23(5): 218-226.

Lowenstein D.M., Walton V.M., 2018. *Halyomorpha halys* (Hemiptera: Pentatomidae) winter survival, feeding activity, and reproduction rates based on episodic cold shock and winter temperature regimes. Journal of Economic Entomology 111: 1210-1218.

Maistrello L., Dioli P., Bariselli M., 2013. Trovata una cimice esotica dannosa per i frutteti. Agricoltura 6: 67-68.

Maistrello L., Dioli P., Bariselli M., Mazzoli G.L., Giacalone-Forini I., 2016. Citizen science and early detection of invasive species: phenology of first occurrences of *Halyomorpha halys* in Southern Europe. Biological Invasions 18: 3109-3116.

Mohekar P., 2016. Brown marmorated stink bug (BMSB), *Halyomorpha halys* taint in wine: impact on wine sensory, effect of wine-processing and management techniques. PhD Thesis, Oregon State University, Oregon, USA, 190 pp.

Mohekar P., Lapis T.J., Wiman N.G., Lim J., Tomasino E., 2017a. Brown marmorated stink bug taint in Pinot noir: detection and consumer rejection thresholds of trans-2-Decenal. American Journal of Enology and Viticulture 68: 120-126.

Mohekar P., Osborne J., Wiman N.G., Walton W., Tomasino E., 2017b. Influence of winemaking processing steps on the amounts of (E)-2-Decenal and Tridecane as off-odorants caused by brown marmorated stink bug (*Halyomorpha halys*). Journal of Agricultural and Food Chemistry 65: 872-878.

Mohekar P., Osborne J., Tomasino E., 2018. Effects of fining agents, reverse osmosis and wine age on brown marmorated stink bug (*Halyomorpha halys*) taint in wine. Beverages 4, doi:10.3390/beverages4010017.

Moore L.C., Tirello P., Scaccini D., Toews M.D., Duso C., Pozzebon A., 2019. Characterizing damage potential of the brown marmorated stink bug (Hemiptera: Pentatomidae) in cherry orchards. Entomologia Generalis in press

Morrison W.R. III, Milonas P., Kapantaidaki D.E., Cesari M., Di Bella E., Guidetti R., Haye T., Maistrello L., Moraglio S.T., Piemontese L., Pozzebon A., Ruocco G., Short B.D., Tavella L., Vétek G., Leskey T.C., 2017. Attraction of *Halyomorpha halys* (Hemiptera: Pentatomidae) haplotypes in North America and Europe to baited traps. Scientific Reports 7: 16941, doi:10.1038/s41598-017-17233-0.

Musolin D.L., Konjević A., Karpun N.N., Protsenko V.Y., Ayba L.Y., Saulich A.K., 2018. Invasive brown marmorated stink bug *Halyomorpha halys* (Stål) (Heteroptera: Pentatomidae) in Russia, Abkhazia, and Serbia: history of invasion, range expansion, early stages of establishment, and first records of damage to local crops. Arthropod-Plant Interactions 12: 517-529.

Nentwig W., 2008. Biological invasions: why it matters (pp. 1-6). In: Biological invasions, Nentwig W. (ed.). Ecological Studies (Analysis and Synthesis), vol. 193. Springer, Berlin, Heidelberg.

Paltrinieri S., Marani G., Francati S., Dindo M.L., Bertaccini A., 2016. Cimice asiatica confermata come insetto vettore di fitoplasmi. L'Informatore Agrario 45: 60-61.

Pimentel D., 2007. Environmental and economic costs of vertebrate species invasions into United States (pp. 2-8). In: Managing Vertebrate Invasive Species: Proceedings of an International

Symposium, Witmer G.W., Pitt W.C., Fagerston K.A, (eds.). US Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, National Wildlife Research Center.

Pimentel D., McNair S., Janecka J., Wightman J., Simmonds C., O'Connell C., Wong E., Russel L., Zern J., Aquino T., Tsomondo T., 2001. Economic and environmental threats of alien plant, animal, and microbe invasions. Agriculture, Ecosystems and Environment, 84: 1-20.

Pimentel D., Zuniga R., Morrison D., 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecological Economics, 52: 273-288.

Rice K.B., Bergh C.J., Bergmann E.J., Biddinger D.J., Dieckhoff C., Dively, G. et al., 2014. Biology, ecology, and management of brown marmorated stink bug (Hemiptera: Pentatomidae). Journal of Integrated Pest Management 5(3): 1-13.

Roques A., Rabitsch W., Rasplus J.-Y., Lopez-Vaamonde C., Nentwig W., Keniset M., 2009. Alien Terrestrial Invertebrates of Europe (pp. 63-79). In: Handbook of Alien Species in Europe, DAISIE. Invading Nature – Springer Series in Invasion Ecology, vol. 3. Springer, Dordrecht.

Sabbatini Peverieri G., Talamas E., Bon M.C., Marianelli L., Bernardinelli I., Malossini G., Benvenuto L., Roversi P.F., Hoelmer K.A., 2018. Two Asian egg parasitoids of *Halyomorpha halys* (Stål) (Hemiptera, Pentatomidae) emerge in northern Italy: *Trissolcus mitsukurii* (Ashmead) and *Trissolcus japonicus* (Ashmead) (Hymenoptera, Scelionidae). Journal of Hymenoptera Research 67: 37-53.

Stahl J., Tortorici F., Pontini M., Bon M.C., Hoelmer K., Marazzi C., Tavella L., Haye T., 2018. First discovery of adventive populations of *Trissolcus japonicus* in Europe. Journal of Pest Science 92: 371-379.

Valentin R.E., Nielsen A.L., Wiman N.G., Lee D.-H., Fonseca, D.M., 2017. Global invasion network of the brown marmorated stink bug, *Halyomorpha halys*. Scientific Reports 7: 9866.

Vaes-Petignat S., Nentwig W., 2014. Environmental and economic impact of alien terrestrial arthropods in Europe. NeoBiota 22: 23-42.

Vandervoet T.F., Bellamy D.E., Anderson D., MacLellan R., 2019. Trapping for early detection of the brown marmorated stink bug, *Halyomorpha halys*, in New Zealand. New Zealand Plant Protection 72: 36-43.

Vétek G., Károlyi B., Mészáros Á., Horváth D., Korányi D., 2018. The invasive brown marmorated stink bug (*Halyomorpha halys*) is now widespread in Hungary. Entomologia Generalis 38: 3-14.

Vilà M., Hulme P.E., 2017. Non-native species, ecosystem services, and human well-being (pp. 1-14). In: Impact of biological invasions on ecosystem services, Vilà M., Hulme P.E. (eds.). Invading Nature – Springer Series in Invasion Ecology, vol. 12. Springer, Cham, Switzerland.

Vilà M., Basnou C., Pyšek P., Josefsson M., Genovesi P., Gollasch S., Nentwig W., Olenin S., Roques A., Roy D., Hulme P.E., DAISIE Partners, 2010. How well do we understand the impacts of alien species on ecosystem services? A pan-European, cross-taxa assessment. Frontiers in Ecology and the Environment, 8: 135-144.

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

Wiman N.G., Walton V.M., Shearer P.W., Rondon S.I., Lee J.C., 2015. Factors affecting flight capacity of brown marmorated stink bug, *Halyomorpha halys* (Hemiptera: Pentatomidae). Journal of Pest Science, 88: 37-47.

Xu J., Fonseca D.M., Hamilton G.C., Hoelmer K.A., Nielsen A.L., 2014. Tracing the origin of US brown marmorated stink bugs, *Halyomorpha halys*. Biological Invasions 16: 153-166.

Zhu GP., Ye Z., Du J., Zhang D.L., Zhen Y., Zheng C., Zhao L., Li M., Bu W.J., 2016. Range wide molecular data and niche modeling revealed the Pleistocene history of a global invader (*Halyomorpha halys*). Scientific Reports 6: 23192.

Chapter 2

Molecular symbiont-host specificity as a tool to track multiple introduction events of the invasive pest *Halyomorpha halys* in northern Italy

Manuscript in preparation as: Molecular symbiont-host specificity as a tool to track multiple introduction events of the invasive pest *Halyomorpha halys* in northern Italy

Isabel Martinez-Sañudo[1], Davide Scaccini[1], Laura Marri[2], Alberto Pozzebon[1], Luca Mazzon[1]

[1]Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova, Italy;

[2]Dipartimento di Scienze della Vita, Università di Siena, Siena, Italy

Davide Scaccini contributed in study conceptualization, data collection and analysis as well as writing of the manuscript.

Abstract

Insect and symbiotic bacteria are widely observed in nature. Symbiotic bacteria can provide numerous advantages to their insect host. These microorganisms can also be used as genetic markers to investigate the phylogeography of its insect host. *Halyomorpha halys* is a globally invasive species, causing significant economic losses to agriculture, which harbors a primary, vertically-transmitted symbiont, '*Candidatus* Pantoea carbekii'. The genetic structure of this primary symbiont combined with that of its host was studied through the analyses of one bacterial marker (the putative pseudogene ΔybgF) and one mitochondrial gene (COI) to elucidate the pathways of expansion of the pest across the invaded area and, in particular, in Italy. New *P. carbekii* haplotypes were retrieved here, and an association pattern between the host and the symbiont haplotypes was observed. Some *H. halys* populations from the northeast of Italy showed the highest values of genetic diversity of the symbiont. These results highlights that northern Italy area could have seen multiple and potential ongoing introductions from native and other areas. Moreover, some of the symbiont-host haplotypes observed were shared only by populations from native areas (especially Japan) and north-eastern Italy. These data suggests that Japan is the most likely source of future introductions into Italy. These findings improve the understanding of the potential origin of multiple introductions in Italy, potentially helping to optimize management strategies.

1. Introduction

Mutualistic associations between insects and microorganisms are widespread in nature. In insect-bacteria associations, symbiotic bacteria provided several adaptive advantages to their host (Moran et al. 2005). They may contribute to the metabolism and digestion of ingested food, give resistance against parasites and natural enemies, affect longevity, and influence social interactions including mating partners' choice (Dillon and Dillon 2004; Douglas 2009; Sharon et al. 2010). Moreover, symbiotic bacteria have the potential to be used as genetic markers to investigate the host biodiversity and biogeographical patterns (Wang et al. 2018; Lashkari et al. 2014; Otero-Bravo et al. 2018). Primary symbionts are transmitted from the mother to their offspring (vertically) and events of co-diversification are often verified; thus they are expected to mirror the history of their hosts, helping to shed additional light on their invasion pathways (Savio et al. 2012; Wang et al. 2018). In some cases, symbiotic bacterial markers show even higher resolution than host loci (Otero-Bravo et al. 2018; Funk et al. 2000; Criscione et al. 2006).

Biological invasions of exotic pests to new areas are often facilitated by increased international trade and human movement during the last century. This change has caused important negative ecological and economic impacts in both agriculture and forestry. Information regarding the invasion pathways of the pest could be useful for improving the knowledge concerning the invasive species, such as colonizing capacity, adaptability and behavior (Brown et al. 2011; Simberloff et al. 2013; Maynard et al. 2017), and consequently defining control strategies.

The invasive pest *Halyomorpha halys* harbours a primary, vertically-transmitted, symbiont inhabiting specialized midgut ceca of the host, '*Candidatus* Pantoea carbekii' (hereafter referred to as *P. carbekii*) which may facilitate survival and development by providing essential nutrients (Kenyon et al. 2015). Native to Southeast Asia, *H. halys* has recently invaded North America and Europe causing substantial damage to agricultural crops and creating nuisance to residents in rural and urban areas (Leskey and Nielsen 2018). In Europe, since its first occurrence in Switzerland in 2004 (Wermelinger et al. 2008), this extremely polyphagous species has rapidly spread to other 12 European countries (Arnold 2009; Heckmann 2012; Callot and Brua 2013; Maistrello et al. 2014; Gariepy et al. 2014; Milonas and Partsinevelos 2014; Vétek et al. 2014; Cesari et al. 2015; Macavei et al. 2015; Rabitsch and Griebe 2015; Šeat 2015; Maurel et al. 2016; Dioli et al. 2016; Simov 2016), as well as Russia, Abkhazia and Georgia (Gapon et al. 2016; Musolin et al. 2018). Based on the newly reported distribution points and recent modeling approaches, its dispersal will likely continue in the coming years (Kriticos et al. 2017; Kapantaidaki et al. 2019). Several efforts have been made to identify the origin and the pathways of *H. halys* dispersal across continents using genetic markers. Most of the studies have focused mainly on the use of the mitochondrial Cytochrome Oxidase I and Cytochrome Oxidase II genes (Cesari et al. 2015; 2018; Xu et al. 2014; Zhu et al. 2016; Valentin et al. 2017; Lee et al. 2018; Kapantaidaki et al. 2019). To our knowledge, only one study has attempted to resolve the genetic structure and origin of this recently invading species considering some pseudogenes of the symbiont (e.g., ΔybgF), *P. carbekii*, as a genetic marker (Otero-Bravo et al. 2018). In this study, the bacterial genetic marker did not explicitly incorporate geographic information among populations probably due to the limited sampling, especially in Europe.

Here, we investigated the genetic structure of the primary symbiont *P. carbekii* of the invasive pest *H. halys* through the analyses of the putative pseudogene ΔybgF. Special attention was given to invasive populations of Italy, where high levels of genetic variability of the insect host have been recently reported (Morrison et al. 2017; Cesari et al. 2018). The obtained data were combined with data regarding the mitochondrial gene (COI) of the insects harboring the analyzed symbiotic bacteria. Both native and newly introduced populations were analyzed to elucidate the pathways of expansion of the pest across the invaded area.

2. Material and Methods

2.1. Sample collection and preparation

Adults of *H. halys* were collected in three successive years (2017–2019) in 3 countries within their native ranges (China, Korea and Japan) and 3 countries of the new invaded areas (United States, Italy and Hungary). Extensive sampling was conducted in Italy that encompassed the northern regions where the pest is mostly present (Emilia-Romagna, Friuli-Venezia Giulia, Liguria, Lombardy, Trentino-Alto Adige and Veneto). In total, 24 localities were sampled. Localities within a 40 km range were grouped and considered as a single population with an average number of at least 9 individuals (Table 1). Samples were preserved in 96% ethanol and shipped to the laboratory where insects were morphologically identified and subsequently stored in 96% ethanol at -20°C until being processed.

The abdomen of the insects was dissected under a stereomicroscope in a laminar flow hood using sterile equipment and sterile water. The tract of the intestine where the symbionts are harboured (V4 region) was extracted, transferred to Eppendorf tubes and kept at -20 °C for further analysis.

Table 1. Geographical localization and collection sites of *Halyomorpha halys* samples and number of specimens analyzed for every molecular marker (ΔybgF for the symbiont and COI for the host) used.

Country	**Region**	**Locality**	**Latitude**	**Longitude**	**n samples *P. carbekii***	**n samples *H. halys***
China	Jingjinji, Beijing	Changping District	40°18'16,3"N	116°11'23.5"E	7	10
		Beijing, Huairou	40°24'39.47"N	116°17'55.32"E	8	10
		Shijingshan District	39°56'29.03"N	116°10'19.37"E	5	7
Hungary	Central Hungary	Budapest	47°28'49.3"N	19°02'27.1"E	18	24
Italy	Emilia Romagna	Bologna, Vill. di Castenaso	44°30'34.0"N	11°26'13.1"E	4	4
		Piacenza	45°02'14.7"N	9°43'49.2"E	16	17
	Friuli-Venezia Giulia	Moruzzo	46°07'20.4"N	13°07'04.5"E	9	9
		Udine	46°01'13.2"N	13°01'45.9"E	6	6
	Liguria	Toirano	44°07'36.8"N	8°11'58.6"E	3	4
	Lombardy	Cremona	45°08'37.5"N	9°59'21.4"E	9	7
		Casolate, Lodi	45°24'02.2"N	9°26'19.4"E	4	6
		Ferno-Milano	45°37'50.8"N	8°43'31.3"E	4	4
	Trentino-Alto Adige	Rovereto	45°53'31.3"N	11°02'49.9"E	15	15
	Veneto	Conegliano	45°53'05.2"N	12°16'36.7"E	3	3
		Legnaro	45°20'49.0"N	11°57'26.2"E	12	11
		Mogliano	45°33'29.81"N	12°16'16.03"E	2	3
		Montechio Maggiore	45°33'05.5"N	11°23'59.7"E	10	11
		Noale	45°29'43.5"N	12°03'26.8"E	11	11
Japan	Ibaraki	Tsuchiura-shi	36°10'17.58"	140°09'46.39"E	12	13
Korea	Gimje	Jeollabuk	35°52'12.68"	126°57'48.52"E	10	12
	Sacheon	Gyeongsangnam	35°03'29.31"	128°05'36.68"E	3	6
USA	California	Central Valley	37°44'49.2"N	121°54'57.2"W	6	8
	Georgia	Tifton	31°28'26.4"N	83°31'50.1"W	2	2
	Oregon	Salem	44°53'59.70"N	123°06'37.60"W	15	16

2.2. Genetic analysis

DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions.

The putative pseudogene ΔybgF previously used by Otero-Bravo et al. (2018) was selected to analyze the genetic variability of the symbiont. Amplifications were performed using the couple of primers dYbg-F and dYbg-R (Otero-Bravo et al. 2018) in 20 µl reactions (1x PCR Go Taq Flexi buffer – Promega, 2.5 mM MgCl2, 0.1 mM dNTPs, 0.5 µM for each primer, 0.5 U of Taq polymerase – Promega, 2 µl DNA template). Thermal cycling conditions were 3 min at 95 °C followed by 30 cycles of 95 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 30 sec with a final extension of 72 °C for 2 min.

The bacterial 16S rRNA gene was also amplified in a representative number of samples for every country by PCR using the bacterial primers designed for *P. carbekii* (Forward symbiont and Reverse symbiont) by Bansal et al. (2014), and a cycling program consisting of a 95 °C 2 min step followed by 35 cycles at 96 °C for 30 s, 56 °C for 30 s, 72 °C for 90 s and a final extension at 72 °C for 10 min.

Genetic diversity of the host *H. halys* was studied on a region of the mitochondrial DNA corresponding to a fragment of the cytochrome c oxidase subunit 1 (COI), which was amplified using the universal primer pairs LCO-1490/HCO-2198 (Folmer et al. 1994) in the same samples where the bacterial symbiont was analysed.

PCR products were checked via electrophoresis on 1.0% agarose gels stained with SYBR® (Invitrogen), purified using Exonuclease and Antarctic Phosphatase (GE Healthcare) and sequenced at the BMR Genomics Service (Padua, Italy).

2.3. Data analysis

Sequences were edited and aligned using MEGA X (Kumar 2018). A GenBank BLAST analysis of the sequences obtained was run through the NCBI website (www.ncbi.nlm.nih.gov) to assess the identity of the sequences.

Haplotype and nucleotide diversity of every population, considering both symbiont and host markers, were calculated with Arlequin 3.5 (Excoffier and Lischer 2010) using a Kimura 2-parameters model.

Sequences of *H. halys* and *P. carbekii* available in the NCBI database were added to our datasets in order to reconstruct a haplotype network for both the symbiont and the host. A statistical parsimony haplotype networks were inferred using the software PopART 1.7 (Leigh and Bryant 2015).

Associations host-symbionts haplotype were represented in a bipartite network generated by the bipartite package (Dormann et al. 2008) in R 3.0.1 software (R Development Core Team – http://www.r-project.org) and the association was compared using generalized linear mixed model with the proc GLIMMIX of SAS (ver. 9.4) with a logit link function and tested using a χ^2 test (α = 0.05).

3. Results

3.1. *P. carbekii* symbiont

In total, 194 sequences targeting the putative pseudogene ΔybgF were obtained. A similarity > 99 % with *P. carbekii* was obtained when comparing all the sequences with the GenBank database. A total of 6 variable sites were observed after aligning the sequences with three of them being parsimony informative sites.

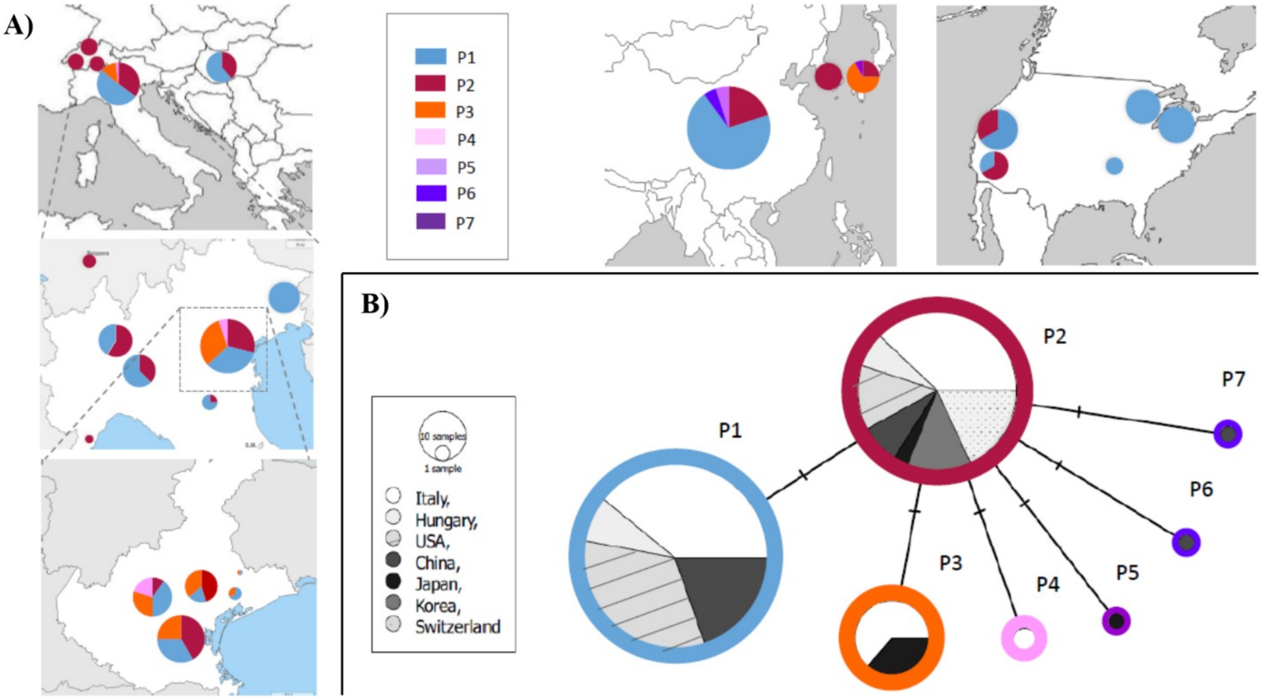
The haplotype diversities (H) of the symbiont populations varied between 0.00 and 0.78, with the population from Montecchio Maggiore (Italy) showing the highest values and populations from Korea, and Udine-Moruzzo (Italy) showing the lowest ones (Table 2). The nucleotide diversities (π) ranged from 0.00% and 0.65%. Population from Montecchio Maggiore (Italy) displayed the highest value, while populations from Korea and Udine-Moruzzo (Italy) showed the lowest variability. These diversity indexes were calculated only in populations with more than five samples.

Table 2. Descriptive statistics of the endosymbiont *Pantoea carbekii* and the host *Halyomorpha halys* with the bacterial and the mitochondrial marker respectively.

Country	**Locality**	***Pantoea carbekii***				***Halyomorpha halys***			
		Haplotypes	**n hap**	**H**	**π (%)**	**Haplotypes**	**n hap**	**H**	**π (%)**
China	Changping District	hA(2), hB(4), hE (1)	4	0.6000	0.38%	h1(4), h3 (3), h7 (1), h65 (2)	5	0.6125	0.22%
	Beijing, Huairou	hA(2), hB(5), hF(1)				h1(7), h3 (2), h64 (1)			
	Shijingshan District	hA(1), hB (4)	2	0.4000	0.19%	h1(5), h3 (1), h13 (1)	3	0.5238	0.12%
Hungary	Budapest	hA(7), hB (11)	2	0.5033	0.24%	h1(21), h3 (3)	2	0.2283	0.05%
Italy	Bologna, Vill. di Castenaso	hA(1), hB (3)	2			h1(3), h3 (1)	2		
	Piacenza	hA(6), hB (10)	2	0.5200	0.25%	h1(9), h3(4), h8(2), h52(1), h54(1)	5	0.6443	0.26%
	Cremona	hA(6), hB (3)				h1(3), h3(3), h8(1)			
	Casolate, Lodi	hA(1), hB (3)	2			h1(3), h3(3)	2		
	Ferno-Milano	hA(3), hB (1)	2			h1(2), h3(2)	2		
	Moruzzo	hB(9)	1	0	0.00%	h1(9)	1	0	0.00%
	Udine	hB(6)				h1(6)	1		0.00%
	Toirano	hA(3)	1			h1(1), h3(1), h52(1), h54(1)	4		
	Rovereto	hA(7), hB (6), hC(2)	3	0.6476	0.37%	h1(8), h3(4), h23(1), h53(2)	4	0.6667	0.47%
	Conegliano	hB(1), hC(1)	2	0.6000	0.58%	h1(1), h53(2)	7	0.5333	0.76%
	Mogliano	hB(2), hC(1)				h1(5), h3(1), h23(1), h53(1), h54(1), h58(1), h59(1)			
	Legnaro	hA(5), hB(4), hC(3)	3	0.6798	0.41%	h1(3)	3	0.8095	0.76%
	Noale	hA(5), hB(2), hC(4)				h1(5), h53(4), h40(2)			
	Montecchio Maggiore	hA(1), hB(4), hC(3), hG(2)	4	0.7778	0.65%	h1(2), h3(1), h23(2), h53(5), h58(1)	5	0.6909	0.81%
Japan	Tsuchiura-shi	hA(3), hC(8), hD(1)	3	0.5303	0.31%	h23(1), h53(1), h57(4), h60(2), h61(1), h62(4)	6	0.8333	0.38%
Korea	Jeollabuk	hA(10)	1	0.0000	0.00%	h2(1), h22(9), h63(1), h66(1)	4	0.4545	0.10%
	Gyeongsangnam	hA(3)	1			h2(2), h22(4)	2	0.1594	0.03%
USA	Center Valley	hA(4), hB(2)	2	0.5333	0.26%	h1(2), h3(6)	2	0.4286	0.09%
	Tifton	hB(2)	1			h1(2)	1		
	Salem	hA(4), hB(11)	2	0.4190	0.20%	h1(3), h3(1), h23 (1), h56(1), h67(1), h79(1), h1017(8)	7	0.7417	0.57%

The alignment of 11 sequences of the 16S rRNA, 741 bp long, did not show any polymorphic site. A statistical parsimony network was constructed, adding to our dataset the sequences of *P. carbekii* available in NCBI. These data showed 7 different haplotypes (Figure 1A). Five of them were retrieved in this study for the first time. The haplotype names followed the nomenclature given by Otero-Bravo et al. (2018). All the haplotypes retrieved were separated by only one mutational step from haplotype P2. Two of these haplotypes (P1 and P2), already identified by Otero-Bravo et al. (2018), included a high number of sequences 100 (38%) and 138 (52%), respectively. Haplotype P3 included 22 samples while P4 was represented by 2 samples. The remaining haplotypes (P5, P6, P7) included only one sequence. The most frequent haplotype (P1) was represented by populations sourced from China (native area) and all populations studied within the newly invaded areas, except Toirano (Italy) and Switzerland populations (Figure 1). Haplotype P2 was found in all the populations analysed in this study except Udine-Moruzzo (Italy). In particular, it was exclusive in populations from Korea, Toirano (Italy) and Switzerland. The third haplotype P3 was present in 4 populations from the northeastern Italy (Rovereto, Conegliano-Mogliano, Legnaro, Noale, and Montecchio Maggiore) and population from Japan. Haplotype P4 included two samples from Montecchio Maggiore (Italy) while P5, P6, and P7 were, rare haplotypes represented by samples from the native area (Figure 1).

Figure 1. Phylogenetic reconstructions of the *Pantoea carbekii* populations and their geographical distribution. A) Map showing the proportional geographic distribution of the *P. carbekii* haplotypes across sampled populations. B) Haplotypes network constructed in PopART. Each haplotype is represented by a circle, with the area of the circle proportional to its frequency. Different colors represent geographic distribution. Hatch mark represents a single mutation while small black dots symbolize intermediate missing or unsampled haplotype.



3.2. Insect host

A total of 218 specimens of *H. halys* representing the 14 populations studied were successfully sequenced and a fragment of 490 bp of COI was obtained for all the individuals. Routinely the analyses of host mitochondrial DNA was performed in the same samples studied for the symbiont 16S rRNA.

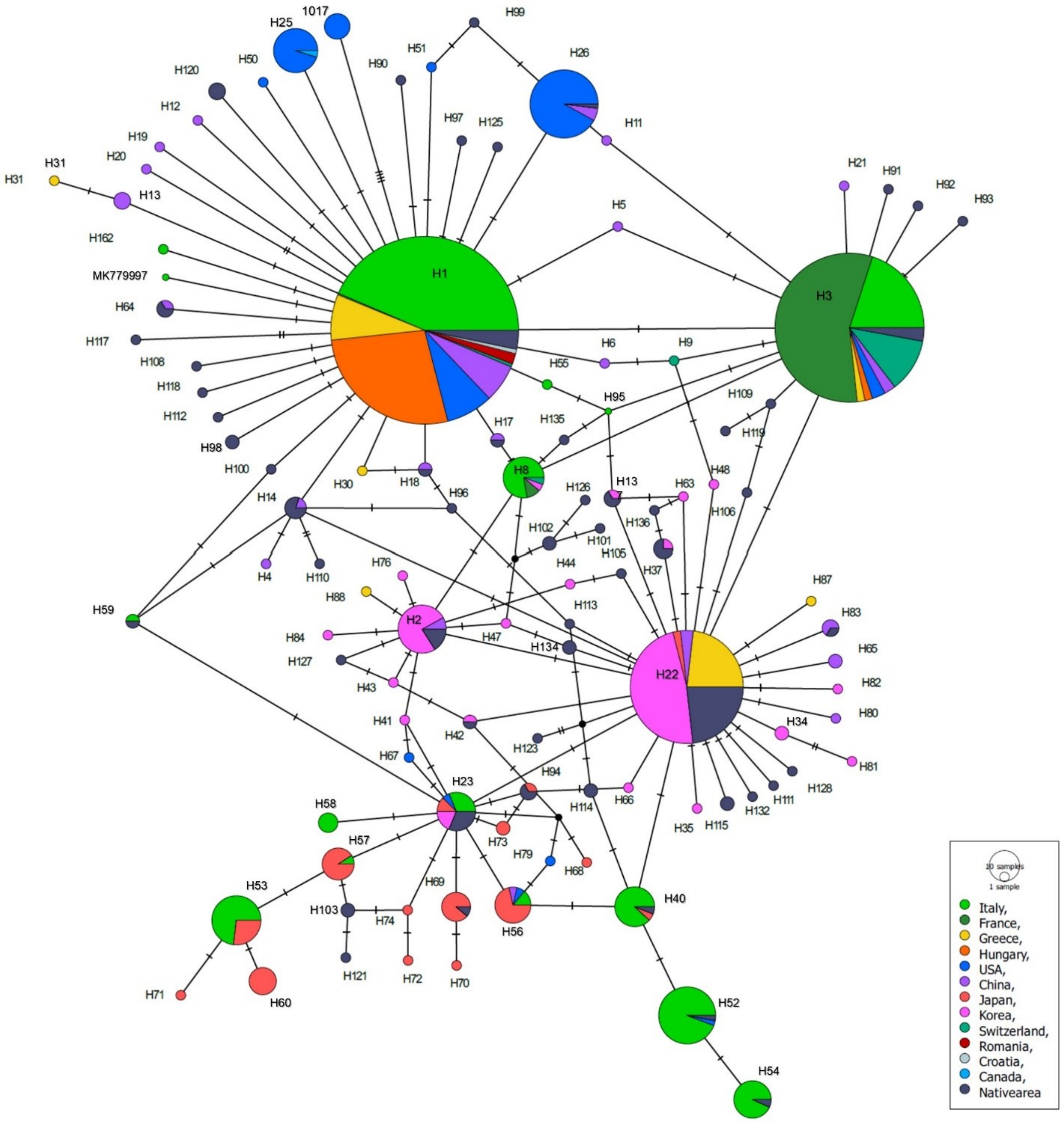
The comparison of all the sequences with GenBank database showed a similarity > 99% with *H. halys*. Alignment of sequences showed a total of 20 polymorphic sites, which resulted parsimony informative. Diversity indexes ranged between 0.23 and 0.83 for the haplotype diversity (H) and between 0.00% and 0.81% for the nucleotide diversity (π; Table 2). Population from Japan followed by Legnaro-Noale (Italy) population, showed the highest H values while population from Hungary and Udine-Moruzzo the lowest. Regarding nucleotide diversity, the population from Montecchio Maggiore (Italy) displayed the highest value and the population from Udine-Moruzzo showed the lowest variability. As for the symbiont data, populations with less than 5 samples were not considered in the former analysis.

Sequences of *H. halys* present in GenBank (Xu et al. 2014; Gariepy et al. 2014; 2015; Cesari et al. 2015; 2017; Morrison et al. 2017; Valentin et al. 2017; Dhami et al. 2016; Zhu et al. 2016; Lee et al. 2018; Kapantaidaki et al. 2019) were added to our dataset to construct a haplotype network obtaining a final data set of 1153 sequences. A total of 123 haplotypes could be observed in the network in which 20 were shared by samples of NCBI and this study, 98 were represented by samples of NCBI and 5 were exclusive of this study.

The topology of the network matched with the one obtained by Valentin et al. (2017). This was characterized by the presence of two frequent and spread haplotypes (H1 and H3) and many rare haplotypes. H1 and H3 included samples from all the countries of the invaded area (Canada, USA, Croatia, France, Greece, Hungary, Italy, Romania and Switzerland) and few samples from China within the native area (Figure 2).

A geographic structure of the native countries studied can be observed in the haplotypes network. The two haplotypes H2 and H22, including samples from Korea, and the two haplotypes H1 and H3 with Chinese samples were separated by only one mutational step. Each of these haplotypes showed several rare haplotypes connected in a star-shape way. Japanese samples were included in many rare haplotypes connected among them and scattered only in a portion of the network. Some of them were shared with samples from Italy (Veneto Region; Figure 2).

Figure 2. Parsimony network created in PopART showing relationships between the different *Halyomorpha halys* populations. Numbers denote haplotype identifiers. Hatch mark represents a single mutation while small black dots symbolize missing intermediate or unsampled haplotypes. Different colors represent geographic distribution.

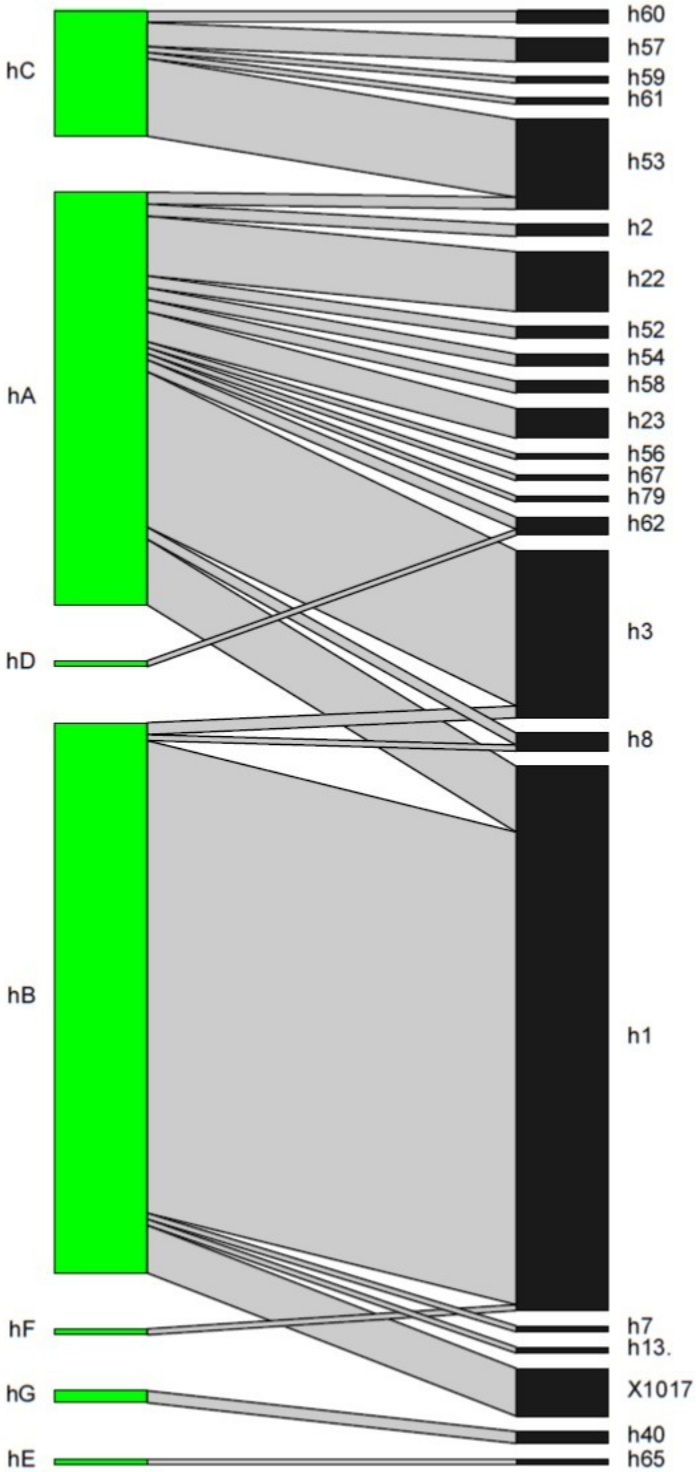


3.3. Pattern of haplotype association host–symbiont

A bipartite interaction matrix between the haplotypes of the host and the symbiont haplotypes was built considering only the samples analyzed in this work. The network of interactions consisted of 7 *P. carbekii* haplotypes and 23 *H. halys* haplotypes and presented a significantly nested pattern ($\chi^2 = 245.29$; $p < 0.0001$; Figure 3).

The most frequent *P. carbekii* haplotype (P1) is linked only by 4 host haplotypes and is associated more frequently with h1 host compare to the other (Figure 3). The haplotype *P. carbekii* P2 is shared by 14 host haplotypes, but the highest frequency was found with h3. The haplotype P3 is shared with five host haplotype, being h53 the most frequent one. Two *P. carbekii* haplotypes (P4 and P6) were one-to-one associations.

Figure 3. Weighted bipartite networks of interactions between the symbiont *Pantoea carbekii* (green bars) and *Halyomorpha halys* host (black bars). The width of the left bars reflects the relative frequency of each of the *P. carbekii* haplotypes, whereas the width of the right bars reflects the relative frequency of *H. halys* haplotypes. The width of the grey lines connecting bars indicates the frequency with which a symbiont's haplotype was found in a given host's haplotype.



4. Discussion

The obligate-associated symbiont of *H. halys, P. carbekii,* was used to better describe the possible invasion routes of the pest. The areas of northern Italy where high levels of genetic variability of *H. halys* have been reported were of particular interest in this study. The association found between the host-symbiont haplotypes and new *P. carbekii* haplotypes retrieved in this study made it possible to to understand the phylogeographical patterns of this economically important pest.

High levels of genetic variability were observed in several populations from both the native and the new invaded areas, regardless of the two markers used (ΔybgF and COI). The slightly lower variability observed in *P. carbekii* can be explained by the vertical model of transmission of the symbiont from mother to offspring. The strong bottlenecks experienced upon this way of transmission through host generations could reduce effective population size and magnified impacts of genetic drift leading to the reduction in the genetic variability (Wernegreen 2002; 2015). Alternatively, the slightly lower genetic variability in *P. carbekii* may be explained by the shorter length of the marker used.

The significant but not perfect association found between the symbiont and host haplotypes lead us to assume the presence of accidental events of horizontal transmission. Offspring acquire the symbiont, previously smeared by the mother, from the egg surface immediately after hatching (Bansal et al. 2014). This could offer chances for possible horizontal symbiont transfer, bypassing the model of strict vertical transmission.

Across the invaded areas, some populations from the northeast of Italy showed the relatively highest values of haplotype and nucleotide diversity considering both the mitochondrial and the *P. carbekii* markers. Genetic variability in invaded area is often increased when multiple introductions occurred (see Dlugosch and Parker 2008). Thus, populations present in northern Italy likely resulted from multiple and ongoing introductions from different parts of the native areas or even from other invaded areas.

Together, the information provided by both the host and the symbiont markers allowed us to observe some dispersion patterns of the invasive pest. The symbiont haplotype P2 linked to the host haplotype h3 included samples from China (within the native areas), Switzerland, Italy (mostly northwestern areas), the USA and Hungary (within the invaded areas). In accordance with what proposed by other authors (Gariepy et al. 2014; Valentin et al. 2017; Cesari et al. 2018) and considering the date of the first reported record of *H. halys* in Europe, the presence of this haplotype in Italy could be the result of its spread from China to Switzerland and then subsequent introductions to the northern Italy.

Moreover, host samples with haplotype h23 harboring the symbiont P2 were found in Veneto, Trentino-Alto Adige and in Japanese populations, suggesting the introduction of *H. halys* samples from Japan or closest native areas into north-eastern Italy (Veneto and Trentino-Alto Adige). Furthermore, the presence of other Italian *H. halys* specimens sharing the same haplotypes than native samples (P2 for the symbiont associated to the host h52, h53 and h54) supports the former hypothesis of further introductions in northeast Italy from native areas.

The endosymbiont's haplotype P1 strongly associated to H1 retrieved in all the China populations was the most spread haplotype across the invaded areas including Europe and USA. The widespread of this symbiont-host haplotypes (h1-P1) in the newly invaded areas may be attributed to a single or few introductions of this successful h1-P1 haplotype that has served as a source of subsequent colorizations of other European and American territories. Moreover, this widespread haplotypes found in new areas may be the result of multiple invasions by samples sharing the same Chinese haplotype (h1-P1), possibly due to the commercial trades.

Among the 5 newly retrieved symbiont haplotypes in this study, P3 was most frequently found. This haplotype was shared between samples from Japan and northeastern Italy, highlighting that possible introductions from Japan to Italy occurred in the recent past. In addition, haplotype P4 included samples from northeastern Italy with host haplotype h40 shared by native and Italian samples strengthening the hypothesis of multiple introductions in northeastern Italy. This trend is particularly evident in the populations from Veneto Region (Montecchio Maggiore) that showed the totality of the symbiont haplotypes retrieved in the invaded area (Table 2). The high diversity retrieved in northeastern Italy could be explained by a large amount of national and international commercial trade. This assertion is also supported by the presence in this area of two of the largest Italian ports importing high volume of solid commodities (Rassati et al. 2015). In fact, the ability to disperse undetected through phytosanitary checks significantly increases the risk of frequent accidental introductions to previously uncolonized regions (Duthie 2012; Haye et al. 2015a; Vandervoet et al. 2019).

Overall, this genetic study focused on the primary symbiont of *H. halys* has improved the understanding of the potential origin of accidental introductions to Italy. Considering these facts, it is of utmost importance to work collaboratively to develop a specific strategic plan to more successfully limit the flow of exotic organisms. The results herein provide a framework for future research that could help to optimize specific monitoring programmes of material trade between national and international authorities. The finding obtained here could have potential implications in recently proposed management tactics targeting symbionts transmission interruption by the use of micronutrient fertilizer with antimicrobial activity (Gonella et al. 2019). Whatever different symbiont haplotypes are related to differences in susceptibility to antimicrobial products could be considered in future studies.

Acknowledgments

We would like to thank all the people helped in collection of *H. halys* specimens from different areas.

This research was partially supported by the Regione Veneto U.O. Fitosanitario and by DAFNAE through project BIRD167802/16 to AP.

References

Arnold K., 2009. *Halyomorpha halys* (Stål, 1855), Eine fur die europaische Fauna neu nachgewiesene Wanzenart (Insecta: Heteroptera: Pentatomidae: Cappaeini). Mitteilungen des Thüringer Entomologogenverbandes 16, pp. 19.

Bansal R., Michel A., Sabree Z., 2014. The crypt-dwelling primary bacterial symbiont of the polyphagous pentatomid pest *Halyomorpha halys* (Hemiptera: Pentatomidae). Environmental Entomology 43(3): 617-625.

Brown J.E., Scholte E.J., Dik M., Den Hartog W., Beeuwkes J., Powell J.R., 2011. *Aedes aegypti* mosquitoes imported into the Netherlands, 2010. Emerging Infectious Diseases 17: 2335.

Callot H., Brua C., 2013. *Halyomorpha halys* (Stål, 1855), la Punaise diabolique, nouvelle espece pour la faune de France (Heteroptera Pentatomidae). L'Entomologiste 69: 69-71.

Cesari M., Maistrello L., Ganzerli F., Dioli P., Rebecchi L., Guidetti R., 2015. A pest alien invasion in progress: potential pathways of origin of the brown marmorated stink bug *Halyomorpha halys* populations in Italy. Journal of Pest Science 88: 1-7.

Cesari M., Maistrello L., Piemontese L., Bonini R., Dioli P., Lee W., Park C.G., Partsinevelos G.K., Rebecchi L., Guidetti R., 2018. Genetic diversity of the brown marmorated stink bug *Halyomorpha halys* in the invaded territories of Europe and its patterns of diffusion in Italy. Biological Invasions 20: 1073-1092.

Criscione C.D., Cooper B., Blouin M.S., 2006. Parasite genotypes identify source populations of migratory fish more accurately than fish genotypes. Ecology 87: 823-828.

Dhami M.K., Dsouza M., Waite D.W., Anderson D., Li D., 2016. Real-time PCR assay for the identification of the brown marmorated stink bug (*Halyomorpha halys*). Frontiers in Molecular Biosciences 3: 5, doi:10.3389/fmolb.2016.00005.

Dillon R.J., Dillon V.M., 2004. The gut bacteria of insects: nonpathogenic interactions. Annual Review of Entomology 49: 71-92.

Dioli P., Leo P., Maistrello L., 2016. Prime segnalazioni in Spagna e in Sardegna della specie aliena *Halyomorpha halys* (Stål, 1855) e note sulla sua distribuzione in Europa (Hemiptera, Pentatomidae). Revista gaditana de Entomología 7(1): 539-548.

Dlugosch K.M., Parker I.M., 2008. Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. Molecular Ecology 17(1): 431-449.

Dormann C.F., Gruber B., Fründ, J., 2008. Introducing the bipartite package: analysing ecological networks. Interaction 8/2: 8-11.

Douglas A.E., 2009. The microbial dimension in insect nutritional ecology. Functional Ecology 23: 38-47.

Duthie C., Michael T., Stephenson B., Yamoah E., McDonald B., 2012. Risk analysis of *Halyomorpha halys* (brown marmorated stink bug) on all pathways. Ministry for Primary Industries, Wellington, New Zealand, 57.

Excoffier L., Lischer H.E.L., 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10: 564-567.

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

Funk D.J., Helbling L., Wernegreen J.J., Moran N.A., 2000. Intraspecific phylogenetic congruence among multiple symbiont genomes. Proceedings of the Royal Society B: Biological Sciences 267: 2517-2521.

Gapon D.A., 2016. First records of the brown marmorated stink bug, *Halyomorpha halys* (Stål, 1855) (Heteroptera: Pentatomidae) in Russia, Abkhazia, and Georgia. Entomological Review 96: 1086-1088.

Gariepy T.D., Haye T., Fraser H., Zhang J., 2014. Occurrence, genetic diversity, and potential pathways of entry of *Halyomorpha halys* in newly invaded areas of Canada and Switzerland. Journal of Pest Science 87: 17-28.

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

Gonella E., Orrù B., Alma A., 2019. Egg masses treatment with micronutrient fertilizers has a suppressive effect on newly emerged nymphs of the brown marmorated stink bug *Halyomorpha halys*. Entomologia Generalis, doi:10.1127/entomologia/2019/0819.

Haye T., Gariepy T., Hoelmer K., Rossi J.P., Streito J.C., Tassus X., Desneux N., 2015. Range expansion of the invasive brown marmorated stinkbug, *Halyomorpha halys*: an increasing threat to field, fruit and vegetable crops worldwide. Journal of Pest Science 88: 665-673.

Heckmann R., 2012. Erster nachweis von *Halyomorpha halys* (Stål, 1855) (Heteroptera: Pentatomidae) für deutschland. Heteropteron 36: 17-18.

Kapantaidaki D.E., Evangelou V.I., Morrison W.R., Leskey T.C., Brodeur J., Milonas P., 2019. *Halyomorpha halys* (Hemiptera: Pentatomidae) genetic diversity in North America and Europe. Insects 10: 174, doi:10.3390/insects10060174.

Kenyon L.J., Meulia T., Sabree Z.L., 2015. Habitat visualization and genomic analysis of "*Candidatus* Pantoea carbekii," the primary symbiont of the brown marmorated stink bug. Genome Biology and Evolution 7(2): 620-635.

Kriticos D.J., Kean J.M., Phillips C.B., Senay S.D., Acosta H., Haye T., 2017. The potential global distribution of the brown marmorated stink bug, *Halyomorpha halys*, a critical threat to plant biosecurity. Journal of Pest Science 90: 1033-1043.

Kumar S., Stecher G., Li M., Knyaz C., Tamura K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution 35(6): 1547-1549.

Lashkari M., Manzari S., Sahragard A., Malagnini V., Boykin L.M. Hosseini R., 2014. Global genetic variation in the Asian citrus psyllid *Diaphorina citri* (Hemiptera: Liviidae) and the endosymbiont Wolbachia: links between Iran and the USA detected. Pest Management Science 70(7): 1033-1040.

Lee W., Guidetti R., Cesari M., Gariepy T.D., Park Y.L., Park C.G., 2018. Genetic diversity of *Halyomorpha halys* (Hemiptera, Pentatomidae) in Korea and comparison with COI sequence datasets from East Asia, Europe, and North America. Florida Entomologist 101(1): 49-55.

Leigh J.W., Bryant D., 2015. popart: full-feature software for haplotype network construction. Methods in Ecology and Evolution 6(9): 1110-1116.

Leskey T.C., Nielsen A.L., 2018. Impact of the invasive brown marmorated stink bug in North America and Europe: history, biology, ecology, and management. Annual Review of Entomology 63: 599-618.

Macavei L., Baetan R., Oltean I., Florean T., Varga M., Costi E., Maistrello L., 2015. First detection of *Halyomorpha halys*, a new invasive species with a high potential of damage on agricultural crops in Romania. Lucrări Ştiinţifice, Universitatea de Stiinte Agricole Şi Medicină Veterinară" Ion Ionescu de la Brad" Iaşi, Seria Agronomie 58: 105-108.

Maistrello L., Dioli P., Vaccari G., Nannini R., Bortolotti P., Caruso S., Costi E., Montermini A., Casoli L., Bariselli M., 2014. First records in Italy of the Asian stinkbug *Halyomorpha halys* a new threat to fruit crops. Atti Giornate Fitopatologiche 1: 283-288.

Maynard A.J., Ambrose L., Cooper R.D., Chow W.K., Davis J.B., Muzari M.O., Hurk A.F.V.D., Hall-Mendelin S., Hasty J.M., Burkot T.R., Bangs M.J., Reimer L.J., Butafa C., Loco N.F., Syafruddin D., Maung Y.N.M., Ahmad R., Beebe N.W., 2017. Tiger on the prowl: invasion history and spatio-temporal genetic structure of the Asian tiger mosquito *Aedes albopictus* (Skuse 1894) in the Indo-Pacific. PLoS Neglected Tropical Diseases 11, e0005546.

Maurel J.P., Blaye G., Valladares L., Roinel É., Cochard P.O., 2016. *Halyomorpha halys* (Stål, 1855), la punaise diabolique en France, a Toulouse (Heteroptera; Pentatomidae). Carnets natures 3: 21-25.

Milonas P.G., Partsinevelos G.K., 2014. First report of brown marmorated stink bug *Halyomorpha halys* Stål (Hemiptera: Pentatomidae) in Greece. Bulletin OEPP/EPPO Bulletin 44: 183-186.

Moran N.A., Tran P., Gerardo N.M., 2005. Symbiosis and insect diversification: an ancient symbiont of sap-feeding insects from the bacterial phylum Bacteroidetes. Applied and Environmental Microbiology 71: 8802-8810.

Morrison W.R. III, Milonas P., Kapantaidaki D.E., Cesari M., Di Bella E., Guidetti R., Haye T., Maistrello L., Moraglio S.T., Piemontese L., Pozzebon A., Ruocco G., Short B.D., Tavella L., Vétek G., Leskey T.C., 2017. Attraction of *Halyomorpha halys* (Hemiptera: Pentatomidae) haplotypes in North America and Europe to baited traps. Scientific Reports 7: 16941, doi:10.1038/s41598-017-17233-0.

Musolin D.L., Konjević A., Karpun N.N., Protsenko V.Y., Ayba L.Y., Saulich A.K., 2018. Invasive brown marmorated stink bug *Halyomorpha halys* (Stål) (Heteroptera: Pentatomidae) in Russia, Abkhazia, and Serbia: history of invasion, range expansion, early stages of establishment, and first records of damage to local crops. Arthropod-Plant Interactions 12: 517-529.

Otero-Bravo A., Sabree Z.L., 2018. Comparing the utility of host and primary endosymbiont loci for predicting global invasive insect genetic structuring and migration patterns. Biological Control 116: 10-16.

Rabitsch W., Griebe G.J., 2015. From the west and from the east? First records of *Halyomorpha halys* (Stål, 1855) (Hemiptera: Heteroptera: Pentatomidae) in Vorarlberg and Vienna, Austria. Beiträge zur Entomofaunistik 16: 115-139.

Rassati D., Faccoli M., Petrucco Toffolo E., Battisti A., Marini L., 2015. Improving the early detection of alien wood-boring beetles in ports and surrounding forests. Journal of Applied Ecology 52(1): 50-58.

Savio C., Mazzon L., Martinez-Sañudo I., Simonato M., Squartini A., Girolami V., 2012. Evidence of two lineages of the symbiont '*Candidatus* Erwinia dacicola' in Italian populations of *Bactrocera oleae* (Rossi) based on 16S rRNA gene sequences. International Journal of Systematic and Evolutionary Microbiology 62(1): 179-187.

Šeat J., 2015. *Halyomorpha halys* (Stål, 1855) (Heteroptera: Pentatomidae) a new invasive species in Serbia. Acta Entomologica Serbica 20: 167-171.

Sharon G., Segal D., Ringo J.M., Hefetz A., Zilber-Rosenberg I., Rosenberg E., 2010. Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. Proceedings of the National Academy of Sciences of the United States of America 107: 20051-20056.

Simberloff D., Martin J.L., Genovesi P., Maris V., Wardle D.A., Aronson J., Martin Courchamp F., Galil B., García-Berthou E., Pascal M., Pyšek P., Sousa R., Tabacchi E., Vilà M., 2013. Impacts of biological invasions: what's what and the way forward. Trends in Ecology and Evolution 28: 58-66.

Simov N., 2016. The invasive brown marmorated stink bug *Halyomorpha halys* (Stål, 1855) (Heteroptera: Pentatomidae) already in Bulgaria. Ecologica Montenegrina 9: 51-53.

Valentin R.E., Nielsen A.L., Wiman N.G., Lee D.-H., Fonseca, D.M., 2017. Global invasion network of the brown marmorated stink bug, *Halyomorpha halys*. Scientific Reports 7: 9866.

Vandervoet T.F., Bellamy D.E., Anderson D., MacLellan R., 2019. Trapping for early detection of the brown marmorated stink bug, *Halyomorpha halys*, in New Zealand. New Zealand Plant Protection 72: 36-43.

Vétek G., Papp V., Haltrich A., Rédei D., 2014. First record of the brown marmorated stink bug, *Halyomorpha halys* (Hemiptera: Heteroptera: Pentatomidae), in Hungary, with description of the genitalia of both sexes. Zootaxa 3780: 194-200.

Wang Y., Lu J., Beattie G.A., Islam M.R., Om N., Dao H.T., Van Nguyen L., Zaka S.M., Guo J., Tian M., Deng X., Tan S., Holford P., He Y., Cen Y., 2018. Phylogeography of *Diaphorina citri* (Hemiptera: Liviidae) and its primary endosymbiont, '*Candidatus* Carsonella ruddii': an evolutionary approach to host–endosymbiont interaction. Pest Management Science 74(9): 2185-2194.

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

Wernegreen J.J., 2002. Genome evolution in bacterial endosymbionts of insects. Nature Reviews Genetics 3(11): 850-861.

Wernegreen J.J., 2015. Endosymbiont evolution: predictions from theory and surprises from genomes. Annals of the New York Academy of Sciences 1360: 1-16.

Xu J., Fonseca D.M., Hamilton G.C., Hoelmer K.A., Nielsen A.L., 2014. Tracing the origin of US brown marmorated stink bugs, *Halyomorpha halys*. Biological Invasions 16: 153-166.

Zhu GP., Ye Z., Du J., Zhang D.L., Zhen Y., Zheng C., Zhao L., Li M., Bu W.J., 2016. Range wide molecular data and niche modeling revealed the Pleistocene history of a global invader (*Halyomorpha halys*). Scientific Reports 6: 23192.

Chapter 3

Seasonal dynamics and damage potential of *Halyomorpha halys* in Italian vineyards

Manuscript in preparation as: Seasonal dynamics and damage potential of *Halyomorpha halys* in Italian vineyards

Davide Scaccini, Diego Fornasiero, Paola Tirello, Simone Vincenzi, Massimo Cecchetto, Ilirjan Allgjata, Carlo Duso, Alberto Pozzebon

Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova, Italy

Davide Scaccini contributed in study conceptualization, data collection and analysis as well as writing of the manuscript.

Abstract

The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) is an invasive polyphagous pest that is often observed in vineyards. In Europe, there is a gap in the knowledge on *H. halys* seasonal dynamics and damage potential on grape that needs to be filled. Here we describe the seasonal dynamics of *H. halys* and its distribution in multi-cultivar vineyards in July–September 2017 and June–September 2018, and we evaluated the damage on grape clusters induced by different infestation densities. In vineyards, the seasonal occurrence of *H. halys* varied across time and grape cultivars, and the pest was most abundant in Cabernet Franc and Merlot, and it was found at comparatively lower incidence in Pinot gris. Overall higher densities of *H. halys* were found on red and late ripening cultivars compared to white and early-ripening varieties. An edge effect was also detected in higher pest incidence close to vineyard borders. From the cage study, *H. halys* infestations caused damage on berries, showing a higher susceptibility for red varieties, in particular Cabernet Franc, and following 50% of grape cluster veraison. *Halyomorpha halys* infestation induced an increase in *Botrytis cinerea* incidence and representing the main issue related to *H. halys* impact on grapevine.

1. Introduction

Wine grape production, *Vitis vinifera* L. (Vitales: Vitaceae), plays a major economic and agricultural role in both Europe and internationally (OIV 2018). Italy is one of the major wine producers in the world (Fregoni 1998; Raimondi 2007; OIV 2018). Vineyards in Italy cover almost 700,000 ha (OIV 2017), or 9% of the global vineyard surface (OIV 2018). The Veneto region in northern Italy is the leading production region within Italy, with annual production levels of more than 1,600,000 Mg (IWC 2019b).

The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) can feed on grapevine creating damage like the internal collapse and necrosis of the fruit tissue in berries (Panizzi 1997; Hori 2000; Bettiga et al. 2013). In the US, *H. halys* is considered a possible minor pest for grape production due to its low preference toward *V. vinifera* compared to other species (Bettiga et al. 2013). This polyphagous pest, which can feed on more than 170 host plant species (Leskey and Nielsen 2018), has been recorded to feed on wild and cultivated *V. vinifera* plants also in Europe

(Maistrello et al. 2016a), and the presence in vineyards of all *H. halys* developmental stages suggests that *V. vinifera* is a reproductive host (Basnet et al. 2015).

Halyomorpha halys can directly damage grape clusters, with damage inlcuding increased levels of dropped berries, discoloration, necrosis and softening. Indirect damage can comprise pathogen and other pest infestations (Nielsen et al. 2016). Indirect damage can also affect the quality of the wine because of *H. halys* the taint emanating from insects during pressing (Mohekar et al. 2016; 2017a,b; 2018). Despite not common (Smith et al. 2014), cases of high infestations in the field were already recorded in vineyards, whit many adults and nymphs found especially on berries in particular in August–October (Nielsen et al. 2016; Musolin et al. 2018).

Despite the known pest status of this pest on many crops, in Europe, a gap in the knowledge on its seasonal dynamics and damage on grape needs to be filled. The aims of this study were: (1) to describe the seasonal dynamics of *H. halys* in vineyards on different grape cultivars and their distribution in vineyard; (2) to evaluate the damage on grape clusters by different infestation densities of *H. halys*.

2. Material and Methods

2.1. Seasonal dynamics of *H. halys* in the vineyards

The seasonal dynamic of *H. halys* was studied in different multi-cultivar vineyards located in Veneto region that were surveyed every 7–10 days from July (2017) or June (2018), following *H. halys* infestations in vineyard, until September, after first harvesting of the early ripening grape varieties. Sampled grapevine cultivars represented the main ones in the region (IWC 2019a) and were Cabernet Franc, Cabernet Sauvignon, Merlot and Raboso with red berry, and Glera, Pinot gris and Tai with white berry. The cultivars Cabernet Sauvignon, Raboso and Tai were sampled only in the second year. Surveys were performed by visual and beat samplings. Sampling points were selected in each vineyard, and, in particular, these were localized at the border and the center of the vineyard rows and consists of four plants. In each vineyard, sampling was performed at least in four points. At each sampling point, plants were inspected for a period of three minutes per plant, counting different life stages of *H. halys* similar to what was performed by Aigner et al. (2016) in soybean fields. Beat sampling was performed on the same plant by shacking the plant canopy and collecting insect on a tray placed below. Also, in this case, all *H. halys* life stages were identified and counted. In the second year only beat sampling was performed.

2.2. Assessment of *H. halys* damage on grape cluster

In vineyards, we established controlled infestations of *H. halys* adults by using insect-proof net cages on the grape clusters on four cultivars: Cabernet Franc, Glera, Merlot and Pinot gris. Cages consisted in 50 x 80 cm white net, mesh 150 µm, installed on the plants early in the season. Each cage included two clusters, and four infestation treatments were established: 0 (control), 0.5, 1 or 2 adults per cluster. Infestation lasted for ten days and each treatment was replicated six times. We replicated the infestation treatment on different clusters during the season. In particular, infestatation were performed following different grapevine phenological stages: bunch closure (BBCH 77), starting veraison (BBCH 81), 50% of veraison (BBCH 83), starting ripening (BBCH 85), and 100% ripened (BBCH 89). During the infestation period, in case of adult's death occurred, the specimen was replaced by a living one. After the infestation, adults were removed and cages kept on the plant until harvesting, when the final assessment was performed in laboratory. For each grape cluster, we considered the presence of damaged berries and the mean weight of berries for each cluster. Damaged berries per each cluster were divided into two categories, i.e., damaged by *H. halys* (fruit presenting discoloration, necrosis and softening of the berry; Nielsen et al. 2016), or with the grey mold, *Botrytis cinerea* (Pers.) (Helotiales: Sclerotiniaceae) and sour rot.

Following the first evaluation, the clusters were manually pressed and the obtained must was analyzed after adding 1 g L^{-1} of potassium metabisulfite for preservation. The further analyses were performed on glucose and fructose, and on tartaric and gluconic acid through High Performance Liquid Chromatography (HPLC). Gluconic acid was used to evaluate the presence of *B. cinerea*, which is one of its secondary metabolite whose presence denotes grey mold infestations (e.g., Donèche 1989; Hong et al. 2012; Albanese et al. 2014; Cinquanta et al. 2015; Kirchert et al. 2019), increasing the accuracy of grey mold assessment than through visual estimations, which may generate bias (Hill et al. 2014).

2.3. Statistical analyses

Data on *H. halys* seasonal dynamics were analyzed with General linear mixed repeated measures model with the MIXED procedure of SAS (ver. 9.4). In this analysis, cultivars, time of sampling, position within vineyard rows (i.e., border vs. center) and their interactions were considered as fixed effect of the models and tested with an F-test ($\alpha = 0.05$). The number of *H. halys* collected using beat sampling or observed in visual sampling during the season were considered as dependent variables made with repeated measures (i.e., sampling dates). Data from both seasons were analyzed separately.

While modeling, different vineyards and rows within vineyards were considered as random effect terms. Differences among cultivars and between the position within vineyard rows was evaluated using a Tukey's test on the least-square means (α = 0.05). In the analysis of the second season data, contrasts were design to compare, using an F test (α = 0.05), *H. halys* numbers between white and red-berry cultivars (i.e., Glera, Pinot gris and Tai vs. Cabernet Franc, Cabernet Sauvignon, Merlot and Raboso), and cultivars categorized according their ripening periods (i.e., early: Pinot gris and Tai; medium: Cabernet Sauvignon, Glera and Merlot; or late season: Cabernet Franc and Raboso). Data were checked for model assumptions prior to the analysis and were log (x+1) transformed before the statistical analysis.

Data obtained from cage experiment (i.e., weight of the cluster, number of berries per cluster, berry weight and the percentage of berries per cluster with *H. halys* damage or with signs of *B. cinerea*) were analyzed through General linear mixed repeated measures model with the MIXED procedure of SAS (ver. 9.4). Cultivars, treatments (i.e., infestation density), time of infestation (i.e., phenological stage) and their interactions were considered as fixed effects and tested using F-tests (α = 0.05) followed by a *post-hoc* Tukey-Kramer test (α = 0.05). Similarly, data obtained from the laboratory HPLC were analyzed through the same model reported before. Red and white cultivars were analyzed separately, in each pair (i.e., Cabernet Franc and Merlot, and Glera and Pinot gris). Transformed data were used prior to the statistical analysis as arcsin (√x) in the case of percentage and as log (x+1) for the others.

3. Results

3.1. Seasonal dynamics of *H. halys* in the vineyards

During 2017, the presence of all life stages of *H. halys* was observed in vineyards. Using beat sampling, higher numbers of insect were observed compared to visual sampling (Tables 1 and 2; Figures 1 and 2), and thus the latter was performed only in 2017. Using beat sampling, a variation of *H. halys* numbers was observed during the season (Figure 2). The infestation number was influenced by cultivar and resulted higher on Pinot gris and Merlot compare to Glera (Table 2; Figure 3).

In 2018, *H. halys* number fluctuated during the surveys increasing at the end of the season particularly in Cabernet F. (Table 3; Figure 4). Overall, differences among cultivars were observed, with Cabernet F. and Merlot showing the highest infestation levels, while Tai, Glera and Cabernet S. the lowest (Figure 5). Pinot gris was infested at the intermediate level. *Halyomorpha halys* numbers were higher on red grape varieties than on white ones (Figure 6), and in late-ripening varieties compare to early ones (Table 4; Figure 7). The distribution along rows was influenced by the position, being higher at the border than the center (Table 4; Figure 8).

Table 1. Results of GLMM model on the seasonal dynamic of *Halyomorpha halys* observed with visual sampling in vineyards during 2017.

Source of variation	df	F	p
Sampling date (D)	4, 214	2.28	0.0616
Position (Pos)	1, 140	1.77	0.1859
Cultivar (Cv)	3, 130	0.80	0.4945
D*Pos	4, 214	1.20	0.3121
D*Cv	12, 231	1.01	0.4386
Pos*Cv	3, 130	0.89	0.4508
D*Pos*Cv	12, 231	0.69	0.7621

Table 2. Results of GLMM model on the seasonal dynamic of *Halyomorpha halys* observed from the beat sampling in vineyards during 2017.

Source of variation	df	F	p
Sampling date (D)	**4, 216**	**3.94**	**0.0042**
Position (Pos)	1, 106	0.06	0.8075
Cultivar (Cv)	**3, 103**	**3.49**	**0.0185**
D*Pos	4, 216	0.46	0.7614
D*Cv	12, 230	1.35	0.1903
Pos*Cv	3, 103	0.89	0.4515
D*Pos*Cv	12, 230	0.57	0.8663

Figure 1. *Halyomorpha halys* seasonal dynamic on different grape varieties observed from visual sampling in northern Italy in July–September 2017.

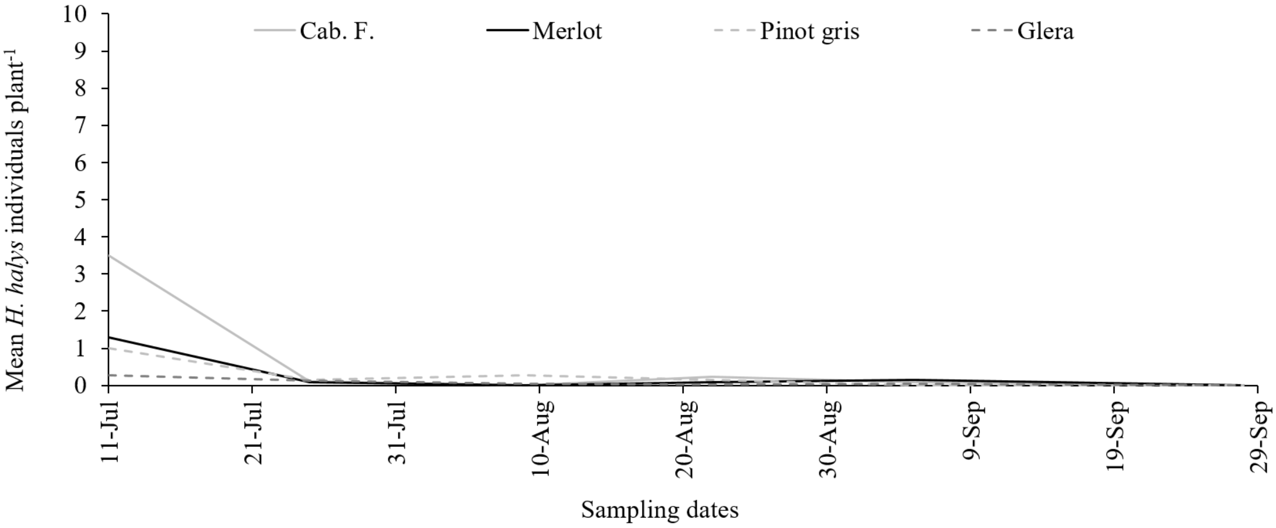


Figure 2. *Halyomorpha halys* seasonal dynamic on different grape varieties observed from beat sampling in northern Italy in July–September 2017.

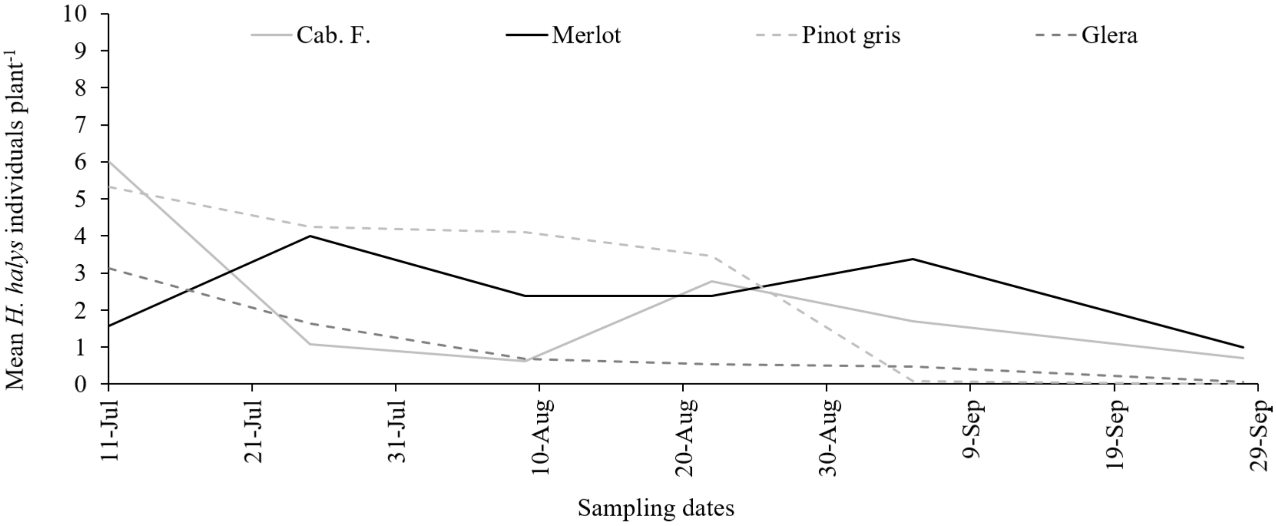


Figure 3. *Halyomorpha halys* number (± SE) of different stages on grape varieties collected with beat sampling in northern Italy in July–September 2017. Back-transformed data from least-square means were used in the figure. Different letters indicate significant differences at the Tukey's test on least-square means (α = 0.05).

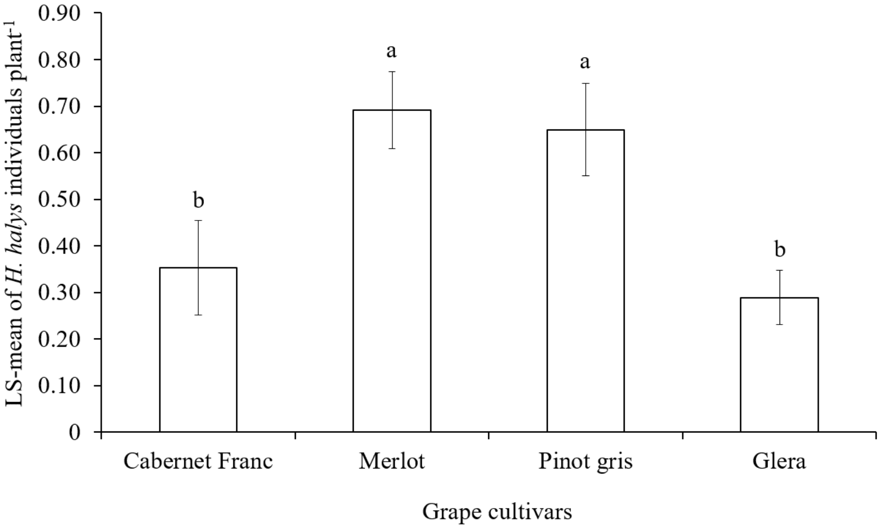


Table 3. Results of GLMM model on the seasonal dynamic of *Halyomorpha halys* observed from the beat sampling in vineyards during 2018.

Source of variation	**df**	**F**	**p**
Sampling date (D)	**10, 339**	**2.32**	**0.0119**
Position (Pos)	**1, 79.1**	**7.06**	**0.0096**
Cultivar (Cv)	**6, 79.1**	**7.36**	**< 0.0001**
D*Pos	10, 339	0.22	0.9944
D*Cv	**60, 345**	**1.81**	**0.0006**
Pos*Cv	6, 79.2	0.34	0.9152
D*Pos*Cv	60, 345	0.56	0.9960

Figure 4. *Halyomorpha halys* seasonal dynamic on different grape varieties observed from beat sampling in northern Italy during June–September 2018.

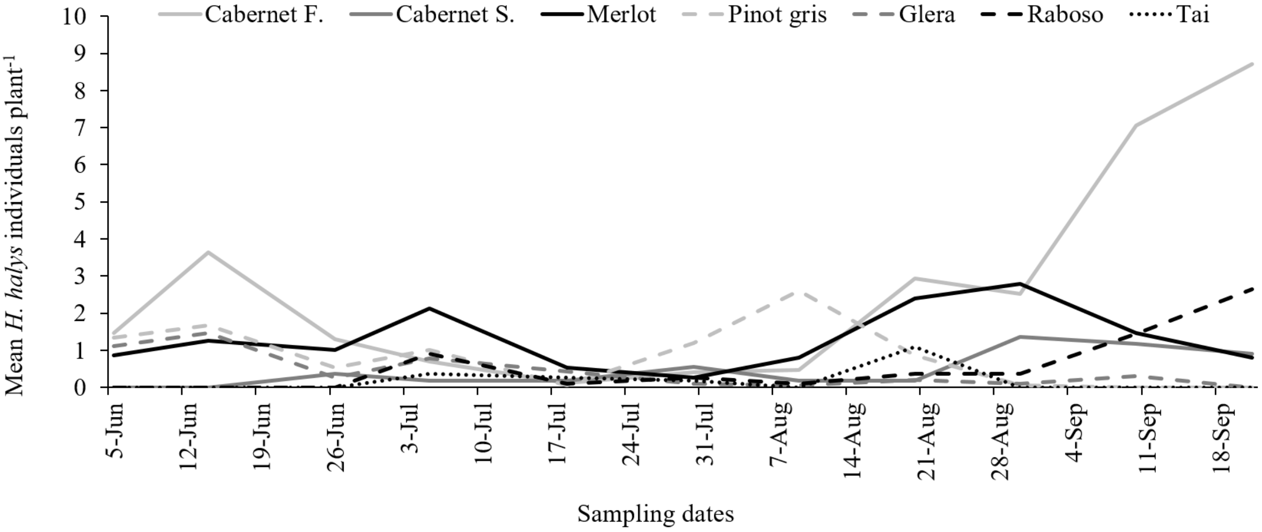


Figure 5. *Halyomorpha halys* number (± SE) of different stages on grape varieties collected with beat sampling in 2018. Back-transformed data from least-square means were used in the figure. Different letters indicate significant differences at the Tukey's test on least-square means (α = 0.05).

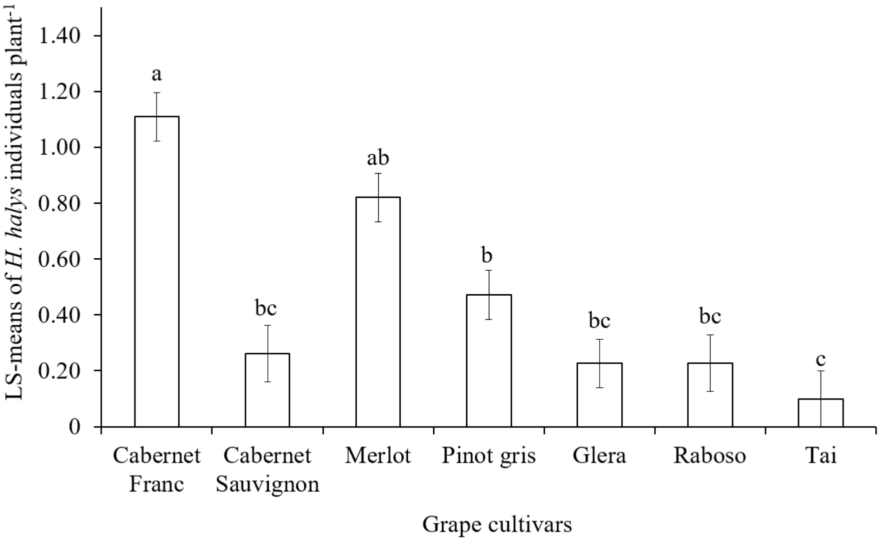


Table 4. Results of contrasts on the seasonal dynamics of *Halyomorpha halys* in vineyards observed in 2018.

Source of variation	**df**	**F**	**p**
Red vs. White	**1, 79.1**	**10.54**	**0.0017**
Early vs. Medium	1, 79.1	1.66	0.2008
Early vs. Late	**1, 79.1**	**7.22**	**0.0088**
Medium vs. Late	1, 79.1	2.83	0.0965

Figure 6. *Halyomorpha halys* abundance (± SE) of different stages in vineyards on white and red grape varieties observed in 2018. Back-transformed data from least-square means were used in the figure. Different letters indicate significant differences at the Tukey's test on least-square means (α = 0.05).

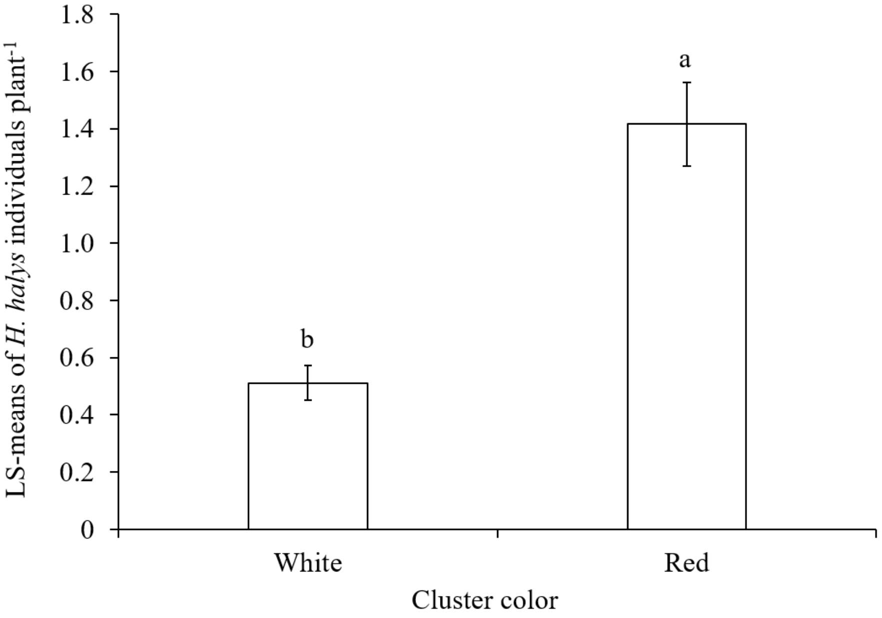


Figure 7. *Halyomorpha halys* abundance (± SE) of different stages observed in 2018 on cultivars with different ripening period. Early ripening cultivars: Pinot gris and Tai; medium ripening: Cabernet Sauvignon, Glera and Merlot; late ripening: Cabernet Franc and Raboso. Back-transformed data from least-square means were used in the figure. Different letters indicate significant differences at the Tukey's test on least-square means (α = 0.05).

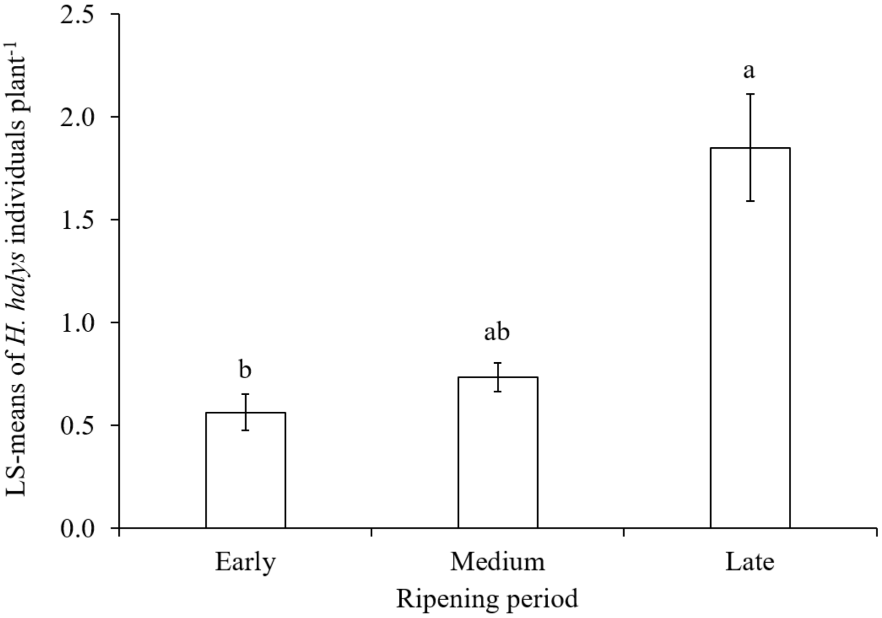
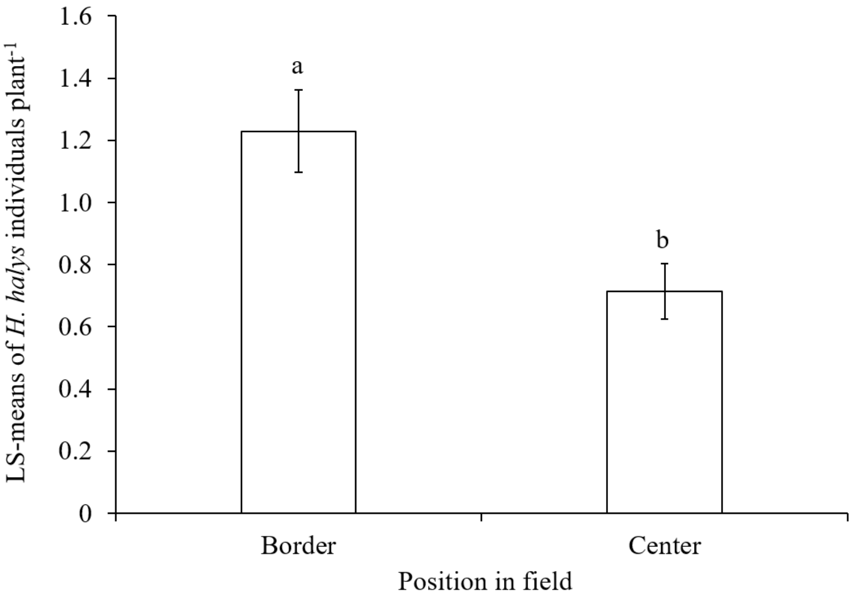


Figure 8. *Halyomorpha halys* abundance (± SE) of different stages observed in 2018 in different position (i.e., border rows vs. center rows of the vineyard). Back-transformed data from least-square means were used in the figure. Different letters indicate significant differences at the Tukey's test on least-square means (α = 0.05).



3.2. Assessment of *H. halys* damage on the grape cluster

In cage experiment, the percentage of berries with feeding damage increased with infestation density in all cultivars (Tables 5 and 6; Figure 9), but a cultivar effect was detected on white- as well as red-berry cultivars. Between white ones, the percentage of damaged berries was higher on Glera than Pinot gris (Table 5; Figure 10), while between red ones, on Cabernet Franc was higher compared to Merlot (Table 6; Figure 11). In red varieties, the effect of infestation level was stronger on Cabernet F. than Merlot (Figure 11). The damage derived by *H. halys* infestation differed from phenological stages of grapevine for white (Table 5) but not for red cultivars (Table 6). On white cultivars, a significant interaction was also found (Table 5). In these cultivars, the highest level of damaged berries on Glera was caused by infestation during 50% of the veraison, while no such effect was observed on Pinot gris (Figure 10).

Table 5. Results of GLMM on the percentage of white cultivars' berries with symptoms of *Halyomorpha halys* feeding.

Source of variation	df	F	p
Cultivar (Cv)	**1, 440**	**52.50**	**< 0.0001**
Phenological stage (Phen)	**4, 440**	**8.75**	**< 0.0001**
***H. halys* density (Dens)**	**3, 440**	**319**	**< 0.0001**
Cv*Phen	**4, 440**	**6.13**	**< 0.0001**
Cv*Dens	**3, 440**	**18.6**	**< 0.0001**
Phen*Dens	12, 440	1.53	0.1094
Cv*Phen*Dens	**12, 440**	**2.08**	**0.0174**

Table 6. Results of GLMM on the percentage of red cultivars' berries with symptoms of *Halyomorpha halys* feeding.

Source of variation	df	F	p
Cultivar (Cv)	**1, 440**	**149.30**	**< 0.0001**
Phenological stage (Phen)	**4, 440**	**2.85**	**0.0235**
***H. halys* density (Dens)**	**3, 440**	**715.17**	**< 0.0001**
Cv*Phen	4, 440	1.00	0.4086
Cv*Dens	**3, 440**	**18.09**	**< 0.0001**
Phen*Dens	12, 440	1.18	0.2945
Cv*Phen*Dens	12, 440	0.71	0.7378

Figure 9. Percentage of damaged berries (± SE) by *Halyomorpha halys* per cluster of white and red grapes with different densities of pest infestations. Different letters indicate significant differences at the Tukey-Kramer test (α = 0.05).

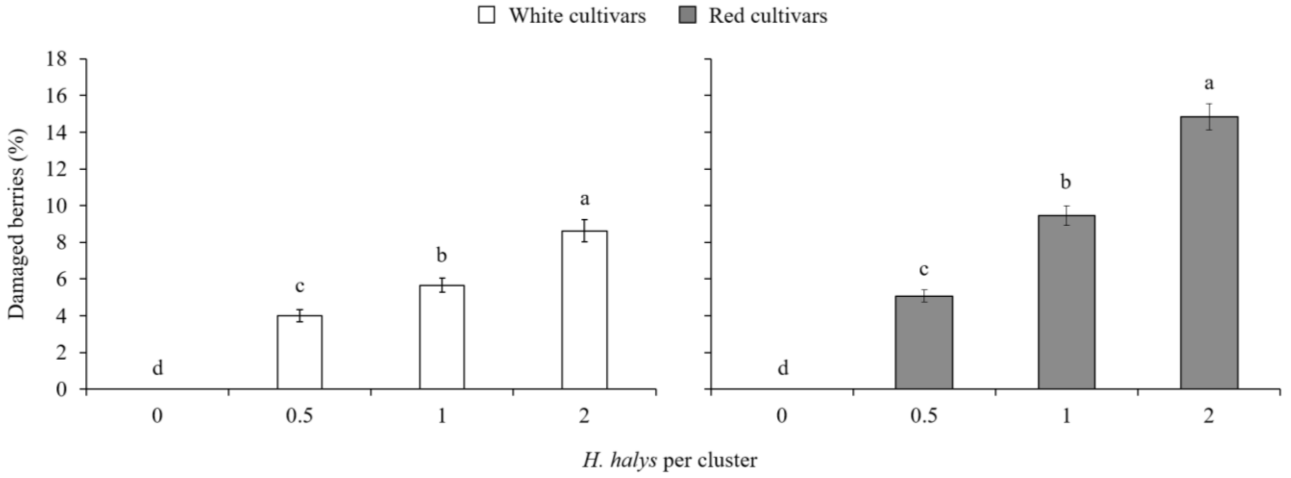


Figure 10. Percentage of damaged berries (± SE) by *Halyomorpha halys* per cluster of white grapes with the interaction grape variety*phenological stage**H. halys* density. Different letters indicate significant differences at the Tukey-Kramer test (α = 0.05).

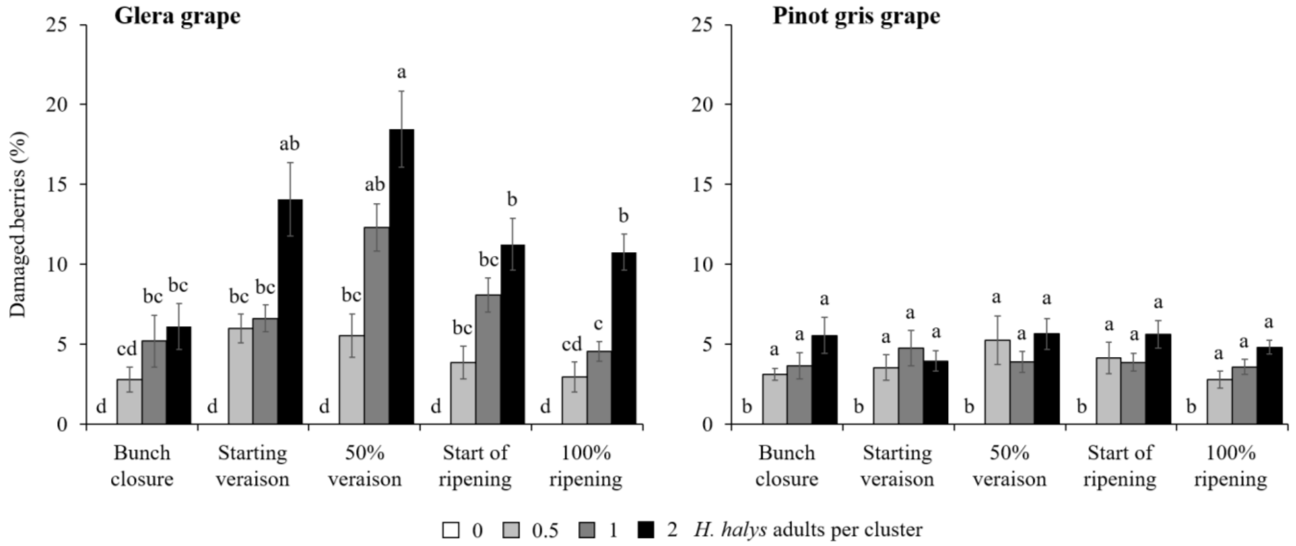
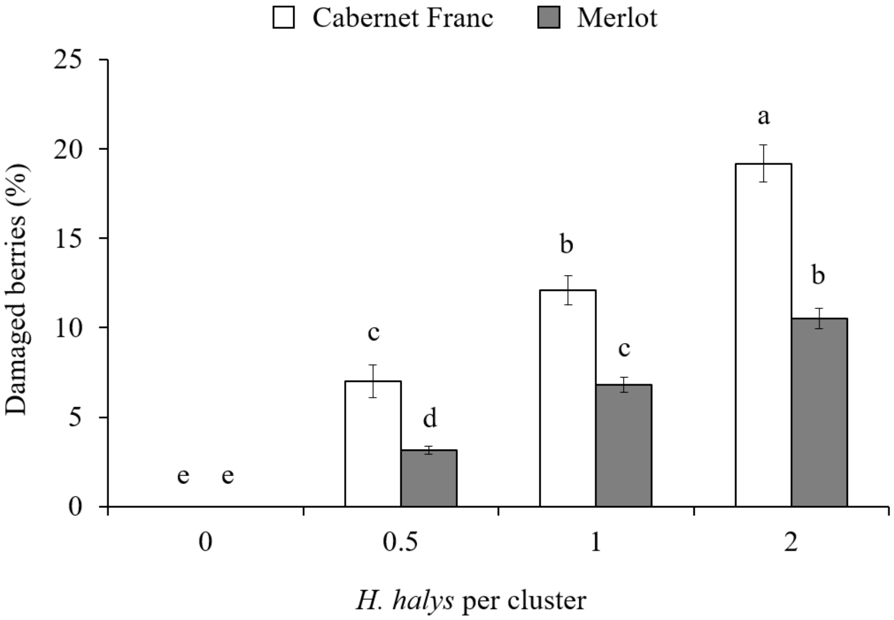


Figure 11. Percentage of damaged berries (± SE) by *Halyomorpha halys* per cluster of white grapes with the interaction grape variety**H. halys* density. Different letters indicate significant differences at the Tukey-Kramer test (α = 0.05).



The percentage of berries with *B. cinerea* was higher in white cultivars' clusters infested by *H. halys*, but this effect was stronger on Pinot gris than Glera (Table 7; Figures 12 and 13). On red varieties, the percentage of berries with signs of the pathogen was different between cultivars, with higher levels on Merlot than Cabernet F. (Table 8; data not shown). On white cultivars, the presence of signs of *B. cinerea* derived by *H. halys* infestation differed among phenological stages with high incidence after infestation during the start of ripening (Figure 14).

Table 7. Results of GLMM on the percentage of white cultivars' berries with signs of *Botrytis cinerea*.

Source of variation	df	F	p
Cultivar (Cv)	**1, 440**	**30.29**	**< 0.0001**
Phenological stage (Phen)	**4, 440**	**5.23**	**0.0004**
***H. halys* density (Dens)**	**3, 440**	**15.32**	**< 0.0001**
Cv*Phen	4, 440	0.57	0.6865
Cv*Dens	**3, 440**	**4.30**	**0.0053**
Phen*Dens	12, 440	1.54	0.1055
Cv*Phen*Dens	12, 440	0.94	0.5113

Table 8. Results of GLMM on the percentage of red cultivars' berries with signs of *Botrytis cinerea*.

Source of variation	df	F	p
Cultivar (Cv)	**1, 440**	**59.04**	**< 0.0001**
Phenological stage (Phen)	4, 440	0.80	0.5232
H. halys density (Dens)	3, 440	1.17	0.3220
Cv*Phen	4, 440	0.95	0.4367
Cv*Dens	3, 440	0.80	0.4967
Phen*Dens	12, 440	0.56	0.8752
Cv*Phen*Dens	12, 440	0.70	0.7493

Figure 12. Percentage of damaged berries (± SE) with signs of *Botrytis cinerea* in white grapes for different *Halyomorpha halys* densities. Different letters indicate significant differences at the Tukey-Kramer test (α = 0.05).

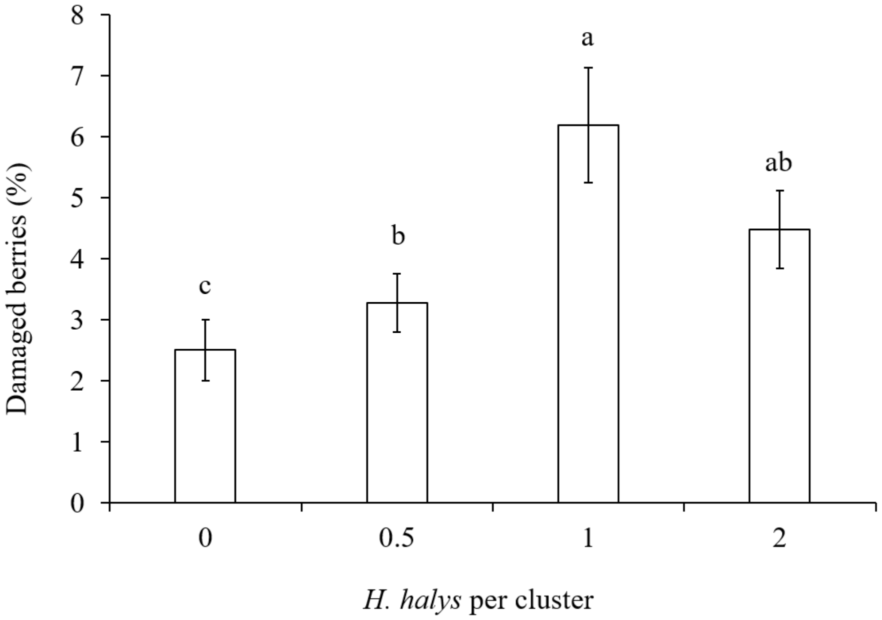


Figure 13. Percentage of damaged berries (± SE) with signs of *Botrytis cinerea* in white grapes for the interaction grape variety**Halyomorpha halys* density. Different letters indicate significant differences at the Tukey-Kramer test (α = 0.05).

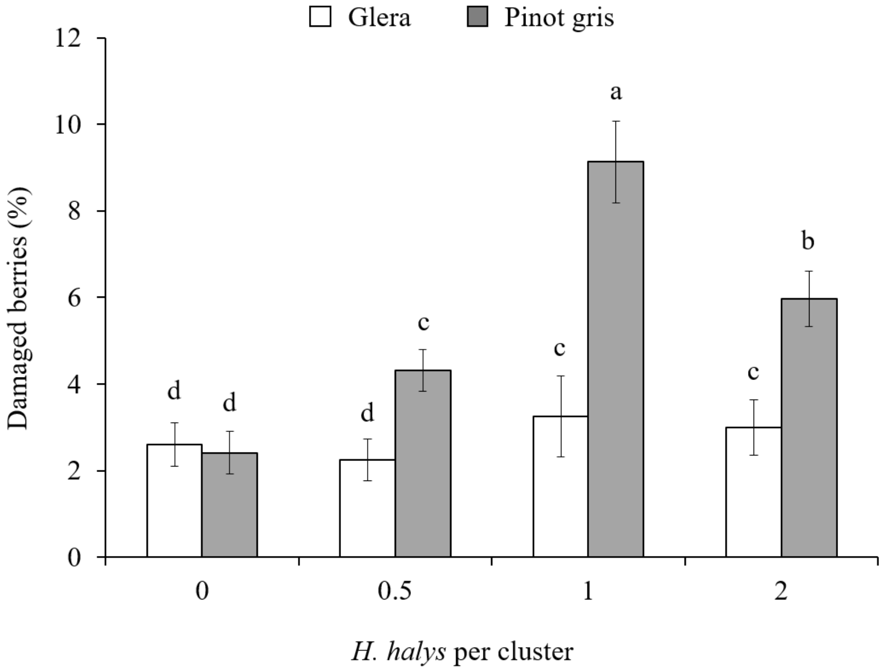
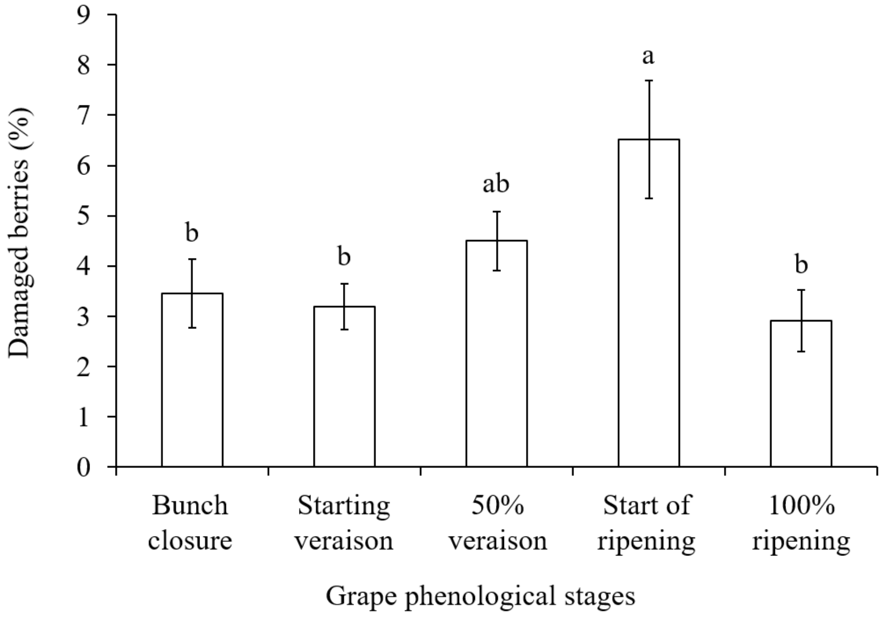


Figure 14. Percentage of damaged berries (± SE) with signs of *Botrytis cinerea* in white grapes for different phenological stages of the plant. Different letters indicate significant differences at the Tukey-Kramer test (α = 0.05).



An effect on the mean weight of berry following *H. halys* infestations emerged on red cultivar but not on white ones (Tables 9 and 10). The weight of the berries was higher with *H. halys* infestations of 0.5 and 1 adult per cluster (Figure 15), and differences were detected when considering the interaction cultivar*phenological stage of the plant (Figure 16).

Table 9. Results of GLMM on the mean weight of berries of white cultivars.

Source of variation	**df**	**F**	**p**
Cultivar (Cv)	**1, 440**	**343.27**	**< 0.0001**
Phenological stage (Phen)	4, 440	1.41	0.2297
H. halys density (Dens)	3, 440	1.17	0.3252
Cv*Phen	4, 440	0.46	0.7132
Cv*Dens	3, 440	0.46	0.7099
Phen*Dens	12, 440	0.69	0.7628
Cv*Phen*Dens	12, 440	0.98	0.4707

Table 10. Results of GLMM on the mean weight of berries of red cultivars.

Source of variation	**df**	**F**	**p**
Cultivar (Cv)	**1, 440**	**13.42**	**0.0003**
Phenological stage (Phen)	4, 440	1.79	0.1302
***H. halys* density (Dens)**	**3, 440**	**10.76**	**< 0.0001**
Cv*Phen	**4, 440**	**2.66**	**0.0322**
Cv*Dens	3, 440	1.22	0.3016
Phen*Dens	12, 440	1.02	0.4259
Cv*Phen*Dens	12, 440	1.01	0.4349

Figure 15. Berry mean weight (± SE) in red grapes for different *Halyomorpha halys* densities. Different letters indicate significant differences at the Tukey-Kramer test (α = 0.05).

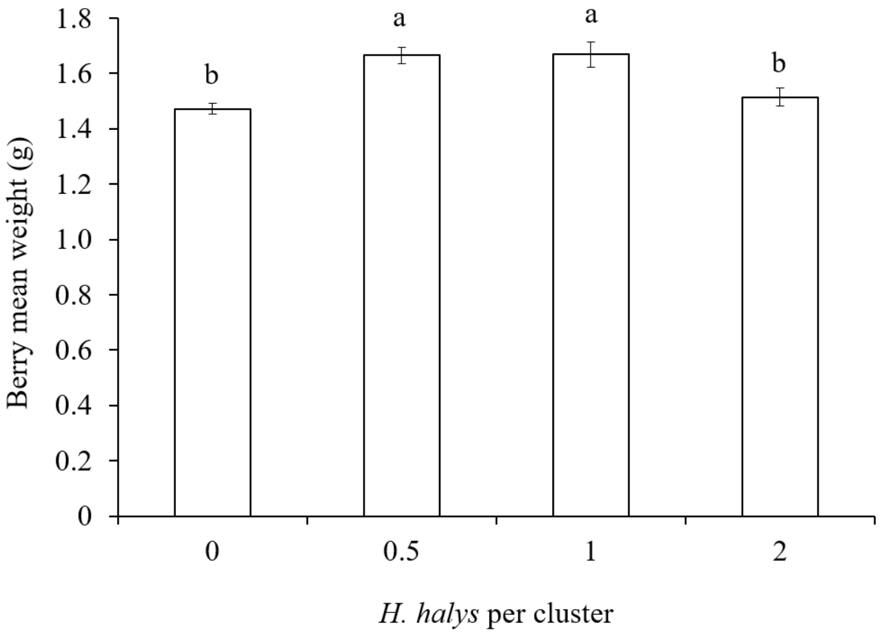
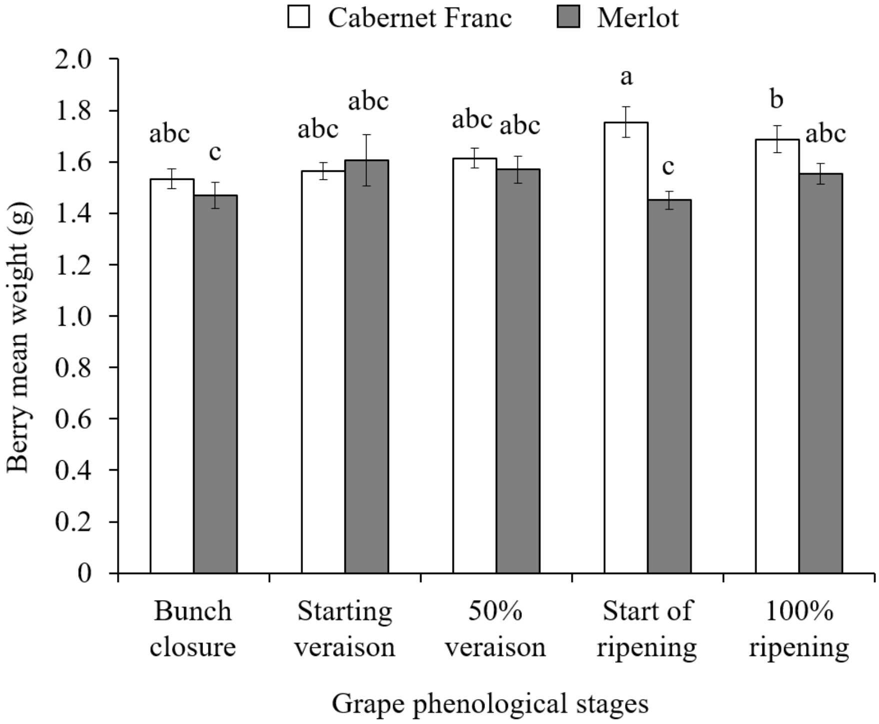


Figure 16. Berry mean weight (± SE) in red grapes for the interaction grape cultivar*phenological stage of the plant. Different letters indicate significant differences at the Tukey-Kramer test (α = 0.05).



In the must, no effect of *H. halys* infestation was observed on glucose concentration, and differences were related to the different cultivars (Tables 11 and 12; data not shown). An increase in fructose concentration was observed in infested berries of white cultivar, but not in red ones (Tables 13 and 14; Figure 17).

Table 11. Results of GLMM on the glucose content of the must of white cultivars.

Source of variation	**df**	**F**	**p**
Cultivar (Cv)	**1, 200**	**924.66**	**< 0.0001**
Phenological stage (Phen)	4, 200	1.70	0.1515
H. halys density (Dens)	3, 200	0.38	0.7661
Cv*Phen	**4, 200**	**6.88**	**< 0.0001**
Cv*Dens	3, 200	0.11	0.9558
Phen*Dens	12, 200	0.93	0.5225
Cv*Phen*Dens	12, 200	1.24	0.2554

Table 12. Results of GLMM on the glucose content of the must of red cultivars.

Source of variation	**df**	**F**	**p**
Cultivar (Cv)	**1, 200**	**29.77**	**< 0.0001**
Phenological stage (Phen)	**4, 200**	**2.69**	**0.0321**
H. halys density (Dens)	3, 200	0.88	0.4520
Cv*Phen	4, 200	0.72	0.5797
Cv*Dens	3, 200	1.75	0.1581
Phen*Dens	12, 200	1.78	0.0541
Cv*Phen*Dens	12, 200	1.39	0.1734

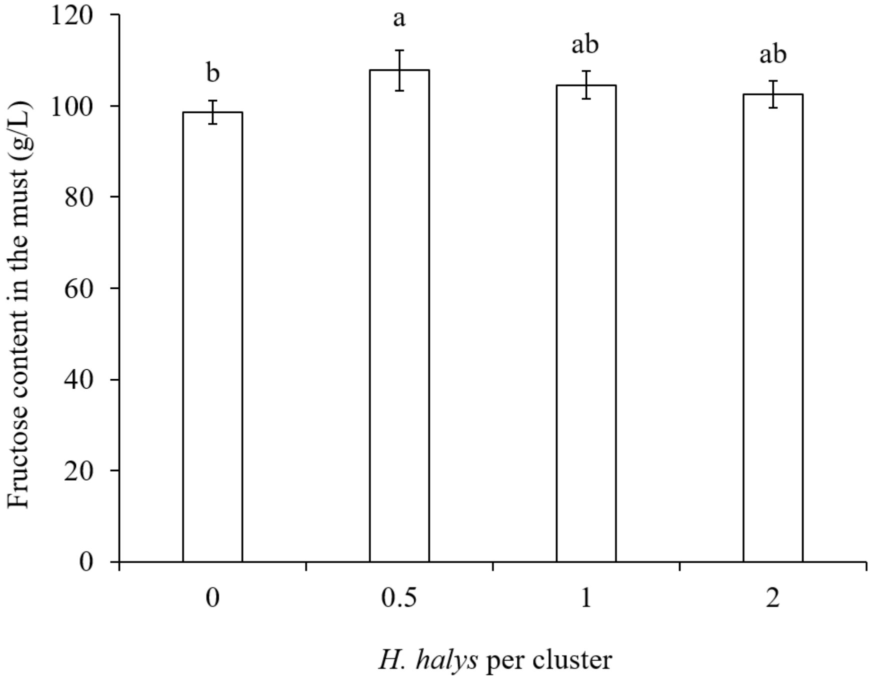
Table 13. Results of GLMM on the fructose content of the must of white cultivars.

Source of variation	**df**	**F**	**p**
Cultivar (Cv)	**1, 200**	**530.37**	**< 0.0001**
Phenological stage (Phen)	4, 200	1.17	0.3263
***H. halys* density (Dens)**	**3, 200**	**3.37**	**0.0195**
Cv*Phen	**4, 200**	**4.82**	**0.0010**
Cv*Dens	3, 200	1.31	0.2714
Phen*Dens	12, 200	1.24	0.2596
Cv*Phen*Dens	12, 200	1.63	0.0860

Table 14. Results of GLMM on the fructose content of the must of red cultivars.

Source of variation	df	F	p
Cultivar (Cv)	**1, 200**	**89.50**	**< 0.0001**
Phenological stage (Phen)	**4, 200**	**3.30**	**0.0120**
H. halys density (Dens)	3, 200	1.94	0.1237
Cv*Phen	4, 200	0.72	0.5767
Cv*Dens	3, 200	0.65	0.5832
Phen*Dens	12, 200	2.16	0.0150
Cv*Phen*Dens	12, 200	0.90	0.5462

Figure 17. Fructose content in must (± SE) of white grapes for different *Halyomorpha halys* densities. Different letters indicate significant differences at the Tukey-Kramer test (α = 0.05).



The tartaric acid concentration in the must obtained from white cultivars was not strongly influenced by *H. halys* infestation, but a reduction in tartaric acid was observed in Pinot gris infested during the starting of the veraison (Table 15; Figure 18). In red cultivars, infestation by *H. halys* was associated with an increase of tartaric acid concentration (Table 16; Figures 19). Other differences were associated with cultivars and phenological stages (Table 16; Figure 20). In both white and red grapevine musts, gluconic acid concentration was influenced by *H. halys* infestation levels and phenological stages of infestation with a significant interaction observed in the case of white grape varieties (Tables 17 and 18; Figures 21–23). Gluconic acid increased along with *H. halys* infestation, and in grape cultivars this increase was associated with infestation established at the beginning of ripening (Tables 17 and 18; Figures 21–23).

Table 15. Results of GLMM on the tartaric acid content of the must of white cultivars.

Source of variation	**df**	**F**	**p**
Cultivar (Cv)	**1, 195**	**182.05**	**< 0.0001**
Phenological stage (Phen)	**4, 195**	**16.03**	**< 0.0001**
H. halys density (Dens)	3, 195	1.73	0.1612
Cv*Phen	**4, 195**	**14.02**	**< 0.0001**
Cv*Dens	**3, 195**	**3.23**	**0.0235**
Phen*Dens	**12, 195**	**3.39**	**0.0002**
Cv*Phen*Dens	**12, 195**	**1.95**	**0.0354**

Table 16. Results of GLMM on the tartaric acid content of the must of red cultivars.

Source of variation	**df**	**F**	**p**
Cultivar (Cv)	**1, 200**	**199.76**	**< 0.0001**
Phenological stage (Phen)	4, 200	2.39	0.0523
***H. halys* density (Dens)**	**3, 200**	**5.95**	**0.0007**
Cv*Phen	**4, 200**	**3.72**	**0.0061**
Cv*Dens	3, 200	0.75	0.5237
Phen*Dens	12, 200	0.80	0.6509
Cv*Phen*Dens	12, 200	1.01	0.4393

Figure 18. Tartaric acid content (± SE) in must white grapes showing the interaction grape variety*phenological stage**Halyomorpha halys* density. Different letters indicate significant differences at the Tukey-Kramer test (α = 0.05).

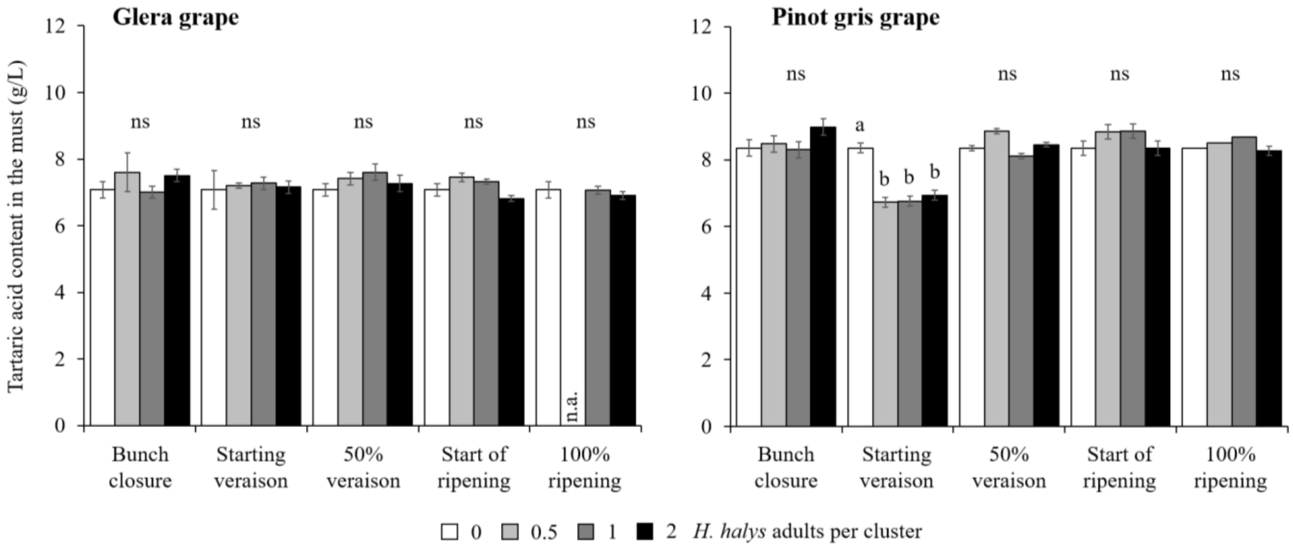


Figure 19. Tartaric acid content (± SE) in must of red grapes for different *Halyomorpha halys* densities. Different letters indicate significant differences at the Tukey-Kramer test (α = 0.05).

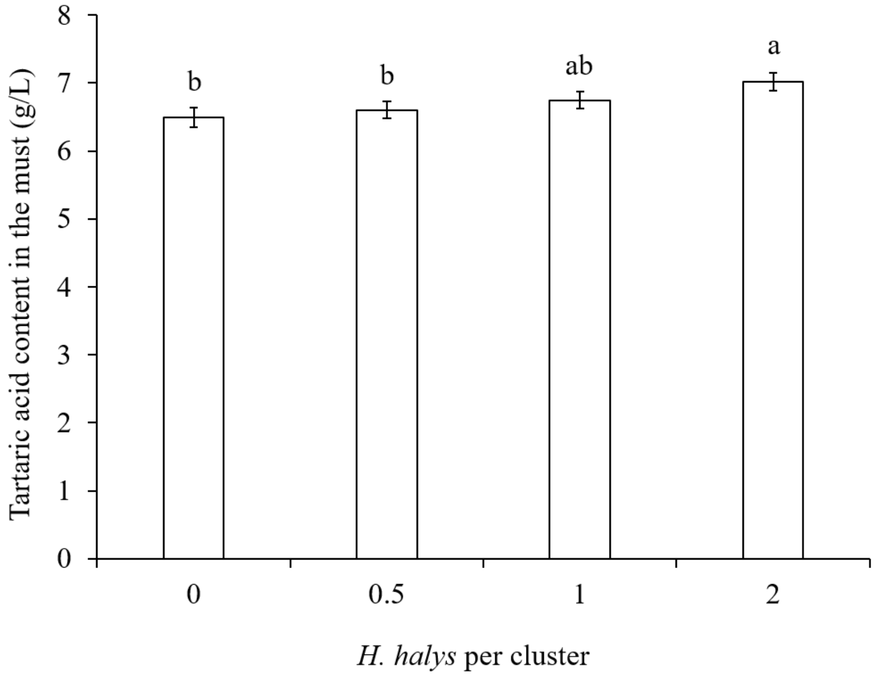


Figure 20. Tartaric acid content (± SE) in must of red grapes showing the interaction grape variety**H. halys* density. Different letters indicate significant differences at the Tukey-Kramer test (α = 0.05).

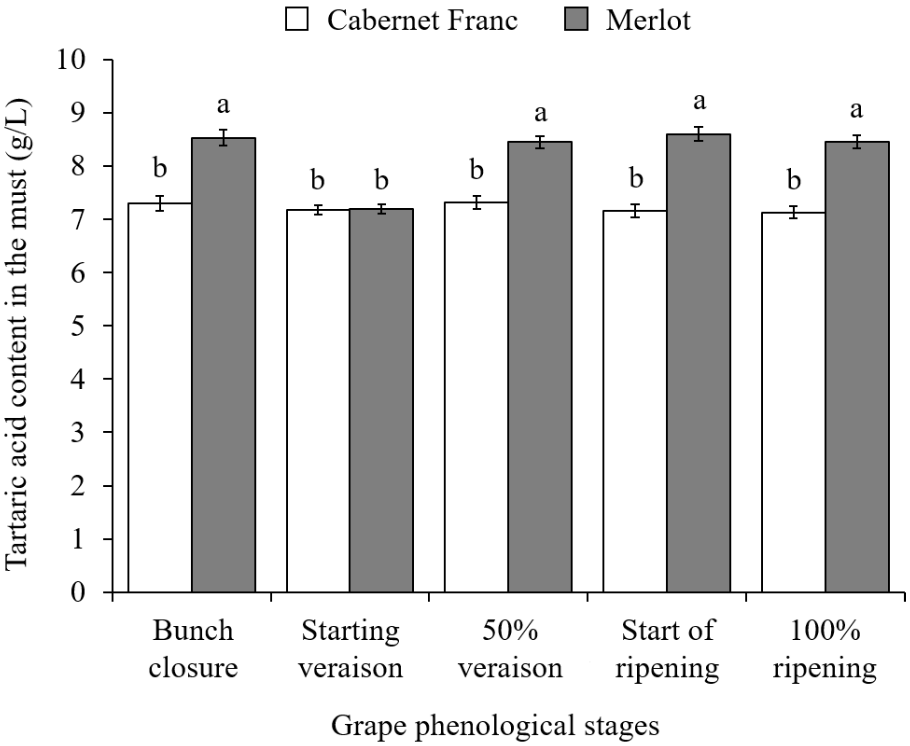


Table 17. Results of GLMM on the gluconic acid content of the must of white cultivars.

Source of variation	**df**	**F**	**p**
Cultivar (Cv)	1, 153	1.37	0.2439
Phenological stage (Phen)	**4, 153**	**5.71**	**0.0003**
***H. halys* density (Dens)**	**3, 153**	**3.63**	**0.0145**
Cv*Phen	4, 153	1.51	0.2007
Cv*Dens	3, 153	0.71	0.5482
Phen*Dens	**12, 153**	**2.19**	**0.0149**
Cv*Phen*Dens	12, 153	0.70	0.7449

Table 18. Results of GLMM on the gluconic acid content of the must of red cultivars.

Source of variation	df	F	p
Cultivar (Cv)	1, 157	0.44	0.5105
Phenological stage (Phen)	**4, 157**	**3.38**	**0.0110**
***H. halys* density (Dens)**	**3, 157**	**4.86**	**0.0029**
Cv*Phen	4, 157	1.77	0.1373
Cv*Dens	3, 157	1.82	0.1454
Phen*Dens	12, 157	1.47	0.1416
Cv*Phen*Dens	12, 157	0.74	0.7109

Figure 21. Gluconic acid content (± SE) in must of white grapes for different *Halyomorpha halys* densities. Different letters indicate significant differences at the Tukey-Kramer test (α = 0.05).

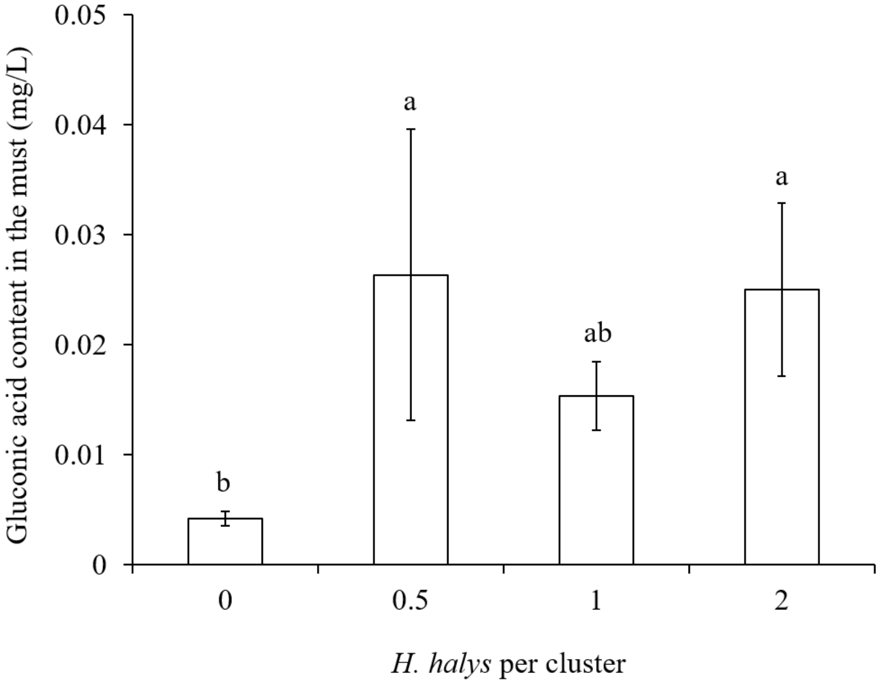


Figure 22. Gluconic acid content (± SE) in must of white grapes in different grape phenological stages*densities of *Halyomorpha halys*. Different letters indicate significant differences at the Tukey-Kramer test (α = 0.05).

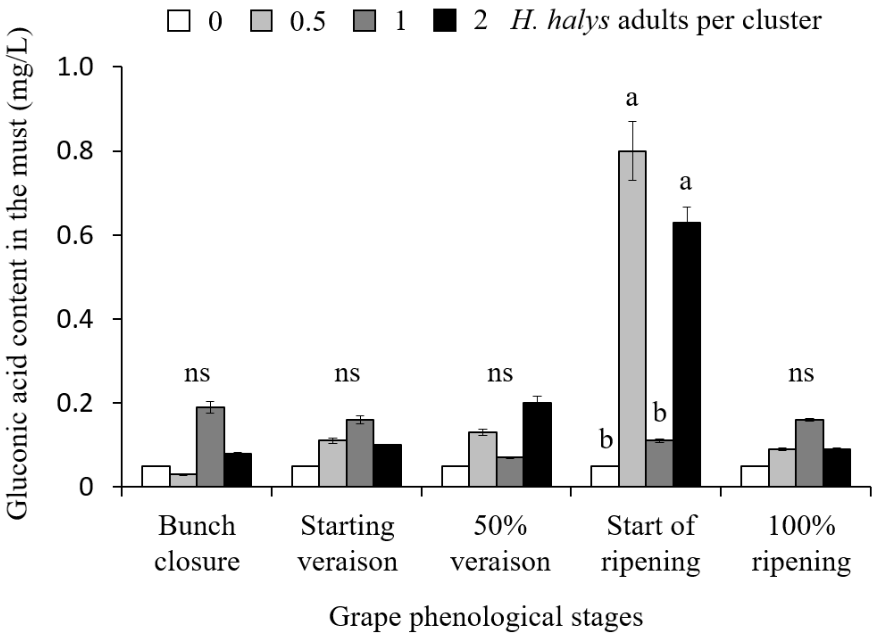
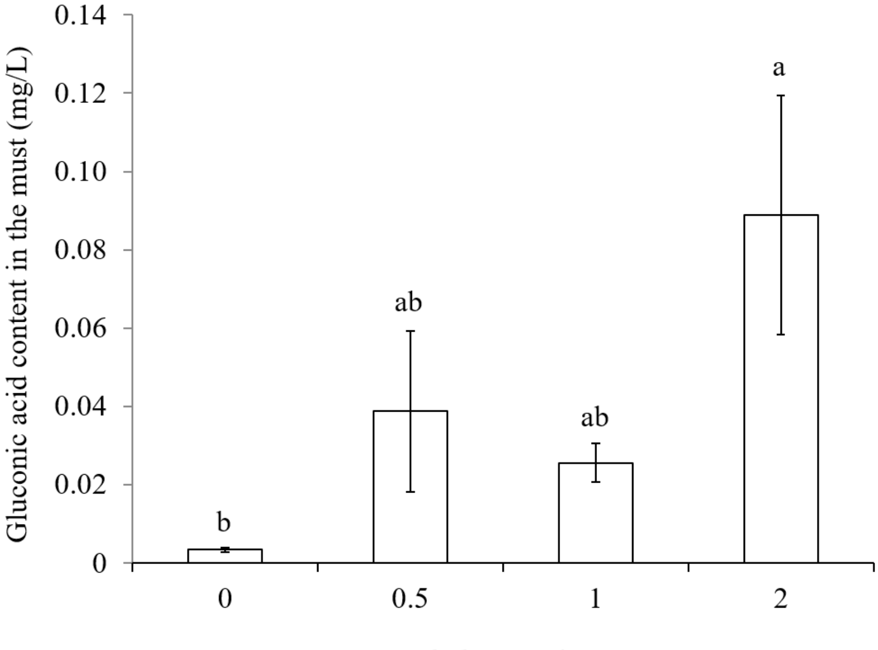


Figure 23. Gluconic acid content (± SE) in must of red grapes for different *Halyomorpha halys* densities. Different letters indicate significant differences at the Tukey-Kramer test (α = 0.05).



4. Discussion

Halyomorpha halys is a pest species known to take advantage of mixed-host diets, which increased nymphal survivorship and fastened developmental duration (Acebes-Doria et al. 2016). Being a greatly mobile species, both at immature and adult stages, it is able to walk for meters (Lee et al. 2014) or to fly for more than 100 km per day (Lee and Leskey 2015). However, its spatial distribution across landscapes as well as the factors responsible for its movement among habitats and hosts are still poorly understood (Wallner et al. 2014). Previous studies from North America found that in vineyards the population of *H. halys* often fluctuates when considering sampling date and location (Basnet 2014; Basnet et al. 2015). In the present study, we found that in vineyards in northern Italy, the seasonal occurrence of *H. halys* varied across time, and *H. halys* showed a preference for some cultivars like Cabernet Franc, Merlot and, with a less extent, Pinot gris compared to the others. The pest is known to invade in particular orchards and row crops, and in previous surveys the grapevine seemed to be less preferred compared to other plant species (e.g., Maistrello et al. 2016b). The presence of all *H. halys* stages on vineyards showed that *V. vinifera* is a suitable host for this stink bug, as reported by Basnet et al. (2015). As already known for row crops (Reeves et al. 2010; Owens 2012; Venugopal et al. 2014; 2015a; Aigner et al. 2017), higher infestation levels of *H. halys* individuals were observed on plants located on margins of patches than in the center of the patch, as also reported in vineyards and orchards (Basnet 2014; Basnet et al. 2015; Venugopal et al. 2015b; Bosco et al. 2018). This information is useful for *H. halys* management, as performing IPM-CPR (Integrated Pest Management – Crop Perimeter Restructuring) practices that are known to reduce the use of insecticides while reducing the pest damage throughout the patch (Blaauw et al. 2015).

Additionally, in vineyards, *H. halys* showed a preference for red grape varieties compared to white ones. Moreover, more individuals were found on cultivars with a late ripening period than early-ripening ones. *Halyomorpha halys* host preference was reported among species, and in particular the movements of adults between hosts overlap with the presence of maturing fruit (Leskey et al. 2012; Leskey and Nielsen 2018). It should be considered that in Italy in the north grapevine is becoming the major perennial crop with a predominant part of the landscape dominated by vineyards. Moreover, grapevine's ripening season is usually at the end of the summer until fall, while other crops susceptible of *H. halys* infestation have a ripening period that is in spring-summer, like cherries and peaches, or late summer like apples (Leskey et al. 2012; Moore et al. 2019). Thus in vineyard dominated landscapes, during the ripening season, grape berry could represent a primarily available food source for *H. halys*. This could explain the increases in *H. halys* number observed in this study at the end of the season.

Halyomorpha halys can have a potential economic impact on grapevine production, especially in case of high infestations. Here we observed damage due to the presence of feeding symptoms. Damaged berries were soft and showed necrotic spots and discolored parts on the skin, as seen in previous studies (Basnet 2014; Smith et al. 2014; Nielsen et al. 2016). Moreover, different grape varieties seemed to be more susceptible to *H. halys* than others, as in the case of Glera compared to Pinot gris, or Cabernet Franc compared to Merlot. Differences among cultivar in susceptibility to *H. halys* were also observed in USA, where the number of feeding punctures on Seyval Blanc was higher than those on Cabernet Sauvignon berries (Basnet 2014). The entity of the damage seems to be related also to the phenological stage of the plant. The previous record of damage on *V. vinifera* berries stated that more feeding punctures occurred in pre-harvest fruit or veraison compared to the other stages, and injuries were more significant when occurred from the veraison stage (Basnet 2014). Here we found that higher susceptibility in term of direct feeding damage was observed when infestations occur during 50% of the veraison, which represent the phenological stage when *H. halys* infestation level increased in the field (Pozzebon et al., unpubl.). Besides, Pinot noir grapes were susceptible to *H. halys* injury in particular from veraison (Nielsen et al. 2016). The damage produced by *H. halys* determined some implications in term of berry weight, and sugar and acid concentration in the must, but these parameters were not dramatically compromised. Beside direct feeding damage, *H. halys* infestation was associated with an increase of damage by *B. cinerea,* particularly on Pinot gris. These differences were supported also by the analysis of gluconic acid that is associated with grey mold infections and was higher in treatments with *H. halys* infestations for both white and red cultivars. The period of infestation can also play a role on the induction of grey mold infection, with an increase of gluconic acid concentration after infestation during the start of ripening. This relation may lead to facilitation in pathogen infestation in case of pest presence, likely due to the opening of wounds in the berry skin. On cherries, *Prunus avium* (L.), *H. halys* infestations increased the number of fruits with fungal infections (Moore et al. 2019), and on fruits and vegetables this pest was able to transmit yeasts (Brust and Rane 2011), other fungi and bacteria (Leskey et al. 2012; Kamminga et al. 2014; Rice et al. 2014; Dobson et al. 2016). The increase of grey mold infection could be considered a threat to grapevine production. The association between grey mold incidence and *H. halys* infestation seem not to be linear with an increase of pathogen incidence induced by lower infestation levels.

In conclusion, our results showed that *H. halys* can infest vineyards and its density is higher at the margins than in the center. *Halyomorpha halys* infestation density was also influenced by grapevine varieties and their ripening time. However, from the cage experiment we found that direct feeding damage is relevant only at high infestation levels, which are not commonly found in the vineyards. This aspect is similar to what was reported by Smith et al. (2014). Notably, these infestation levels that determine consistent direct feeding damage were not observed here in field conditions or were localized at vineyard margins. However, together with the above-mentioned direct damage, we found secondary damage related to the increase of grey mold incidence. This represents an important implication of this pest presence in vineyards since grey mold is considered among the main disease problem of grapevine (Elmer and Michailides 2007). It should be mentioned that the vineyards where the experiments were performed was subject to standard application of fungicides and specific management against grey mold was applied. Additionally, the incidence of grey mold infections can be influenced by climatic conditions during the ripening period, and at the time of the experiments the conditions were not particularly favorable to grey mold development; hence, we can expect higher incidence of this pathogen if more favorable conditions occur. This represents the main issue related to *H. halys* effect on the grapevine and should be considered for its management.

Acknowledgments

This research was partially supported by the Regione Veneto U.O. Fitosanitario and by DAFNAE through project BIRD167802/16 to AP.

Part of the data were included in the Bachelor degree thesis of Ilirjan Allgjata (population seasonal dynamics) and Massimo Cecchetto (cage experiment study) at University of Padova.

References

Acebes-Doria A.L., Leskey T.C., Bergh J.C., 2016. Host plant effects on *Halyomorpha halys* (Hemiptera: Pentatomidae) nymphal development and survivorship. Environmental Entomology 45(3): 663-670.

Aigner B.L., Herbert D.A., Dively G.P., Venugopal D., Whalen J., Cissel B., Kuhar T.P., Brewster C.C., Hogue J.W., Seymore E., 2016. Comparison of two sampling methods for assessing *Halyomorpha halys* (Hemiptera: Pentatomidae) numbers in soybean fields. Journal of Economic Entomology 109(6): 2586-2589.

Aigner B.L., Kuhar T.P., Herbert D.A., Brewster C.C., Hogue J.W., Aigner J.D., 2017. Brown marmorated stink bug (Hemiptera: Pentatomidae) infestations in tree borders and subsequent patterns of abundance in soybean fields. Journal of Economic Entomology 110(2): 487-490.

Albanese D., Malvano F., Sannini A., Pilloton R., Di Matteo M., 2014. A doped polyaniline modified electrode amperometric biosensor for gluconic acid determination in grapes. Sensors, 14:11097-11109, doi:10.3390/s140611097.

Aldrich J.R., Hoffmann M.P., Kochansky J.P., Lusby W.R., Wilson L.T., Zalom F.G., 1995. Pheromone compositions for attracting *Euschistus* spp. insects. U.S. Patent No. 5,447,718.

Basnet S., 2014. Biology and pest status of brown marmorated stink bug (Hemiptera: Pentatomidae) in Virginia vineyards and raspberry plantings. MSc Thesis, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA, 67 pp.

Barbagallo M.G., Guidoni S., Hunter J.J., 2011. Berry size and qualitative characteristics of *Vitis vinifera* L. cv. Syrah. South African Journal for Enology and Viticulture 32: 129-136.

Basnet S., Kuhar T.P., Laub C.A., Pfeiffer D.G., 2015. Seasonality and distribution pattern of brown marmorated stink bug (Hemiptera: Pentatomidae) in Virginia vineyards. Journal of Economic Entomology 108: 1902–1909.

Bettiga L.J., Varela L.G., Bentley W.J., 2013. Brown marmorated stink bug. In: Grape Pest Management – 3rd Edition, Bettiga L.J. (ed.). University of California – Agriculture and Natural Resources, California, pp. 187-188.

Blaauw B.R., Polk D, Nielsen A.L., 2015. IPM-CPR for peaches: incorporating behaviorally-based methods to manage *Halyomorpha halys* and key pests in peach. Pest Management Science 71: 1513-1522.

Bosco L., Moraglio S., Tavella L., 2018. *Halyomorpha halys*, a serious threat for hazelnut in newly invaded areas. Journal of Pest Science 91: 661-670.

Brust G.E., Rane K.K., 2011. Transmission of the yeast *Eremothecium coryli* to fruits and vegetables by the brown marmorated stink bug. University of Maryland Extension. https://extension.umd.edu/learn/transmission-yeast-eremothecium-coryli-fruits-andvegetables-brown-marmorated-stink-bug. Accessed on 31 August 2019.

Cinquanta L., Albanese D., De Curtis F., Malvano F., Crescitelli A., Di Matteo M., 2015. Rapid assessment of gray mold (*Botrytis cinerea*) infection in grapes using biosensors system. American Journal of Enology and Viticulture 66(4):502-508.

Dobson R.C., Rogers M., Moore J.L.C., Bessin R.T., 2016. Exclusion of the brown marmorated stink bug from organically grown peppers using barrier screens. HortTechnology 26: 191-198.

Donèche B., 1989. Carbohydrate metabolism and gluconic acid synthesis by *Botrytis cinerea*. Canadian Journal of Botany 67(10):2888-2893.

Elmer P.A.G., Michailides T.J., 2007. Epidemiology of *Botrytis cinerea* in orchard and vine crops. In: *Botrytis*: Biology, Pathology and Control, Elad Y., Williamson B., Tudzynski P., Delen N. (eds.). Springer, Dordrecht, The Netherlands, pp. 243-272.

Fregoni M., 1998. Viticoltura di qualità. Ed. l'Informatore Agrario S.r.l., Piacenza, VIII + 707 pp.

Hill G.N., Evans K.J., Beresford R.M., Dambergs R.G., 2014. Comparison of methods for the quantification of botrytis bunch rot in white wine grapes. Australian Journal of Grape and Wine Research 20:432-441.

Hong Y.-S., Martinez A., Liger-Belair G., Jeandet P., Nuzillard J.-M., Cilindre C., 2012. Metabolomics reveals simultaneous influences of plant defence system and fungal growth in *Botrytis cinerea*-infected *Vitis vinifera* cv. Chardonnay berries. Journal of Experimental Botany 63(16):5773-5785.

Hori K., 2000. Possible causes of disease symptoms resulting from the feeding of phytophagous Heteroptera. In: Heteroptera of economic importance, Schaefer C.W., Panizzi A.R. (eds.). CRC Press, Boca Raton, Florida, pp. 11-35.

IWC (Italian Wine Central), 2019a. Get Into Italia Wine. https://italianwinecentral.com/region/veneto/. Accessed on 30 August 2019.

IWC (Italian Wine Central), 2019b. Winegrape Production by Region. https://italianwinecentral.com/grape-production-by-region/. Accessed on 30 August 2019.

Kamminga K., Herbert D.A., Toews M. D., Malone S., Kuhar T., 2014. *Halyomorpha halys* (Hemiptera: Pentatomidae) feeding injury on cotton bolls. Journal of Cotton Science 18: 68-74.

Kirchert S., Kaiserb R.E., Morlock G.E., 2019. In-process quality control of wine by planar chromatography *versus* micro planar chromatography. Journal of Chromatography A 1588:137-149.

Lee D.-H., Leskey T.C., 2015. Flight behavior of foraging and overwintering brown marmorated stink bug, *Halyomorpha halys* (Hemiptera: Pentatomidae). Bulletin of Entomological Research 105: 566-573.

Lee D.-H., Nielsen A.L., Leskey T.C., 2014. Dispersal capacity and behavior of nymphal stages of *Halyomorpha halys* (Hemiptera: Pentatomidae) evaluated under laboratory and field conditions. Journal of Insect Behavior 27: 639-651.

Leskey T.C., Hamilton G.C., Nielsen A.L., Polk D.F., Rodriguez-Saona C., Bergh J.C. et al., 2012. Pest status of the brown marmorated stink bug, *Halyomorpha halys* in the USA. Outlooks on Pest Management 23(5): 218-226.

Leskey T.C., Nielsen A.L., 2018. Impact of the invasive brown marmorated stink bug in North America and Europe: history, biology, ecology, and management. Annual Review of Entomology 63: 599-618.

Maistrello L., Dioli P., Bariselli M., Mazzoli G.L., Giacalone-Forini I., 2016a. Citizen science and early detection of invasive species: phenology of first occurrences of *Halyomorpha halys* in Southern Europe. Biological Invasions 18: 3109-3116.

Maistrello L., Costi E., Caruso S., Vaccari G., Bortolotti P., Nannini R., Casoli L., Montermini A., Bariselli M., Guidetti R., 2016b. *Halyomorpha halys* in Italy: first results of field monitoring in fruit orchards. IOBC-WPRS Bulletin 112: 1-5.

Moore L.C., Tirello P., Scaccini D., Toews M.D., Duso C., Pozzebon A., 2019. Characterizing damage potential of the brown marmorated stink bug (Hemiptera: Pentatomidae) in cherry orchards. Entomologia Generalis in press.

Musolin D.L., Konjević A., Karpun N.N., Protsenko V.Y., Ayba L.Y., Saulich A.K., 2018. Invasive brown marmorated stink bug *Halyomorpha halys* (Stål) (Heteroptera: Pentatomidae) in Russia, Abkhazia, and Serbia: history of invasion, range expansion, early stages of establishment, and first records of damage to local crops. Arthropod-Plant Interactions 12: 517-529.

Mohekar P., 2016. Brown marmorated stink bug (BMSB), *Halyomorpha halys* taint in wine: impact on wine sensory, effect of wine-processing and management techniques. PhD Thesis, Oregon State University, Oregon, USA, 190 pp.

Mohekar P., Lapis T.J., Wiman N.G., Lim J., Tomasino E., 2017a. Brown marmorated stink bug taint in Pinot noir: detection and consumer rejection thresholds of trans-2-decenal. American Journal of Enology and Viticulture 68: 120-126.

Mohekar P., Osborne J., Wiman N.G., Walton W., Tomasino E., 2017b. Influence of winemaking processing steps on the amounts of (E)-2-decenal and tridecane as off-odorants caused by brown marmorated stink bug (*Halyomorpha halys*). Journal of Agricultural and Food Chemistry 65: 872-878.

Mohekar P., Osborne J., Tomasino E., 2018. Effects of fining agents, reverse osmosis and wine age on brown marmorated stink bug (*Halyomorpha halys*) taint in wine. Beverages 4, doi:10.3390/beverages4010017.

Musolin D.L., Konjević A., Karpun N.N., Protsenko V.Y., Ayba L.Y., Saulich A.K., 2018. Invasive brown marmorated stink bug *Halyomorpha halys* (Stål) (Heteroptera: Pentatomidae) in Russia, Abkhazia, and Serbia: history of invasion, range expansion, early stages of establishment, and first records of damage to local crops. Arthropod-Plant Interactions 12: 517-529.

Nielsen A.L., Rivera M., Polk D., Leskey T., Morrison R., Dalton D., Hedstrom C., Tomasino E., Walton V., Wiman N., Saunders M., Pfeiffer D., 2016. Integrated Pest Management for Brown Marmorated Stink Bug in Grapes. https://www.stopbmsb.org/managing-bmsb/management-by-crop/grapes/. Accessed on 30 August 2019.

OIV (International organization of vine and wine), 2017. Focus OIV 2017 – Distribution of the world's grapevine varieties. http://www.oiv.int/public/medias/5888/en-distribution-of-the-worlds-grapevine-varieties.pdf. Accessed on 30 August 2019.

OIV (International organization of vine and wine), 2018. OIV Statistical Report on World Vitiviniculture – 2018 World Vitiviniculture Situation. http://www.oiv.int/public/medias/6371/oiv-statistical-report-on-world-vitiviniculture-2018.pdf. Accessed 30 August 2019.

Owens D.R., 2012. Behavior of and crop injury induced by native and exotic stink bugs in Mid-Atlantic soybean. MSc Thesis, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA, 107 pp.

Panizzi A.R., 1997. Wild hosts of Pentatomids: ecological significance and role in their pest status on crops. Annual Review of Entomology 42: 99-122.

Raimondi S., 2007. Vite e vino nel mondo. In: La vite e il vino, Angelini R. (coord.). Coltura e Cultura, Bayer CropScience S.r.l. – ART Servizi Editoriali S.p.A., Milan and Bologna, pp. 548-579.

Reeves R., Greene J., Reay-Jones F., Toews M., Gerard P., 2010. Effects of adjacent habitat on populations of stink bugs (Heteroptera: Pentatomidae) in cotton as part of a variable agricultural landscape in South Carolina. Environmental Entomology 39: 1420-1427.

Rice K.B., Bergh C.J., Bergmann E.J., Biddinger D.J., Dieckhoff C., Dively, G. et al., 2014. Biology, ecology, and management of brown marmorated stink bug (Hemiptera: Pentatomidae). Journal of Integrated Pest Management 5(3): 1-13.

Roby G., Matthews M., 2004. Relative proportions of seed, skin and flesh, in ripe berries from Cabernet Sauvignon grapevines grown in a vineyard either well irrigated or under water deficit. Australian Journal of Grape and Wine Research 10: 74-82.

Roby G., Harbertson J.F., Douglas A.A., Matthews M.A., 2004. Berry size and vine water deficits as factors in wine grape composition: anthocyanins and tannins. Australian Journal of Grape and Wine Research 10: 100-107.

Smith J.R., Hesler S.R., Loeb G.M., 2014. Potential impact of *Halyomorpha halys* (Hemiptera: Pentatomidae) on grape production in the Finger Lakes Region of New York. Journal of Entomological Science 49: 290-303.

Venugopal P.D., Coffey P.L., Dively G.P., Lamp W.O., 2014. Adjacent habitat influence on stink bug (Hemiptera: Pentatomidae) densities and the associated damage at field corn and soybean edges. PLoS ONE 9(10): e109917, doi:10.1371/journal.pone.0109917.

Venugopal P.D., Dively G.P., Lamp W.O., 2015a. Spatiotemporal dynamics of the invasive *Halyomorpha halys* (Hemiptera: Pentatomidae) in and between adjacent corn and soybean fields. Journal of Economic Entomology 108(5): 2231-2241.

Venugopal P.D., Martinson H.M., Bergmann E.J., Shrewsbury P.M., Raupp M.J., 2015b. Edge effects influence the abundance of the invasive *Halyomorpha halys* (Hemiptera: Pentatomidae) in woody plant nurseries. Environmental Entomology 44(3): 474-479.

Walker R.R., Blackmore D.H., Clingeleffer P.R., Kerridge G.H., Rühl E.H., Nicholas P.R., 2005. Shiraz berry size in relation to seed number and implications for juice and wine composition. Australian Journal of Grape and Wine Research 11: 2-8.

Wallner A.M., Hamilton G.C., Nielsen A.L., Hahn N., Green E.J., Rodriguez-Saona C.R., 2014. Landscape factors facilitating the invasive dynamics and distribution of the brown marmorated stink bug, *Halyomorpha halys* (Hemiptera: Pentatomidae), after arrival in the United States. PLoS ONE 9(5): e95691, doi: 10.1371/journal.pone.0095691.

Chapter 4

Lethal effects of high temperatures on *Halyomorpha halys* adults before and after overwintering

Published as:

Scaccini D., Duso C., Pozzebon A., 2019. Lethal effects of high temperatures on brown marmorated stink bug adults before and after overwintering. *Insects* 10(10): 355, doi:10.3390/insects10100355.

(This Chapter represents an early version of the published paper.)

Davide Scaccini, Carlo Duso, Alberto Pozzebon

Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova, Italy

Davide Scaccini contributed in study conceptualization, data collection and analysis as well as writing of the manuscript.

Abstract

The invasive brown marmorated stink bug, *Halyomorpha halys* is causing economic and ecological damage in invaded areas. Its overwintering behavior warrants mitigation practices in warehouses and shipping operations. The aim of this study was to characterize the mortality response curves of *H. halys* adults to short-term high temperature exposure. Here we compared field-collected individuals entering (ENA) and exiting diapause (EXA). EXA adults displayed higher susceptibility to high temperatures compared to ENA individuals. Complete mortality of all tested individuals were obtained after 10 minutes of exposure at 50.0 °C, and after 15 (EXA) or 20 minutes (ENA) at 47.5 °C. The nutritional status of these insects had no effect to high temperature tolerance. The results obtained here underlined the relative inability of *H. halys* to tolerate upper temperature limits compared to lower limits. The mortality-curves obtained here may be used to provide cost-effective heat treatments aimed at the *H. halys* for phytosanitory control.

1. Introduction

Invasive insect species cause significant economic and ecological damage in newly-invaded areas (Kenis and Branco 2009; Kenis et al. 2010), resulting in high economic costs (Pimentel et al. 2005; Kenis et al. 2017). In the recent past, the brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), started to spread worldwide from the native range in East Asia. It is an invasive phytophagous pest, already recorded to feed on more than 170 plant species worldwide (Leskey and Nielsen 2018). The first *H. halys* was recorded in the 2000s in Switzerland (Wermelinger et al. 2008), and it is now widespread throughout Europe (Haye et al. 2015; Leskey and Nielsen 2018; Vétek et al. 2018). In Italy, *H. halys* rapidly became an agricultural pest resulting in damage to crops and causing nuisance problems for the human population (Maistrello et al. 2013; 2016; Bariselli et al. 2016).

In autumn, adult *H. halys* field populations invade buildings searching for shelters to overwinter (Inkley 2012; Cambridge et al. 2015). As a result, adults can be found in warehouses and in food industries that are present in the area of invasion, with potential risks of contamination of goods that could be distributed worldwide (Nicoli Aldini and Fraschini 2017; Acebes-Doria et al. 2018). This aspect plays a key role in the invasion process of this pest. Overwintering aggregations are frequently intercepted in freightliners transporting cargo and this is likely to represent an important invasion pathway for *H. halys* worldwide (Haye et al. 2015; Nixon et al. 2018; 2019).

In disinfestation programs, insects' control in food industries can be achieved by modifying the environmental temperature below or above their critical temperature levels. The use of extreme temperatures can be a viable method in quarantine security in order to avoid the introduction of exotic pests into other countries. Treatments using suboptimal low temperatures (i.e., freezing) are less commonly used due to the high energetic costs, while high temperatures are extensively used since the last century (e.g., Dean 1911; 1913; Chapman 1932; Burges and Burrell 1964) to kill insect pests of stored products (Fields and White 2002; Phillips and Throne 2010).

Acute changes in temperature result in significant metabolic changes in insects. These changes include anaerobiotic metabolism increases; enzyme activity is affected; cellular membranes are altered by changing the fluidity of the phospholipid bilayer; external structures can be modified, as arises for the cuticular wax of the insect; changes in the behavior and in nervous and endocrine systems also occur (Neven 2000). Above 40.0 °C these effects become more critical (Neven 2000). Generally short exposures up to temperatures of 50.0 °C are lethal for almost all tested insects (Hammond 2015). Similar high temperature treatments are used for quarantine treatments of goods (Mangan and Hallman 1998), where exposures of 50–60 °C for 24 hours are required in disinfestation programs (Phillips and Throne 2010).

For *H. halys*, it is known that consistently low temperature is required to kill adults (Cira et al. 2016; 2018; Lowenstein and Walton 2018; Chapter 5), making cold treatments a less suitable practice in disinfestation programs. Conversely, heat treatments have been proposed to manage *H. halys* in closed environments by exposing goods possibly containing insects to 50.0 °C for 15 minutes or more (Aigner and Kuhar 2016). However, more detailed information is necessary to optimize heat treatments, in order to reduce energetic costs and efficacy in *H. halys* control.

Additionally, treatments aimed at managing 'hitchhiking' pest populations within shipped goods potentially involve insects in the different physiological states. These may include fall aggregations to others that are close or within to their overwintering sites but are ready to move into the field. Indeed, *H. halys* can overwinter as aggregations of adults in non-feeding and non-reproductive state within hidden and protected sites (Watanabe et al. 1994; Lee et al. 2014). Such insects typically display gradual springtime emergence and field dispersion patterns. This transition from overwintering to the overwintered state is associated with non-reproductive to reproductive physiological changes (Nielsen et al. 2014; 2016). During overwintering the nutritional levels of such insect populations decline (Skillman et al. 2018b) but then display a gradual increase as the season progress through the field season (Skillman et al. 2018a). Different physiological and nutritional statuses can have a potential effect on resistance to high temperatures, however this information is not available for *H. halys* since previous research was performed using laboratory maintained colonies (Aigner and Kuhar 2016).

The aim of this study was to characterize short-term (2.5 minutes to 1 hour) high-temperature mortality response curves of *H. halys* adults. We also tested if the effect of heat exposure changes between entering and exiting diapause adults. In a specific experiment, we tested whether the nutritional status of insects influenced their mortality induced by heat exposure.

2. Materials and methods

2.1. Insects

Both entering diapause (September to October; hereafter ENA) and exiting diapause adults (April to May; hereafter EXA) were hand-collected in Legnaro, Italy (45.344872 N, 11.956208 E) in 2017. ENA adults are insects that developed in the current year (two generations per year are recorded in northern Italy; Costi et al. 2017). EXA adults were collected from artificial overwintering units placed in outdoor conditions under shade. Artificial overwintering units consisted of five plastic boxes (50 x 35 x 15 cm) containing wooden cage (34 x 19 x 10 cm) with a 34 x 1 cm slit along one side. Cardboards and paper were placed inside wooden cages to provide shelter for insects. 250 *H. halys* adults were placed in each box in fall 2016. These units were monitored three times per week from November 2016 to May 2017. Adults were considered exiting diapause when they were found outside the wooden cages in the plastic boxes. Diapause was confirmed by insect dissection, where no eggs were recorded in ENA or EXA females, which were in the 'one immature oocyte per ovariole' rank as described by Nielsen et al. (2017).

2.2. Lethal high temperatures with short exposure times

Laboratory experiments were performed using a thermocryostat (LAUDA Alpha, RA 12®) for heat treatment. Adults of *H. halys* were placed singly in glass vials (7-ml volume) and were sealed by a cotton swab to allow gas exchange. The available air volume of vials for each adult was about 5.5 ml. The starting relative humidity was 50 (± 2)%, and fluctuated to 56 (± 4)% during the experiment. At least 30 adults per temperature-time combination were tested, in replicates of 10 vials. Before treatment, adults were collected from buildings (ENA) or from overwintering units (EXA) and kept for two hours in boxes at room temperature (23 °C). Vials were placed in the thermocryostat and exposed to temperatures ranging from 32.5 to 60.0 °C, with a 2.5 °C step, for a period of 2.5, 5, 10, 15, 30 or 60 minutes – covering different short-time exposures to high temperatures similarly to those performed by Waddell et al. (2000) and Aigner and Kuhar (2016). The lower tested temperature was set to include 2.5 °C below temperature where European *H. halys* adults failed to develop (Haye et al. 2014). To better describe the mortality curves, the following temperatures not included in the 2.5 °C step were also performed only for the ENA adults: 41.5, 45.5, 46.0, 46.5, 47.0, and 56.5 °C. The temperature within the vials was checked using a thermocouple (RhOS, 4-Channel Digital Thermometer Thermocouple Sensor). Heating rate was 15.0 °C minute^{-1}, with heating times ranging from 0.6 to 2.5 minutes depending on the set temperature. After reaching the set temperature, the exposure was maintained for the time required (i.e., 2.5, 5, 10, 15, 30 or 60 minutes). Then, the insects were removed from vials, kept in bug dorms at room temperature and followed for 24 hours to assess the mortality. Control ENA and EXA insects were subjected to room temperature (maintained at 23 °C during all the procedures).

2.3. Nutrient index and *H. halys* mortality after high temperature exposures

Prior to heat exposure, we weighted and evaluated the nutrient status of each insect as described by Funayama (2004) and Skillman et al. (2018a,b), considering their prothorax width and body mass [weight (mg)/prothorax width (mm)^3]. We tested if the weight or the level of nutrient index have an effect on adult mortality after heat exposure. 240 ENA and EXA females and males were exposed at the set temperatures for 15, 30 or 60 minutes using the procedures described above. After heat treatment insects were processed following the same procedure described above.

2.4. Data analysis

Data on the effect of lethal high temperatures were analyzed with a probit regression using the PROBIT procedure of SAS (ver. 9.4) and interpolating the observed data to mortality curves. Lethal temperatures for 50% (LT50) and 99% (LT99) mortality of adults, for any exposure time, were estimated. For brevity we run probit regression on data obtained with 2.5, 15, 30 and 60 minutes exposure times. Comparisons between the two groups of adults (i.e., EXA and ENA) for the lethal temperature levels (i.e., LT50 and LT99) were done by using the Lethal Dose ratios method (α = 0.05) based on their 95% confidence limits, depending on the intercepts and the slopes of the probit lines and considering the variance-covariance matrices as described by Robertson et al. (2007).

The time causing mortality (MT) was studied through the probit regression analysis for 47.5 and 50.0 °C and using data from all exposure times. The probit analysis was conducted as stated before but considering time as independent variable, evaluating the MT50 and MT99 levels and using the Lethal Dose ratios method (Robertson et al. 2007) ($\alpha = 0.05$) for comparisons between overwintering states within the same temperature.

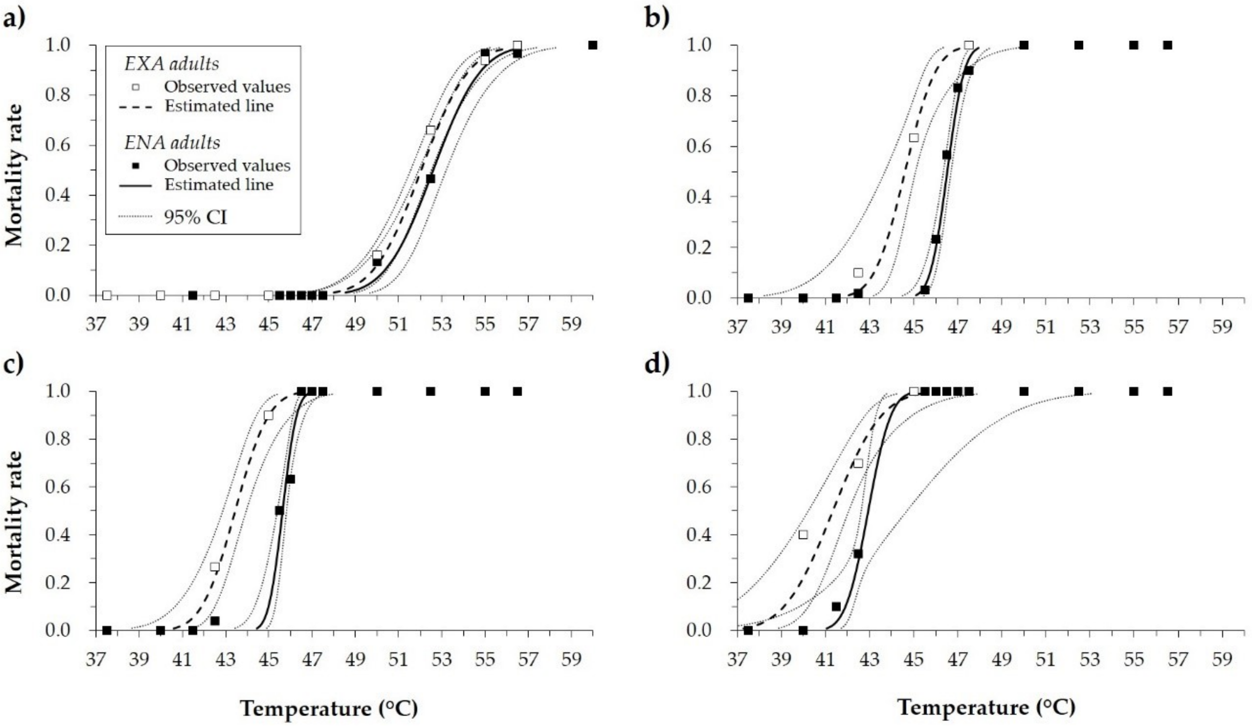
Finally, data obtained in the last experiment were analyzed using a General Linear Mixed Model, with the MIXED procedure of SAS (ver. 9.4) with an F-test ($\alpha = 0.05$) followed by a Tukey-Kramer test ($\alpha = 0.05$) to determine if there were differences in nutrient index or weight between dead and alive insect after exposure to 42.5 or 45.0 °C for 15, 30 or 60 minutes. In this analysis we considered the status of the insect (i.e., dead or alive), sex, state of diapause (i.e., EXA or ENA), temperature (i.e., 42.5 or 45.0 °C) and their interactions as source of variation. Data on mortality after 15, 30 or 60 minutes were analyzed separately. Data were checked for model assumptions prior to the analysis and untransformed data were used.

3. Results

An increase in insect mortality was associated with high temperature exposure and exposure time. Shorter exposure times required higher temperatures to result in similar *H. halys* adults mortality levels. With 2.5 minutes of exposure, the minimum temperature to induce *H. halys* mortality was 50.0 °C for both EXA and ENA adults (Figure 1a). The lowest temperature inducing adult mortality was 42.5 °C for 15 and 30 minutes respectively (Figures 1b and 1c). At 40.0 °C and 41.5 °C, 60 minutes were needed to kill the first adults of EXA and ENA, respectively (Figure 1d).

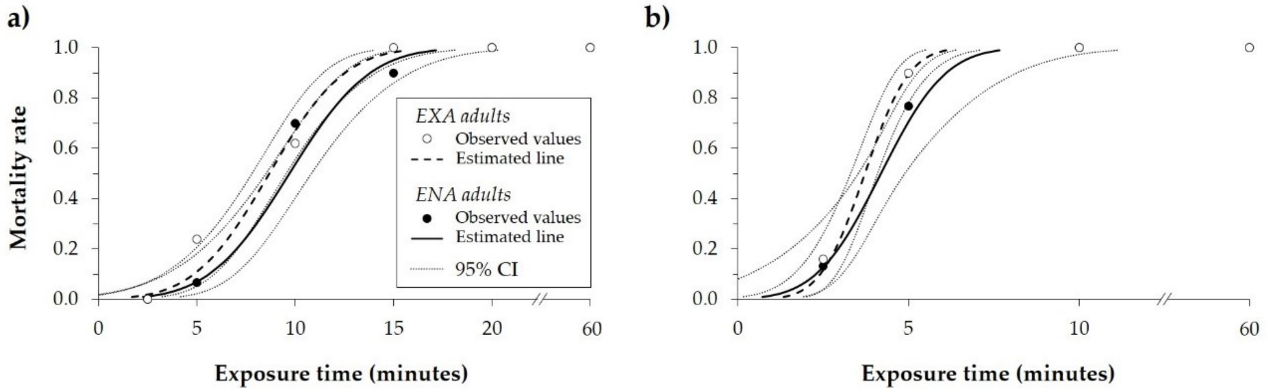
Considering all the exposure times tested, LT50 ranged from 41.3 to 52.6 °C and LT99 from 44.8 to 56.7 °C. Mortality curves were different between EXA and ENA adults; at the same exposure time, LTs were lower for EXA adults, which were more susceptible to high temperatures than ENA ones (excepted for LT99 after 60 minutes; Table 1).

Figure 1. Mortality rate [observed values, estimates of the curves and 95% confidence interval (CI)] of *Halyomorpha halys* adults (EXA and ENA) after exposure to high temperatures for 2.5 (a), 15 (b), 30 (c) and 60 (d) minutes.



For the two temperatures of 47.5 and 50.0 °C, some *H. halys* adults died after just 2.5 minutes when exposed to 50.0 °C, and 5 minutes when exposed to 47.5 °C. The mortality rate was 100% after 10 minutes of exposure at 50.0 °C, and after 15 (EXA) or 20 minutes (ENA) at 47.5 °C. No survivorship was recorded for adults exposed to these two temperatures for 20 minutes or more (Figure 2). The time required to kill 50% of the adults (MT50) was 8.67 and 9.69 minutes, while the MT99 was 3.72 and 4.19 minutes for EXA and ENA, respectively (Table 2). For the exposure to the same temperature, the Lethal Dose ratios method did not show differences between the EXA and ENA curves for the MT50, while MT99 was higher for ENA than EXA at both tested temperatures (Table 2).

Figure 2. Mortality rate [observed values, estimates of the curves and 95% confidence interval (CI)] of *Halyomorpha halys* adults (EXA and ENA) calculated after different exposure times at the constant temperature of 47.5 °C (a) and 50.0 °C (b).



The mortality rate of *H. halys* after exposure to 42.5 and 45.0 °C for 15, 30 and 60 minutes was not different considering insect weight or nutrient index (Figures 3, 4; Table 3). Weight and nutrient index values were higher for ENA than for EXA adults, and weight was higher for females than for males (Figure 3; Table 3).

Figure 3. Mean (± SE) of *Halyomorpha halys* weight in relation to their status (dead or alive), overwintering state and sex. Data on *H. halys* exposed to high temperature for 15 (a: females; b: males), 30 (c: females; d: males) and 60 (e: females; f: males) minutes. Different letters indicate significant differences at Tukey-Kramer test (α = 0.05).

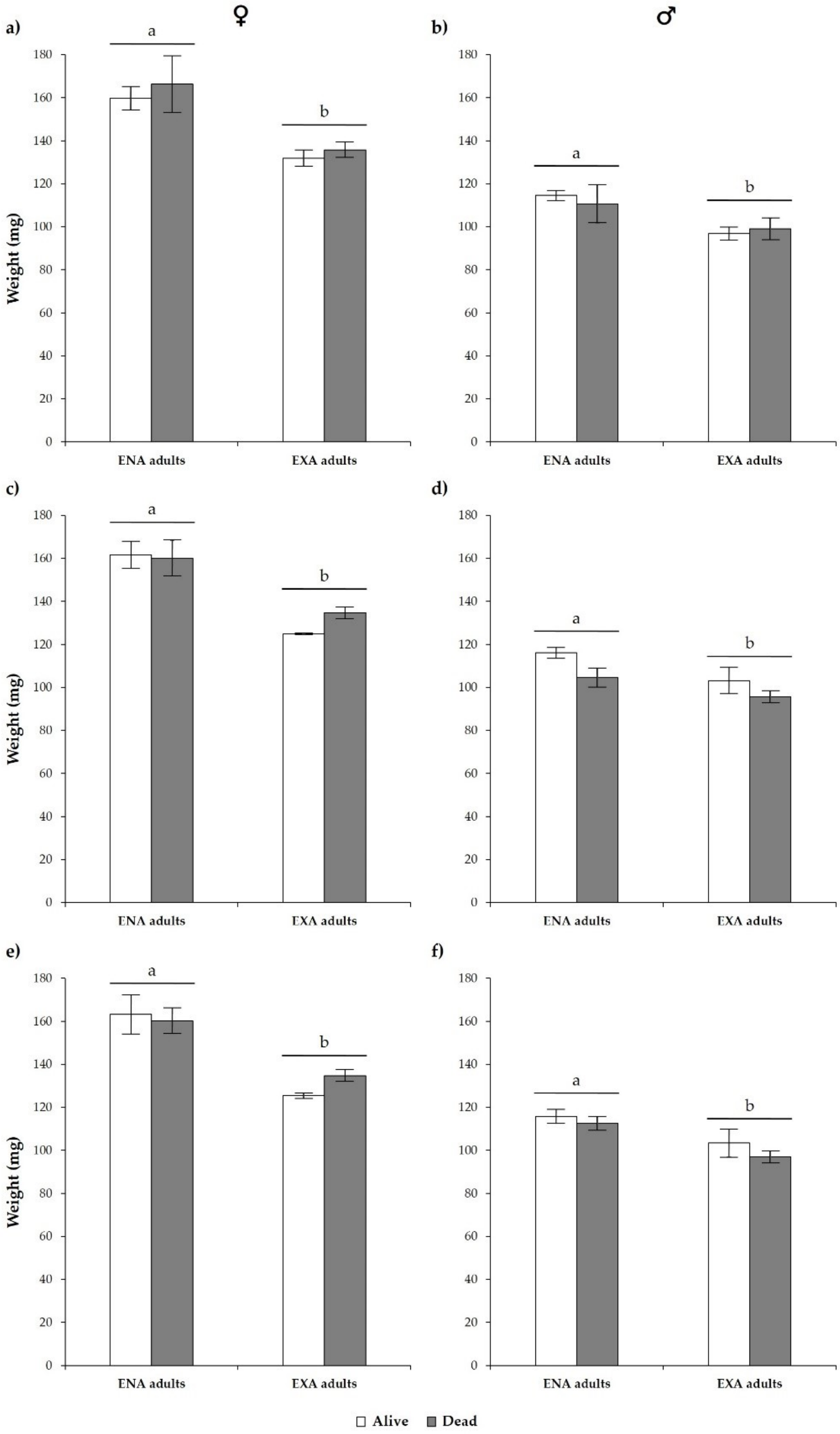


Figure 4. Mean (± SE) of *Halyomorpha halys* nutrient index in relation to their status (dead or alive), overwintering state and sex. Data on *H. halys* exposed to high temperature for 15 (a: females; b: males), 30 (c: females; d: males) and 60 (e: females; f: males) minutes. Different letters indicate significant differences at Tukey-Kramer test (α = 0.05).

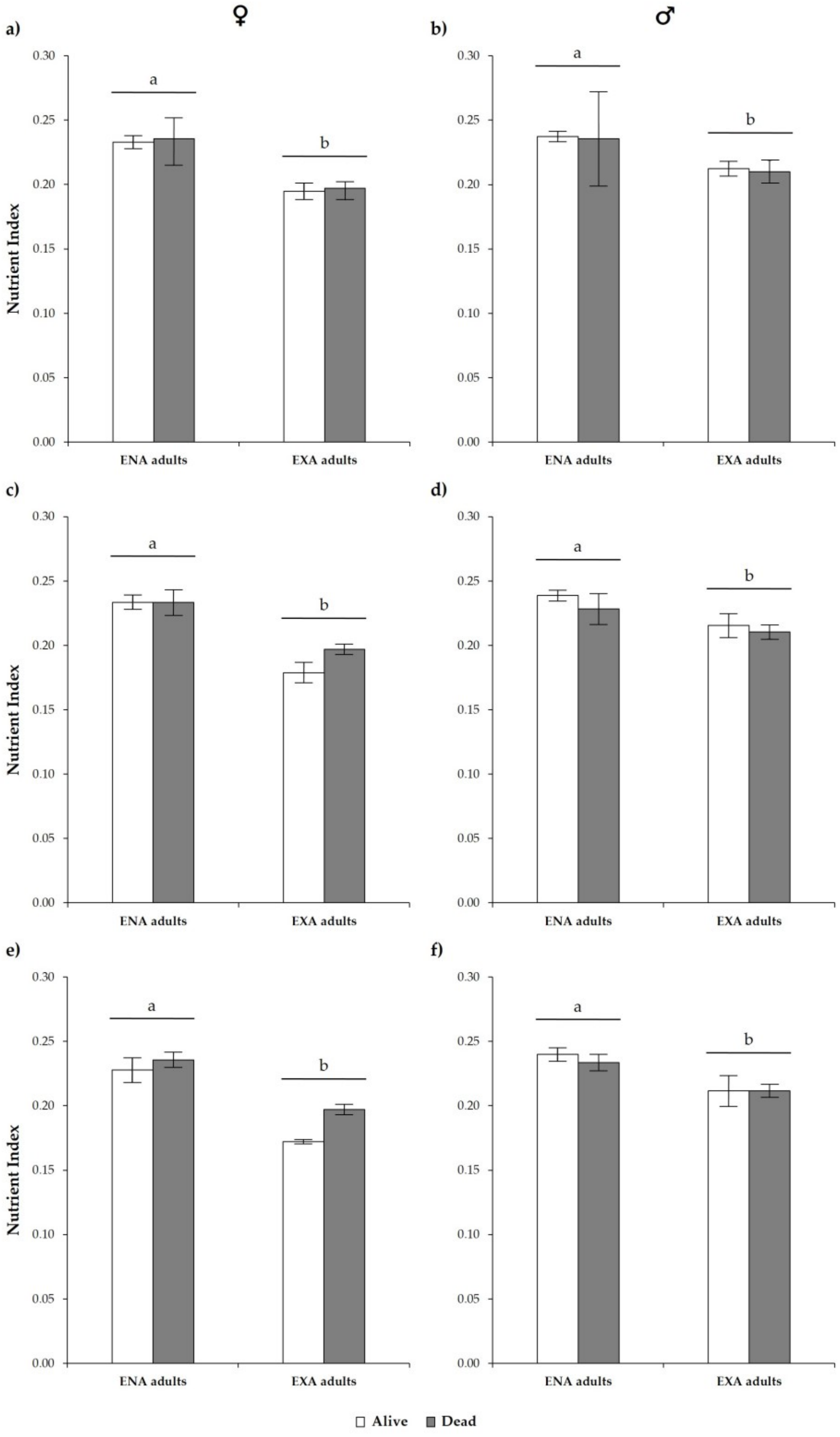


Table 1. Lethal high temperatures (LT) 50 and 99 with 95% confidence interval (CI) for *Halyomorpha halys* adults of the two investigated states (i.e., ENA and EXA), with probit regression parameters.

Adults' state	**Exposure time (minutes)**	**n**	**LT50 (°C) ***		**95% $\mathrm{CI}_{\mathrm{LT50}}$ (°C)**		**LT99 (°C) ***		**95% $\mathrm{CI}_{\mathrm{LT99}}$ (°C)**		**Intercept**	**$\mathrm{SE}_{\mathrm{Intercept}}$**	**Slope**	**$\mathrm{SE}_{\mathrm{Slope}}$**	**X^2 ****	**df**
					Lower	**Upper**			**Lower**	**Upper**						
EXA	2.5	310	52.04	b	51.54	52.50	56.07	b	55.22	57.39	-30.01	3.70	0.58	0.07	32.94	29
ENA	2.5	400	52.61	a	51.94	53.20	56.66	a	55.68	58.30	-73.15	4.51	1.55	0.09	26.16	35
EXA	15.0	310	44.60	b	43.69	45.09	47.17	b	46.33	49.81	-40.47	11.49	0.91	0.25	8.77	29
ENA	15.0	400	46.53	a	46.36	46.69	47.95	a	47.62	48.51	-76.34	10.92	1.64	0.23	24.66	35
EXA	30.0	310	43.44	b	42.86	43.96	46.29	b	45.44	48.01	-35.44	6.81	0.82	0.16	10.97	29
ENA	30.0	400	45.64	a	45.38	45.81	46.83	a	46.52	47.53	-89.54	19.04	1.96	0.41	14.17	35
EXA	60.0	310	41.34	b	40.23	42.05	45.39	a	44.26	47.94	-23.74	5.31	0.57	0.13	11.01	29
ENA	60.0	400	42.95	a	42.62	44.70	44.83	b	43.78	53.05	-53.09	20.83	1.24	0.49	4.61	35

* Within a column, LT values for each exposure time pair with the same letter are not significantly different ($p > 0.05$) according to the Lethal Dose ratios method (Robertson et al. 2007).

** All χ^2 values fit the model at $p > 0.05$.

Table 2. Mortality-time (MT) resulting in 50 and 99% kill rates with 95% confidence interval (CI) for *Halyomorpha halys* adults exposed to high temperatures, with probit regression parameters.

Adults' state	Temperature (°C)	n	MT50 (minutes) *		95% CI$_{MT50}$ (minutes)		MT99 (minutes) *		95% CI$_{MT99}$ (minutes)		Intercept	SE$_{Intercept}$	Slope	SE$_{Slope}$	X^2 **	df
					Lower	Upper			Lower	Upper						
EXA	47.5	400	8.67	a	7.80	9.54	15.65	b	14.06	18.13	-2.89	0.38	0.33	0.04	33.28	43
ENA	47.5	310	9.69	a	8.53	10.83	17.14	a	15.22	20.39	-3.03	0.48	0.31	0.05	22.14	28
EXA	50.0	400	3.72	a	3.31	4.09	6.10	b	5.50	7.12	-3.64	0.61	0.98	0.15	9.05	34
ENA	50.0	310	4.19	a	3.53	4.90	7.67	a	6.40	11.18	-2.80	0.72	0.67	0.17	2.43	28

* Within a column, MT values for each exposure temperature pair (i.e., 47.5 or 50.0 °C) with the same letter are not significantly different ($p > 0.05$) according to the Lethal Dose ratios method (Robertson et al. 2007).

** All χ^2 values fit the model at $p > 0.05$.

Table 3. Effects of *Halyomorpha halys* weight on tested variables and their interactions. Significant *p* values are indicated in bold.

Source of variation	15 minutes			30 minutes			60 minutes		
	F value	df	P value	F value	df	P value	F value	df	P value
Status (dead or alive)	0.10	1, 210	0.7517	0.38	1, 210	0.5404	0.17	1, 210	0.6850
Temperature (Temp)	3.47	1, 210	0.0639	2.47	1, 210	0.1173	1.20	1, 210	0.2739
Status*Temp	0.50	1, 210	0.4804	0.66	1, 210	0.4173	0.00	1, 210	0.9954
Sex	**92.77**	**1, 210**	**< 0.0001**	**54.81**	**1, 210**	**< 0.0001**	**46.79**	**1, 210**	**< 0.0001**
Status*Sex	0.31	1, 210	0.5793	1.47	1, 210	0.2273	0.27	1, 210	0.6033
Sex*Temp	0.46	1, 210	0.4968	0.27	1, 210	0.6066	0.83	1, 210	0.3621
Status*Sex*Temp	0.14	1, 210	0.7090	0.73	1, 210	0.3950	0.18	1, 210	0.6732
Overwintering state (OS)	**22.12**	**1, 210**	**< 0.0001**	**13.88**	**1, 210**	**0.0003**	**16.35**	**1, 210**	**< 0.0001**
Status*OS	0.08	1, 210	0.7759	0.41	1, 210	0.5202	0.30	1, 210	0.5815
OS*Temp	0.11	1, 210	0.7391	0.45	1, 210	0.5037	1.29	1, 210	0.2579
Status*OS*Temp	0.51	1, 210	0.4763	0.21	1, 210	0.6442	1.86	1, 210	0.1744
Sex*OS	0.76	1, 210	0.3844	1.84	1, 210	0.1765	1.37	1, 210	0.2439
Status*Sex*OS	0.07	1, 210	0.7867	0.18	1, 210	0.6746	0.62	1, 210	0.4324
Sex*OS*Temp	0.93	1, 210	0.3366	0.69	1, 210	0.4071	0.24	1, 210	0.6227
Status*Sex*OS*Temp	0.03	1, 210	0.8633	0.27	1, 210	0.6029	0.01	1, 210	0.9118

Table 4. Effects of *Halyomorpha halys* nutrient index on tested variables and their interactions. Significant *p* values are indicated in bold.

Source of variation	**15 minutes**			**30 minutes**			**60 minutes**		
	F value	**df**	**P value**	**F value**	**df**	**P value**	**F value**	**df**	**P value**
Status (dead or alive)	0.14	1, 210	0.7120	0.00	1, 210	0.9877	0.69	1, 210	0.4066
Temperature (Temp)	1.94	1, 210	0.1652	2.55	1, 210	0.1118	0.98	1, 210	0.3223
Status*Temp	0.16	1, 210	0.6930	0.84	1, 210	0.3595	0.02	1, 210	0.9024
Sex	1.34	1, 210	0.2481	2.87	1, 210	0.0915	3.88	1, 210	0.0501
Status*Sex	0.03	1, 210	0.8519	1.65	1, 210	0.1999	1.93	1, 210	0.1657
Sex*Temp	0.94	1, 210	0.3343	1.42	1, 210	0.2353	0.28	1, 210	0.6002
Status*Sex*Temp	0.67	1, 210	0.4144	0.17	1, 210	0.6766	0.13	1, 210	0.7237
Overwintering state (OS)	**22.5**	**1, 210**	**< 0.0001**	**17.77**	**1, 210**	**< 0.0001**	**20.34**	**1, 210**	**< 0.0001**
Status*OS	0.02	1, 210	0.8758	0.90	1, 210	0.3431	1.03	1, 210	0.3110
OS*Temp	0.29	1, 210	0.5913	0.81	1, 210	0.3701	0.49	1, 210	0.4832
Status*OS*Temp	0.32	1, 210	0.5732	0.62	1, 210	0.4313	0.19	1, 210	0.6646
Sex*OS	0.38	1, 210	0.5361	2.70	1, 210	0.1016	1.80	1, 210	0.1814
Status*Sex*OS	0.01	1, 210	0.9237	0.42	1, 210	0.5154	0.28	1, 210	0.5991
Sex*OS*Temp	3.88	1, 210	0.0501	1.80	1, 210	0.1817	0.53	1, 210	0.4692
Status*Sex*OS*Temp	0.00	1, 210	0.9530	0.00	1, 210	0.979	0.46	1, 210	0.5000

4. Discussion

The mortality parameters from different time and temperature combinations here provide significant new insights into effective heat treatments aimed at *H. halys* control. These data provide scenarios where high levels of *H. halys* mortality were obtained by using relatively limited energetic costs. Such heat treatments can be effectively used as part of quarantine procedures for *H. halys* contamination. Both ENA and EXA adults are likely *H. halys* life stages that could be effectively targeted by such heat treatments. These insects are in a non-reproductive status (Nielsen et al. 2016; 2017), as confirmed by the absence of eggs in ENA and EXA females in our study. ENA adults enter into overwintering sites in autumn, while EXA adults emerge from these sites in spring (Costi et al. 2017). In general, these *H. halys* adults do not have to cope with high-temperature stress because such high temperatures do not normally occur in autumn or spring. On the other hand, adults developed during the warmer period of the year (which move to overwintering sites at the end of the summer; Inkley 2012; Cambridge et al. 2015) may have some forms of tolerance to high temperatures. This topic however fell outside the scope of this study and should be investigated in future.

Halyomorpha halys adults start emerge from overwintering sites with more than 10.0 °C of ambient temperature, and flight activity strongly increase and with temperature above 15.0 °C (Lee et al. 2013; Lee and Leskey 2015; Bergh et al. 2017). Nixon et al. (2019) showed that increases in the mortality of overwintering *H. halys* were associated to high temperature (30.0 °C at the peak), than induced the mobility of many individuals, which died probably because of the lack of food. Previous published data on *H. halys* development also showed that European populations failed to develop under controlled conditions at 35.0 °C or more (Haye et al. 2014), implying possible restrictions in the distribution area of the species and influencing its phenology in areas where unsuitable climate conditions and heatwaves occur (Ingels and Daane 2018). Aigner and Kuhar (2016) found that a minimum temperature of 45.0 °C for 15 minutes or 35.0 °C for 4 hours are required to kill *H. halys* adults in the laboratory, but precise minimum threshold cannot be derived by their data. Additionally they used insect from an artificial mass rearing colony and do not assessed potential effect of adult state. In the present manuscript we observed that mortality of the insects starts with 40.0 °C and 41.5 °C for 60 minutes for EXA and ENA adults, respectively. Using data from probit regression we can determine a minimum threshold for *H. halys* mortality that is 41.1 °C for ENA adults and 37.3 °C for EXA adults considering a 60 minutes exposure, while higher thresholds are obtained with 2.5 minutes exposure: 48.6 °C and 48.0 °C for ENA and EXA adults respectively.

Notably, our results showed that EXA adults are more sensitive to high temperatures than ENA ones, possibly due to the physiological status of the insect, as entering diapause adults have a reduction of nutritional levels and a different energetic fitness than exiting ones (Skillman et al. 2018a,b); EXA adults had a lower weight and nutrient index than ENA ones. However, the lipid, glycogen and sugar content do not seem to be related to the nutrient index of *H. halys* adults (Skillman et al. 2018b). In our study, the state of diapause well explained the mortality levels showed by *H. halys* adults while the nutrient index or the weight did not, confirming that the adult's state of diapause more than their nutrient index should be taken into account during the optimization of heat treatment against *H. halys*.

Furthermore, all the tested *H. halys* adults died with exposures to at least 48.0 °C for 15 minutes, or 45.5 °C for 1 hour. In the USA, *H. halys* high temperature mortality showed no adult survival with the exposure to 50.0 °C for at least 15 minutes, or to 45.0 °C for 1 hour or more (Aigner and Kuhar 2016). Here we found that the time required to kill 99% of the adults was ~17 or ~7.5 minutes for 47.5 or 50 °C, respectively (considering ENA adults as the most conservative case), highlighting what are the temperature-time combinations that may be considered for practical uses as for heat treatment at quarantine facilities. Differences on mortality detected between studies may be related to the use of laboratory-reared insects (as in Aigner and Kuhar 2016) versus field collected ones (as in this study), but also by genetic features of the tested insects (Loeschcke 1994), or the methodology performed (Chown et al. 2009; Terblanche et al. 2011; Overgaard et al. 2012).

The results of the present study, and in particular data on minimum thresholds for *H. halys* mortality obtained with 1 hour exposure, can also be of importance in forecasting geographical distribution of this invasive pest. Heatwaves, that are extreme short-term climatic events defined as prolonged periods of excessive heat, are globally increasing in their frequency (Perkins et al. 2012; Perkins and Alexander 2013). Typically during heatwaves, in several part of the word, maximum daily temperatures can exceed 35–40 °C for several days (WMO 2018a,b; 2019). This temperatures range pose risks for the survival of *H. halys* in some of these area (e.g., southern Europe and Australia) where heatwaves can be associated with temperature above 35.0 °C for hours. Here we found that temperatures higher than 37.3 °C for 1 hour can reduce the survival of *H. halys* adults. This type of information can be used to update current models on dynamics and geographic distribution of this pest (Zhu et al. 2012; Nielsen et al. 2016; Zobel et al. 2016; Kriticos et al. 2017) accounting for the effect of high environmental temperatures on mortality. Data provided here are not complete for the understanding of high environmental temperature impact on *H. halys*, but may represent a starting point for future research that should investigate the effect of high temperatures on young stages and simulating longer exposure time.

Data on *H. halys* mortality can be used to compare the heat tolerance of this insect with other arthropod pests. *Halyomorpha halys* appeared less resistant to high temperature than *Cimex lectularius* L. (Hemiptera: Cimicidae), which showed a LT99 at 48.3 °C with 90 minutes of exposure (Kells and Goblirsch 2011). As previously stated, the methodological approach has to be considered in LT studies; in the present study, we used a 'step function' heating (*sensu* Clarke 1967), i.e., modifying the temperature as fast as possible, reaching the tested temperature at insect level within 2.5 minutes from the immersion in the water bath. A rapid heating rate in spiders of the genus *Loxosceles* Heineken & Lowe (Araneae: Sicariidae) resulted in higher LT50 values than a shorter heating (Fischer and Vasconcellos-Neto 2003; Cramer and Zagar 2015). Similarly, slow heating rates by hot-water immersion showed higher mortality in *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) eggs (Waddell et al. 2000). Based on this evidence, LT values with rapid heating may be considered conservative in disinfestation practices with high temperature exposures (Terblanche et al. 2007), but see Kay and Whitford (1978), Neven (1998) and Haye et al. (2014) for different results. Furthermore, insects' heat tolerance can be influenced by the acclimation to a moderately high temperature exposure, altering insect heat resistance (Bowler and Terblanche 2008). In particular, previous study on the southern green stink bug, *Nezara viridula* (L.) (Hemiptera: Pentatomidae) indicated that acclimation to a relatively high temperature can increase the survival of the pest to extreme temperature (Chanthy et al. 2012), and these results have to be considered for heat treatments of the goods as well.

The results obtained here underlined the relative inability of *H. halys* to tolerate upper temperature limits compared to lower limits. These data provide important parameters that can be used as heat shock treatments for *H. halys* control in quarantine methods for disinfestation of goods and in export fresh food industries. Heat treatments with short-time exposure should be targeted on the *H. halys* physiological status, which seems to be related to the overwintering state. The definition of cost-effective heat treatments aimed at the *H. halys* control may be performed also following mortality-curves here reported.

Acknowledgments

This research was partially supported by Bottega s.p.a. (Godega di S. Urbano, Italy).

References

Acebes-Doria A.L., Morrison W.R., Short B.D., Rice K.B., Bush H.G., Kuhar T.P., Duthie C., Leskey T.C., 2018. Monitoring and biosurveillance tools for the brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae). Insects 9: 1-17, doi:10.3390/insects9030082.

Aigner J.D., Kuhar T.P., 2016. Lethal high temperature extremes of the brown marmorated stink bug (Hemiptera: Pentatomidae) and efficacy of commercial heat treatments for control in expert shipping cargo. Journal of Agricultural and Urban Entomology 32: 1-6, doi:10.3954/1523-5475-32.1.1.

Bariselli M., Bugiana R., Maistrello L., 2016. Distribution and damage caused by *Halyomorpha halys* in Italy. Bulletin OEPP/EPPO Bulletin 46: 332-334.

Bergh J.C., Morrison III W.R., Joseph S.V., Leskey T.C., 2017. Characterizing spring emergence of adult *Halyomorpha halys* using experimental overwintering shelters and commercial pheromone traps. Entomologia Experimentalis et Applicata 162: 336-345.

Bowler K., Terblanche J.S., 2008. Insect thermal tolerance: what is the role of ontogeny, ageing and senescence? Biological Review 83: 339-355.

Burges H.D., Burrell N.J., 1964. Cooling bulk grain in the British climate to control storage insects and to improve keeping quality. Journal of the Science of Food and Agriculture 15: 32-50, doi:10.1002/jsfa.2740150106.

Cambridge J., Payenski A., Hamilton G.C., 2015. The distribution of overwintering brown marmorated stink bugs (Hemiptera: Penatatomidae) in college dormitories. Florida Entomologist 98: 1257-1259.

Chanthy P., Martin R.J., Gunning R.V., Andrew N.R., 2012. The effects of thermal acclimation on lethal temperatures and critical thermal limits in the green vegetable bug, *Nezara viridula* (L.) (Hemiptera: Pentatomidae). Frontiers in Physiology 3: 1-8, doi:10.3389/fphys.2012.00465.

Chapman R.N., 1932. Insects infesting stored food products. Minnesota Bulletin 198: 1-83.

Chown S.L., Jumbam K.R., Sørensen J.G., Terblanche J.S., 2009. Phenotypic variance, plasticity and heritability estimates of critical thermal limits depend on methodological context. Functional Ecology 23: 133-140.

Cira T.M., Venette R.C., Aigner J., Kuhar T., Mullins D.E., Gabbert S.E., Hutchison W.D., 2016. Cold tolerance of *Halyomorpha halys* (Hemiptera: Pentatomidae) across geographic and temporal scales. Environmental Entomology 45: 484-491.

Cira T.M., Koch R.L., Burkness E.C., Hutchison W.D., Venette R.C., 2018. Effects of diapause on *Halyomorpha halys* (Hemiptera: Pentatomidae) cold tolerance. Environmental Entomology 47: 997-1004.

Clarke K.U., 1967. Insects and temperature. In Thermobiology, Rose A.H., Ed. Academic Press: London, pp. 293-352.

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

Cramer K.L., Zagar L.M., 2015. Heat tolerance of the brown recluse spider (Araneae: Sicariidae): potential for pest control. Journal of Economic Entomology 109: 284-287.

Dean G.A., 1911. Heat as a means of controlling mill insects. Journal of Economic Entomology 4: 142-161.

Dean G.A., 1913. Further data on heat as a means of controlling mill insects. Journal of Economic Entomology 6: 40-55.

Fields P.G., White N.D.G., 2002. Alternatives to methyl bromide treatments for stored-product and quarantine insects. Annual Review of Entomology 47: 331-359.

Fischer M.L., Vasconcellos-Neto J., 2003. Determination of the maximum and minimum lethal temperatures (LT50) for *Loxosceles intermedia* Mello-Leitão, 1934 and *L. laeta* (Nicolet, 1849) (Araneae, Sicariidae). Journal of Thermal Biology 28: 563-570.

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

Hammond D., 2015. Heat treatment for insect control: developments and applications, Woodhead Publishing Series in Food Science, Technology and Nutrition: Number 241; Woodhead Publishing: Amsterdam, Netherlands, pp. 1-120.

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

Haye T., Gariepy T., Hoelmer K., Rossi J.-P., Streito J.-C., Tassus X., Desneux N., 2015. Range expansion of the invasive brown marmorated stinkbug, *Halyomorpha halys*: an increasing threat to field, fruit and vegetable crops worldwide. Journal of Pest Science 88: 665-673.

Ingels C.A., Daane K.M., 2018. Phenology of brown marmorated stink bug in a California urban landscape. Journal of Economic Entomology 111: 780-786.

Inkley D.B., 2012. Characteristics of home invasion by the brown marmorated stink bug (Hemiptera: Pentatomidae). Journal of Entomological Science 47: 125-130.

Kay C.A.R., Whitford W.G., 1978. Critical thermal limits of desert honey ants: possible ecological implications. Physiological Zoology 51: 206-213.

Kells S.A., Goblirsch M.J., 2011. Temperature and time requirements for controlling bed bugs (*Cimex lectularius*) under commercial heat treatment conditions. Insects 2: 412-422.

Kenis M., Auger-Rozenberg M.-A., Roques A., Timms L., Péré C., Cock M.J.W., Settele J., Augustin S., Lopez-Vaamonde C., 2009. Ecological effects of invasive alien insects. Biological Invasions 11: 21-45.

Kenis M., Branco M., 2010. Chapter 5: Impact of alien terrestrial arthropods in Europe. In: Alien terrestrial arthropods of Europe, Roques A., Kenis M., Lees D., Lopez-Vaamonde C., Rabitsch W., Raspulus J.-Y., Roy D. (eds.). BioRisk 4: 51-71.

Kenis M., Roques A., Santini A., Liebhold A.M., 2017. Impact of non-native invertebrates and pathogens on market forest tree resources. In Impact of biological invasions on ecosystem services, Vilà M., Hulme P.E., Eds.; Invading Nature – Springer Series in Invasion Ecology, Springer: Cham, Switzerland; Volume 12, pp. 103-117.

Kriticos D.J., Kean J.M., Phillips C.B., Senay S.D., Acosta H., Haye T., 2017. The potential global distribution of the brown marmorated stink bug, *Halyomorpha halys*, a critical threat to plant biosecurity. Journal of Pest Science 90: 1033-1043.

Lee D.-H., Cullum J.P., Anderson J.L., Daugherty J.L., Beckett L.M., Leskey T.C., 2014. Characterization of overwintering sites of the invasive brown marmorated stink bug in natural landscapes using human surveyors and detector canines. PLoS ONE 9: e91575, doi:10.1371/journal.pone.0091575.

Lee D.-H., Leskey T.C., 2015. Flight behavior of foraging and overwintering brown marmorated stink bug, *Halyomorpha halys* (Hemiptera: Pentatomidae). Bulletin of Entomological Research 105: 566-573.

Lee D.-H., Short B.D., Joseph S.V., Bergh J.C., Leskey T.C., 2013. Review of the biology, ecology, and management of *Halyomorpha halys* (Hemiptera: Pentatomidae) in China, Japan, and the Republic of Korea. Environmental Entomology 42: 627-641.

Leskey T.C., Nielsen A.L., 2018. Impact of the invasive brown marmorated stink bug in North America and Europe: history, biology, ecology, and management. Annual Review of Entomology 63: 599-618.

Loeschcke V., Krebs R.A., Barker J.S.F., 1994. Genetic variation for resistance and acclimation to high temperature stress in *Drosophila buzzatii*. Biological Journal of the Linnean Society 52: 83-92.

Lowenstein D.M., Walton V.M., 2018. *Halyomorpha halys* (Hemiptera: Pentatomidae) winter survival, feeding activity, and reproduction rates based on episodic cold shock and winter temperature regimes. Journal of Economic Entomology 111: 1210-1218.

Maistrello L., Dioli P., Bariselli M., 2013. Trovata una cimice esotica dannosa per i frutteti. Agricoltura 6: 67-68.

Maistrello L., Dioli P., Bariselli M., Mazzoli G.L., Giacalone-Forini I., 2016. Citizen science and early detection of invasive species: phenology of first occurrences of *Halyomorpha halys* in Southern Europe. Biological Invasions 18: 3109-3116.

Mangan R.L., Hallman G.J., 1998. Temperature treatments for quarantine security: new approaches for fresh commodities. In Temperature sensitivity in insects and application in integrated pest management; Hallman G.J., Denlinger D.L. (eds.). Westview Press: Boulder, Colorado, pp. 201-236.

Neven L.G., 1998. Effects of heating rate on the mortality of fifth instar codling moth. Journal of Economic Entomology 91: 297-301.

Neven L.G., 2000. Physiological responses of insects to heat. Postharvest Biology and Technology 21: 103-111.

Nicoli Aldini R., Fraschini A., 2017. Gestione della difesa antiparassitaria in un'industria conserviera del nord Italia. In Programma e Riassunti, Proceedings of the X Simposio – La Difesa Antiparassitaria nelle Industrie Alimentari e la Protezione degli Alimenti, Piacenza, Italy, 20–22 September 2017: 47.

Nielsen A.L., Chen S., Fleischer S.J., 2016. Coupling developmental physiology, photoperiod, and temperature to model phenology and dynamics of an invasive heteropteran, *Halyomorpha halys*. Frontiers in Physiology 7: 165, doi:10.3389/fphys.2016.00165.

Nielsen A.L., Fleischer S., Hamilton G.C., Hancock T., Krawczyk G., Lee J.C., Ogburn E., Pote J.M., Raudenbush A., Rucker A., Saunders M., Skillman V.P., Sullivan J., Timer J., Walgenbach J., Wiman N.G., Leskey T.C., 2017. Phenology of brown marmorated stink bug described using female reproductive development. Ecology and Evolution 7: 6680-6690.

Nixon L.J., Morrison W.R., Rice K.B., Brockerhoff E.G., Leskey T.C., Guzman F., Khrimian A., Goldson S., Rostás M., 2018. Identification of volatiles released by diapausing brown marmorated stink bug, *Halyomorpha halys* (Hemiptera: Pentatomidae). PLoS ONE 13: e0191223, doi:10.1371/journal.pone.0191223.

Nixon L.J., Tabb A., Morrison W.R., Rice K.B., Brockerhoff E.G., Leskey T.C., van Koten C., Goldson S., Rostás M., 2019. Volatile release, mobility, and mortality of diapausing *Halyomorpha halys* during simulated shipping movements and temperature changes. Journal of Pest Science 92: 663-641.

Overgaard J., Kristensen T.N., Sørensen J.G., 2012. Validity of thermal ramping assays used to assess thermal tolerance in arthropods. PLoS ONE 7: e32758, doi:10.1371/journal.pone.0032758.

Perkins S.E., Alexander L.V., 2013. On the measurement of heat waves. Journal of Climate 156: 4500-4517.

Perkins S.E., Alexander L.V., Nairn J.R., 2012. Increasing frequency, intensity and duration of observed global heatwaves and warm spells. Geophysical Research Letters 39: L20714, doi:10.1029/2012GL053361.

Phillips T.W., Throne J.E., 2010. Biorational approaches to managing stored-product insects. Annual Review of Entomology 55: 375-397.

Pimentel D., Zuniga R., Morrison D., 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecological Economics 52: 273-288.

Robertson J.L., Savin N.E., Preisler H.K., Russell R.M., 2007. Bioassays with Arthropods, 2nd ed.; CRC Press: Boca Raton, Florida, 2007, pp. 1-224.

Skillman V.P., Wiman N.G., Lee J.C., 2018a. Monitoring nutrient status of Brown Marmorated Stink Bug adults and nymphs on Summer Holly. Insects 9: 120, doi:10.3390/insects9030120.

Skillman V.P., Wiman N.G., Lee J.C., 2018b. Nutrient declines in overwintering *Halyomorpha halys* populations. Entomologia Experimentalis et Applicata 166: 778-789.

Terblanche J.S., Deere J.A., Clusella-Trullas S., Janion C., Chown S.L., 2007. Critical thermal limits depend on methodological context. Proceedings of the Royal Society B: Biological Sciences 274: 2935-2942.

Terblanche J.S., Hoffmann A.A., Mitchell K.A., Rako L., le Roux P.C., Chown S.L., 2011. Ecologically relevant measures of tolerance to potentially lethal temperatures. Journal of Experimental Biology 214: 3713-3725.

Vétek G., Károlyi B., Mészáros Á., Horváth D., Korányi D., 2018. The invasive brown marmorated stink bug (*Halyomorpha halys*) is now widespread in Hungary. Entomologia Generalis 38: 3-14.

Waddell B.C., Jones V.M., Petry R.J., Sales F., Paulaud D., Maindonald J.H., Laidlaw W.G., 2000. Thermal conditioning in *Bactrocera tryoni* eggs (Diptera: Tephritidae) following hot-water immersion. Postharvest Biology and Technology 21: 113-128.

Watanabe M., Arakawa R., Shinagawa Y., Okazawa T., 1994. Overwintering flight of brown-marmorated stink bug, *Halyomorpha mista* to the buildings. Japanese Journal of Sanitary Zoology 45: 25-31.

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

WMO (World Meteorological Organization), 2018a. WMO Statement on the State of the Global Climate in 2018. URL https://library.wmo.int/doc_num.php?explnum_id=5789. Accessed on 29 September 2019.

WMO (World Meteorological Organization), 2018b. European heatwave sets new temperature records. URL https://public.wmo.int/en/media/news/european-heatwave-sets-new-temperature-records. Accessed on 29 September 2019.

WMO (World Meteorological Organization), 2019. 2019 starts with extreme, high-impact weather. URL https://public.wmo.int/en/media/news/2019-starts-extreme-high-impact-weather. Accessed on 29 September 2019.

Zhu G., Bu W., Gao Y., Liu G., 2012. Potential geographic distribution of brown marmorated stink bug invasion (*Halyomorpha halys*). PLoS ONE 7: e31246, doi:10.1371/journal.pone.0031246.

Zobel E.S., Hooks C.R.R., Dively G.P., 2016. Seasonal abundance, host suitability, and feeding injury of the brown marmorated stink bug, *Halyomorpha halys* (Heteroptera: Pentatomidae), in selected vegetables. Journal of Economic Entomology 109: 1289-1302.

Chapter 5

Lethal and sub-lethal effects of low-temperature exposures on *Halyomorpha halys* adults before and after overwintering

Manuscript in preparation as: Lethal and sub-lethal of low-temperature exposures on *Halyomorpha halys* adults before and after overwintering

Davide Scaccini, Luka Vanishvili, Paola Tirello, Carlo Duso, Alberto Pozzebon

Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova, Italy

Davide Scaccini contributed in study conceptualization, data collection and analysis as well as writing of the manuscript.

Abstract

Cold winter temperatures can influence insects' survival in temperate zones. Insects are however adapted to cold temperatures typically found in such temperate regions by going into diapause. The brown marmorated stink bug, *Halyomorpha halys* (Stål) is a relatively chill-intolerant species that overwinters as the adult stage in buildings as in other man-made structures. In this study, we characterized low temperature mortality rates of *H. halys* adults of entering (ENA) and exiting (EXA) diapause. We considered different duration of cold exposure, but also wanted to determine the impact of the nutritional status of the insect on cold tolerance. We evaluated the effects of exposure to low temperature on longevity and fecundity of surviving adults. Mortality of ENA and EXA adults was observed from temperatures reaching -2.5 °C for 6 hours, and 2.5 °C for 2 hours, respectively. EXA compared to ENA populations were more sensitive to low-temperature exposure periods (LT50 for 2 hours of exposure was -5.73 °C vs. -10.42 °C). The nutritional status of EXA *H. halys* influenced survival rates at low temperatures with lower survival for insects that have a low nutritional index. Finally, low-temperature exposures increased longevity, but reduced fecundity of ENA females. These findings showed to which extent low winter and spring temperatures can influence the survival rate and population fecundity levels of *H. halys*. The results highlight that spring frost events can result in significant mortality levels of surviving late dormant populations of this pest.

1. Introduction

In the temperate zones, cold winter and spring temperature events can affect the life table parameters of surviving late-dormant diapausing insect populations. Dormancy is an adaptive state characterized by suppressed development, usually accompanied with metabolic suppression and arrested activity. Diapause is a sub-type of dormancy (Koštál 2006), and in insects its induction is mediated in particular by photoperiod regimes and temperature changes (Saunders 2002; Koštál 2006; Tougeron 2019). In temperate climate zones, these environmental cues strongly influence and trigger stink bug (Hemiptera: Pentatomidae) physiological processes, which usually undergo a facultative diapause (Saulich and Musolin 2012). This is the case of the brown marmorated stink bug, *Halyomorpha halys* (Stål), where in fall, after the photoperiod reduction (Niva and Takeda 2003; Nielsen et al. 2016), adults exhibit aggregation behavior and orient towards suitable overwintering places inlcuding buildings and other structures (Inkley 2012; Cambridge et al. 2015). *Halyomorpha halys* is an invasive pest species native from East Asia and now widespread in North America, Europe including Russia, recently recorded in Chile (Leskey and Nielsen 2018), and intercepted in points-of-entry as in New Zealand (Duthie 2012; Vandervoet et al. 2019). Along with survival, low temperatures regulate population growth and range distribution of a species, as well as its establishment and spread (Addo-Bediako et al. 2000; Sinclair et al. 2015; Halbritter et al. 2018). Climate conditions bias the distribution of *H. halys* in invaded areas, and models have shown possible threats for countries in the Southern Hemisphere and extending throughout most of the horticultural areas (Kriticos et al. 2017).

Depending on the capability to survive to intracellular ice formation, insects are classified as chill-intolerant (i.e., they die before freezing), freeze-intolerant (i.e., they live until they freeze), or freeze-tolerant (i.e., they are able to live after the formation of ice in the body; Lee 2010; Sinclair et al. 2015). Recently, studies on supercooling point (i.e., the temperature at which body fluids start to freeze) on *H. halys* showed that the species has to be considered as chill-intolerant; thus, death occurs at temperatures above intracellular ice formation (Cira et al. 2016).

Ice crystal formation may cause physical damage, and survival can be affected by osmotic stress, anoxia and damage in a fraction of fat body cells. Insect survival is also related to dehydration of the organism, which is caused by unfavorable changes in protoplasmic proteins and the surrounding water, as well as osmoses of water out of structures to the surrounding formed ice structures during the freezing process (Asahina 1970; Storey and Storey 1987; Danks 2000). Among the sub-lethal frost injuries, the failure in metamorphosis completion is probably the most common (Asahina 1970). Exposure to low temperatures may however also have an impact on post-diapause survival, fecundity or behavior, but these can vary depending on the Pentatomidae species (Musolin 2007; Saulich and Musolin 2007; 2012; Takeda et al. 2010; Lowenstein and Walton 2018).

Information on the effects of low temperatures on *H. halys* and identification of those critical for its survival can be used to model seasonal population dynamics. In this study we performed experiment aimed at: (1) characterization of low-temperature mortality response curves of entering and exiting diapause *H. halys* adults; (2) quantification of the effect of insect nutritional status on tolerance to low temperature exposures; (3) evaluation the effects of exposure to low temperature on longevity and fecundity of surviving adults.

2. Material and Methods

2.1. Insects

Halyomorpha halys adults searching for overwintering sites (ENA) were collected on building walls in Legnaro, Italy (45.344872 N, 11.956208 E), in September–October 2017 and 2018. More than 250 of these insects were placed in five artificial overwintering units built with a wooden cage (34 x 19 x 10 cm) with slit (34 x 1 cm) along one side and containing cardboards and paper as shelter placed inside a plastic box (50 x 35 x 15 cm). The overwintering units were maintained under shaded outdoor conditions during the two winters and were monitored daily to detect insects exiting overwintering sites (EXA). Insects were collected in April–May as soon as they were found outside the wooden box and moving within the plastic box, and promptly used in the experiments. Insect dissection confirmed that EXA and ENA females were in the 'one immature oocyte per ovariole' rank as described by Nielsen et al. (2017).

2.2. Temperature-mortality curves

Temperature-mortality curves were calculated to characterize the response in term of mortality of *H. halys* adults to the exposure to low temperatures for different times. Low temperatures used in the experiment were +2.5 (only for EXA), 0, -2.5, -5.0, -8.0, -10.0, -12.0, -14.0, and -16.0 °C (only for ENA), for 2, 4 or 6 hours as exposure times. Insects were singly placed in a glass vial of 7-ml volume, closed by a cotton swab to allow gas exchange (available volume: about 5.5 ml after placing the cotton swab). Low-temperature exposures were performed by the immersion of vials containing the insect in thermostatic liquid (KRYO 30®, LAUDA DR. R. WOBSER GMBH & CO. KG, Lauda-Königshofen, Germany) using a cooling thermostat (Alpha, RA 12®, LAUDA DR. R. WOBSER GMBH & CO. KG, Lauda-Königshofen, Germany). During the exposure, the relative humidity within vials ranged from 46 to 54%. Cooling to the set temperature was from -2.5 to -7.5 °C minute^{-1}. A thermocouple (RhOS, 4-Channel Digital Thermometer Thermocouple Sensor) was used to check the temperature in vials. At least 20 (EXA) or 35 (ENA), up to 45, adults for each temperature-time combination were tested, in replicates of 5 vials. After low-temperature exposure, adults were removed from vials and transferred in cages at room temperature and checked for 24 hours for mortality assessment. A control treatment was performed by keeping ENA and EXA adults at room temperature.

2.3. Effects of low temperature on surviving insects

To study the sub-lethal effect on *H. halys*, survived insects exposed to low temperatures were paired and placed in bug dorm under controlled conditions at a temperature of 23 ± 1 °C. All the insects were reared with carrots (*Daucus carota* L.), green beans (*Phaseolus vulgaris* L.), and shelled sunflower seeds (*Helianthus annuus* L.), replaced every week (carrots and green beans) or when molding/rotting or too dry (sunflower seeds). Water was supplied ad libitum on a cotton swab. Pieces of little pieces of paper were used as oviposition substrate and shelters. Laid eggs were removed and placed separately in a plastic box to assess the number of eggs produced and the hatching rate. The collected data were used for the calculation of female longevity after treatment, length of the pre-oviposition period, number of egg masses and mean number of eggs per egg mass.

2.4. Data analysis

Data on the lethal effect of low temperatures were analyzed with a probit regression using the PROBIT procedure of SAS (ver. 9.4), interpolating the observed data to mortality curves. For any exposure time, lethal temperatures causing 50% (LT50; Leather et al. 1993) and 99% (LT99) of adults' mortality were estimated. Comparisons were made between the two groups of adults (i.e., EXA and ENA) for the LT50 and LT99 following the Lethal Dose ratios method ($\alpha = 0.05$), which is based on their 95% confidence limits and depends on intercepts and slopes of the probit lines, also considering the variance-covariance matrices (Robertson et al. 2007).

To determine if there were differences in weight or nutrient index [NI, which is a proxy of nutritional levels, calculated as weight (mg)/prothorax width (mm)^3 (Funayama 2004, Skillman et al. 2018a,b)] between dead and alive insect after exposure to low temperatures for 2, 4 or 6 hours, these parameters were calculated before the treatment for a subsets of insects (480 ENA and 300 EXA adults) that were exposed to low temperatures as described before and analyzed using a General Linear Mixed Model, with the MIXED procedure of SAS (ver. 9.4) with an F-test ($\alpha = 0.05$), followed by a Tukey-Kramer test ($\alpha = 0.05$). The status of the insect (i.e., dead or alive), state of diapause (i.e., EXA or ENA), time of exposure (i.e., 2, 4 or 6 hours) and their interactions were considered as sources of variation. Data were checked for model assumptions prior to the analysis, and untransformed data were used.

Finally, the effects of low-temperature exposure on surviving females were evaluated through a Generalized Linear Mixed Models with the procedure GLIMMIX of SAS (ver. 9.4). The effect of temperature application, exposure time and their interaction were considered as independent variables. Their effect was tested with an F test ($\alpha = 0.05$) and means were separated using a Tukey-

Kramer test (α = 0.05) on least-square means. Due to the low number of survived females exposed to -12 °C and -10 °C, these data were not included in the analysis. Data were transformed in log (x+1) to meet model assumptions.

3. Results

Low-temperature exposure was associated with increased mortality of *H. halys* adults (Figures 1–3). Mortality of ENA adults was observed from -5.0 °C for 2 and 4 hours, and -2.5 °C for 6 hours (Figures 1–3), while for EXA adults, mortality initiated at temperature reaching 2.5 °C for 2 hours (10% mortality rate), and increased with longest exposure time up to 20% (Figure 1–3). LT50s varied from -10.42 °C (2h; Table 1) to -7.57 °C (6h) for ENA adults, while for EXA adults the same parameter varied between -5.73 °C (2h) and -3.34 °C (6h). LT99 values ranged between -15.42 °C (2h) and -16.02 °C (6h; Table 1) for ENA adults, whilst for EXA LT99 ranged from -14.17 °C (2h) to -13.49 °C (6h). LT50s of EXA adults were always lower than those of ENA adults, while for LT99 differences emerged only at 4 hours of exposure (Table 1).

Weight of *H. halys* adults differed between ENA and EXA individuals, but its variation was not associated to differences in *H. halys* mortality rate due to low-temperature exposure (Table 2; Figure 4a). The nutrient index was higher in ENA than EXA adults (Table 2; Figure 4). In EXA adult, insects with a lower NI were found dead after low-temperature exposure independently of exposure time. These differences were not significant in ENA adults (Table 2; Figure 4b).

Effects of exposure ENA and EXA adults to low temperature were evaluated on the pre-oviposition period, number of egg masses, eggs per egg mass and hatching rate. The low-temperature effect was significant on insect longevity of ENA adults: exposure to temperature lower than 0 °C was associated with increased longevity (Table 3; Figure 5). Such differences were not observed for EXA ones (Table 3; Figure 5). An effect of low-temperature exposure emerged on the number of eggs per egg mass laid by ENA females, in particular insect exposed to -8.0 °C showed a lower number of eggs per egg mass compared to other treatments (Figure 5b).

Figure 1. Mortality rate [observed and estimated values 95% confidence interval (CI)] of EXA and ENA *Halyomorpha halys* adults after exposure to controlled low temperatures for 2 hours.

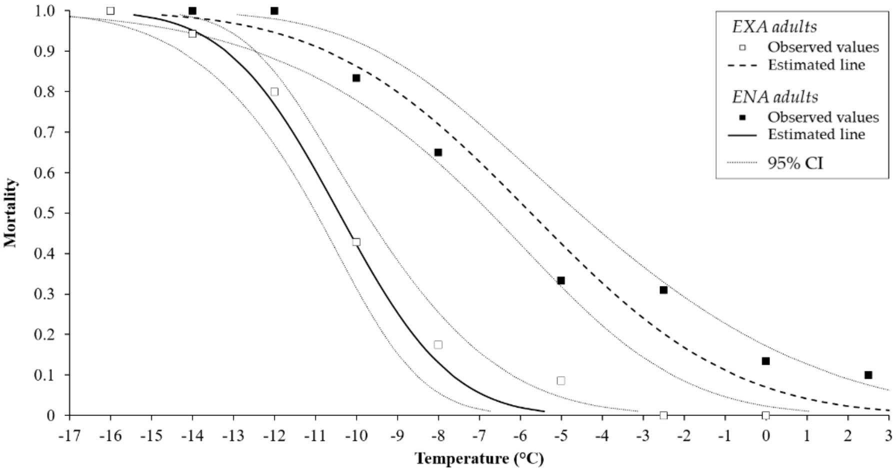


Figure 2. Mortality rate [observed and estimated values 95% confidence interval (CI)] of EXA and ENA *Halyomorpha halys* adults after exposure to controlled low temperatures for 4 hours.

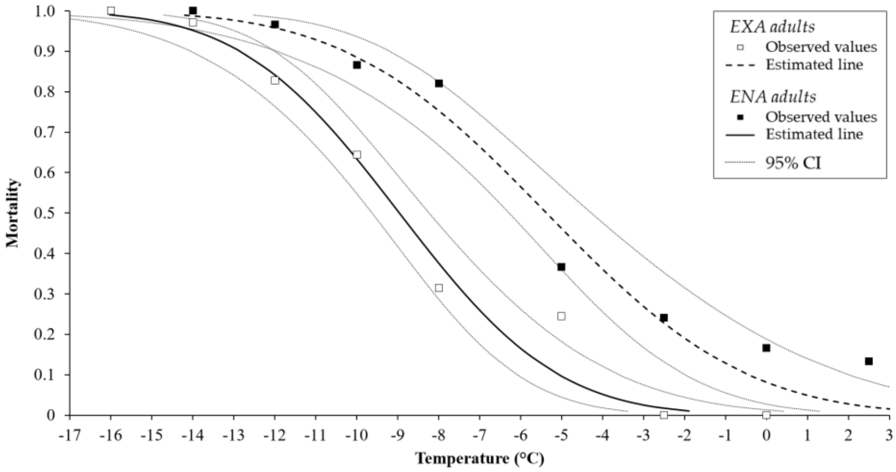


Figure 3. Mortality rate [observed and estimated values 95% confidence interval (CI)] of EXA and ENA *Halyomorpha halys* adults after exposure to controlled low temperatures for 6 hours.

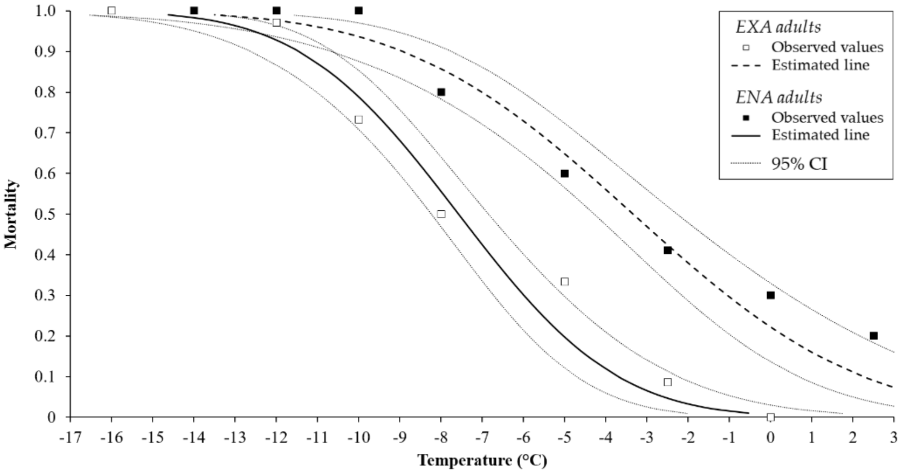


Table 1. Lethal low temperatures (LT) 50 and 99 with 95% confidence interval (CI) and probit regression parameters for ENA and EXA *Halyomorpha halys* adults exposed for the three duration times (2, 4 and 6 hours).

Adults' state	Exposure time	n	LT50 (°C) *		95% CI_{LT50} (°C)		LT99 (°C) *		95% CI_{LT99} (°C)		Intercept	$SE_{Intercept}$	Slope	SE_{Slope}	χ^2 **	df
					Lower	Upper			Lower	Upper						
EXA	2h	220	-5.7331	a	-6.7300	-4.5745	-14.7794	a	-17.8056	-12.9101	-1.4743	0.2680	-0.2572	0.0342	5.7715	6
ENA	2h	285	-10.4234	b	-10.9874	-9.7738	-15.4251	a	-17.3399	-14.3061	-4.8481	0.7488	-0.4651	0.0675	4.7132	6
EXA	4h	248	-5.3761	a	-6.2781	-4.2754	-14.3180	a	-17.2263	-12.5417	-1.3987	0.2628	-0.2602	0.0349	5.4501	6
ENA	4h	300	-8.9576	b	-9.6253	-8.2295	-16.0277	b	-18.0063	-14.7096	-2.9474	0.3705	-0.3290	0.0370	10.2708	6
EXA	6h	259	-3.3432	a	-4.2764	-2.2652	-13.4989	a	-16.4673	-11.5920	-0.7658	0.1666	-0.2291	0.0277	6.3769	6
ENA	6h	305	-7.5746	b	-8.2368	-6.8462	-14.6200	a	-16.5422	-13.3306	-2.5011	0.3218	-0.3302	0.0366	8.1116	6

* Within a column, LT values for each exposure time pair with the same letter are not significantly different ($\alpha = 0.05$) according to the Lethal Dose ratios method (Robertson et al. 2007).

** All χ^2 values fit the model at $\alpha = 0.05$.

Table 2. Statistics of GLMM models (α = 0.05) on the weight and nutrient index measured on *Halyomorpha halys* exposed to low temperatures.

Source of variation	**Weight**			**Nutrient Index**		
	F value	**df**	**P-value**	**F value**	**df**	**P-value**
Status (dead or alive)	**5.54**	**1, 194**	**0.0196**	**23.23**	**1, 194**	**< 0.0001**
Overwintering state (OS)	**8.64**	**1, 194**	**0.0037**	**25.51**	**1, 194**	**< 0.0001**
OS*Status	1.18	1, 194	0.2795	**4.28**	**1, 194**	**0.0399**
Hours	0.73	2, 194	0.4843	0.40	2, 194	0.6729
Hours*Status	0.59	2, 194	0.5526	0.92	2, 194	0.4000
Hours*OS	0.09	2, 194	0.9142	0.01	2, 194	0.9882
Hours*OS*Status	0.17	2, 194	0.8446	0.05	2, 194	0.9553

Figure 4. Mean (± SE) of *Halyomorpha halys* weight (a) and nutrient index (b) exposed to low temperatures in relation to their status (dead or alive) and overwintering state. Different letters indicate significant differences at the Tukey-Kramer test (α = 0.05).

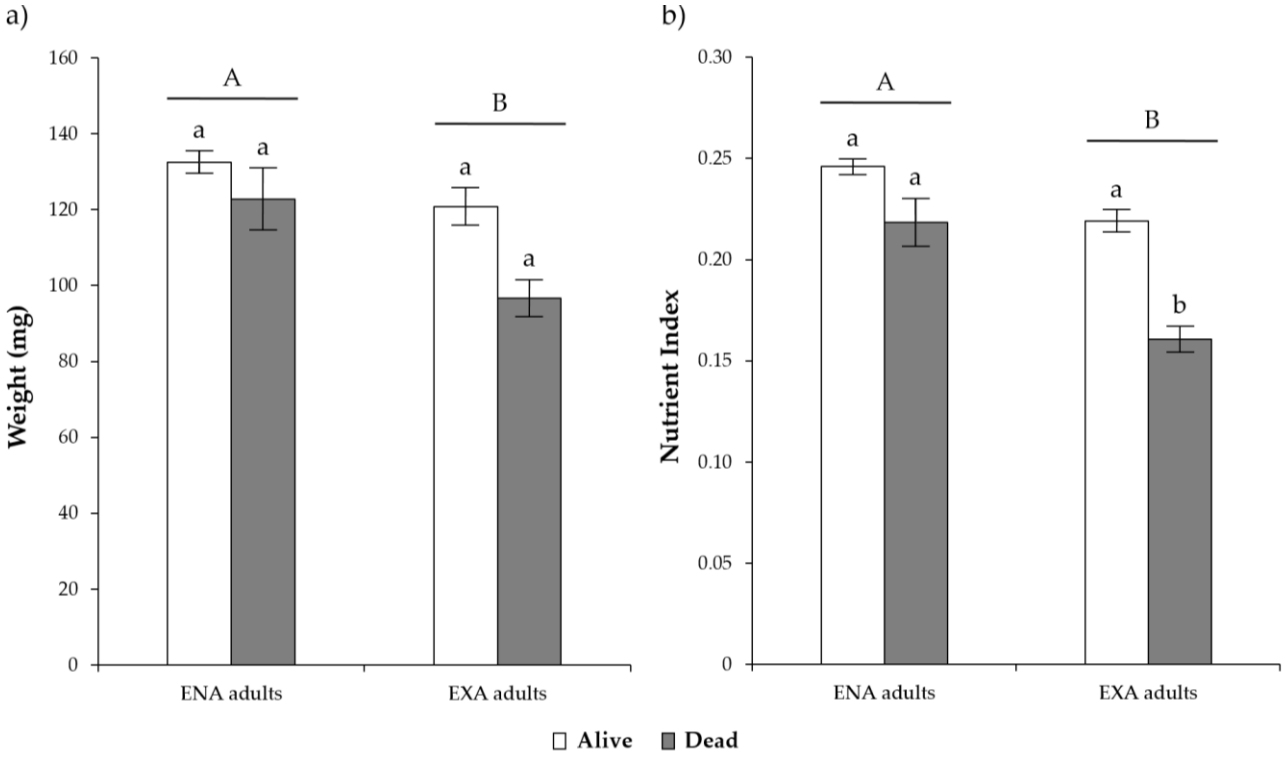


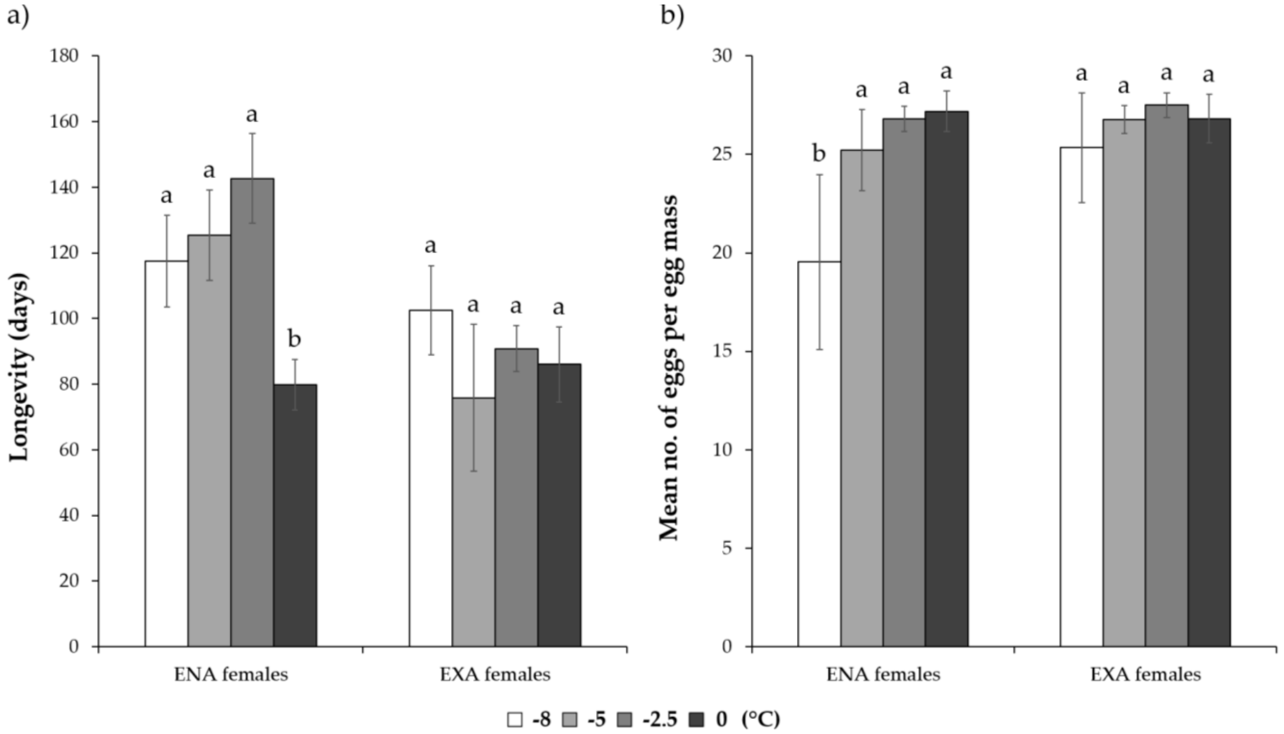
Table 3. Statistics of GLMM models (α = 0.05) on *Halyomorpha halys* female pre-oviposition period and longevity. Data were analyzed separately by overwintering state.

Overwintering state	**Effect**	**Pre-oviposition period**			**Female lifespan**		
		F value	**df**	**P-value**	**F value**	**df**	**P-value**
ENA	Temperature (Temp)	0.47	3, 43	0.7030	**6.20**	**3, 70**	**0.0008**
ENA	Exposure time (Hours)	0.62	2, 43	0.5419	0.78	2, 70	0.4626
ENA	Temp*Hours	1.26	6, 43	0.2939	0.51	6, 70	0.8012
EXA	Temp	1.28	4, 27	0.3010	0.79	4, 30	0.5431
EXA	Hours	0.43	2, 27	0.6573	0.63	2, 30	0.5372
EXA	Temp*Hours	1.89	8, 27	0.1031	1.33	8, 30	0.2684

Table 4. Statistics of GLMM models (α = 0.05) on *Halyomorpha halys* fecundity (i.e., number of egg masses laid, the mean number of eggs per egg mass) and egg hatching rate. Data were analyzed separately by overwintering state.

Overwintering state	**Effect**	**no. of egg masses**			**Mean no. of eggs per egg mass**			**% of hatching**		
		F value	**df**	**P value**	**F value**	**df**	**P value**	**F value**	**df**	**P value**
ENA	Temperature (Temp)	1.78	3, 47	0.1642	**3.22**	**3, 41**	**0.0325**	0.95	3, 47	0.4241
ENA	Exposure time (Hours)	1.84	2, 47	0.1702	1.79	2, 41	0.1802	1.70	2, 47	0.1941
ENA	Temp*Hours	0.94	6, 47	0.4783	1.13	6, 41	0.3628	1.58	6, 47	0.1737
EXA	Temp	0.41	4, 30	0.7975	0.87	4, 30	0.4959	0.54	4, 31	0.7068
EXA	Hours	0.34	2, 30	0.7166	0.47	2, 30	0.6314	0.64	2, 31	0.5322
EXA	Temp*Hours	0.71	8, 30	0.6828	0.44	8, 30	0.8847	1.93	8, 31	0.0900

Figure 5. Mean (± SE) of *Halyomorpha halys* female longevity (a) and mean (± SE) number of eggs laid per egg mass (b) at different low-temperature exposures, in relation to their overwintering state. Different letters indicate significant differences at the Tukey-Kramer test (α = 0.05).



4. Discussion

The results obtained on the effect of controlled low-temperature exposures on *H. halys* adults gave valuable information on the mortality levels of this pest for different temperature-time combinations. In our study, high mortality levels (i.e., 99%) were obtained with temperature lower than -13.45 °C, but this temperature has to last at least for 6 hours. Previous results on *H. halys* low-temperature exposures showed supercooling points levels from about -17.06 to -13.90 °C depending on the examined population (Cira et al. 2016), and our results showed similar killing temperature levels. The supercooling point is extensively used in studies on insect low-temperature mortality, with lower supercooling point meaning higher supercooling capacity for an insect, and vice versa (Lee 1991; 2010). It is known that supercooling is affected by many factors like body size, life stage or age of the insect, where death commonly occurs at temperatures above this threshold (Andreadis and Athanassiou 2017), and for *H. halys* adults it occurs before reaching the freezing point (Cira et al. 2016).

The results obtained here show that there are differences in cold tolerance between insects entering and exiting diapause (i.e., ENA vs. EXA). Considering the LT50s, we found most significant differences as regards to diapause state; for instance with 2 hours of exposure LT50 of ENA was -10.42 °C, while was -5.73 °C for EXA, with 6 hours -7.60 °C was calculated for ENA, while -3.40 °C for EXA. This is not surprising since during diapause insect reduce water loss by converting carbohydrates into fats (Danks 2000; Skillman 2017), also enhancing desiccation resistance and energy conservation (Ciancio 2018). Furthermore, the ability of an organism to survive at low temperature, called cold-hardness (Leather et al. 1993), can change following rapid cold hardening in insects (Lee et al. 1987; Ju et al. 2011), and it is a necessary trait for insects survival when exposed to temperatures that go to values below 0 °C in some periods of the year; this may involve different responses of the insect to low-temperature exposures (Andreadis and Athanassiou 2017). However the data obtained here highlight that at extreme temperature close to supercooling points the diapause mechanism used to cope with this conditions are not effective, and have clear implications in forecasting the mortality of *H. halys* after exposure to low winter temperature or spring frost. By looking at mortality curves obtained interpolating data on ENA individuals, we can suggest that mortality of overwintering *H. halys* occurs with temperature lower than -0.5 °C for 2 hours. Additionally, previous research showed that for *H. halys* adults exposed to an episodic and gradual cold shock and allowing them to conclude the overwintering, post-diapause survival was less than 25% if exposed to -6.0 °C, and 40–50% if exposed from -2.0 to +2.0 °C (Lowenstein and Walton 2018).

Data on EXA individuals suggest that for these insect mortality can occurs round 3.0 °C for 2 hours. The findings obtained here can be placed in a climate change context. On one hand, climate warming will be associated with warmer winter that are likely to decrease the possibility of *H. halys* mortality during this period, on the other hand warmer spring are expected to anticipate plants and insects phenology with a potentially increased risk of spring frost damage (Badeck et al. 2004; Wilby and Perry 2006; Kiritani 2007; Saulich and Musolin 2007; Musolin et al. 2010; Takeda et al. 2010; Forrest 2016). While winter temperatures regulate population growth rates of insects (Crozier and Dwyer 2006), frost risk in spring impacts on plants (Rigby and Porporato 2008) and insects (Crozier and Dwyer 2006) influencing their seasonal dynamic and eventually infestation levels in the following season. Frost occurrence has both direct (i.e., mortality by low-temperature exposures) and indirect effects (e.g., loss of food sources) on animals and plants distribution and their growth and reproduction (Inouye 2000). In the case of *H. halys*, adults emergence from overwintering sites starts with an ambient temperature higher than 10.0 °C, and flight activity starts with 15.0 °C or more (Lee et al. 2013; Lee and Leskey 2015; Bergh et al. 2017). Especially when warm conditions arise early in spring, *H. halys* adults can eventually leave overwintering sites, but according to our findings in case of spring frost with temperature lower than 0 °C a certain level of mortality is expected with an impact of overwintered population abundance. This impact showed a general higher mortality rate of *H. halys* adults when exposed to lower temperatures and for longer periods.

Our results highlighted that nutrient index could contribute in explaining the variation in resistance to low temperature, while it did not for high temperature (Chapter 4) and, particularly for exiting diapause adults, higher susceptibility to low temperatures was here related to lower value of this index. Changes in nutritional levels were reported, and a nutrient decline was showed during overwintering, which is a costly process (Leather et al. 1993), and to gradually increase as the season progress through the field season (Skillman et al. 2018a,b). An impact on the adult's vitality may also be measured, and possibly for the ones with lower nutritional levels that are often found in the post-diapause population (Skillman et al. 2018a,b). We found that nutritional index of the insects influenced the survival of EXA individuals and thus we can suggest that *H. halys* adults exiting diapause being able to reach a food source and feed on it have a higher probability of survival compared to the ones that do not feed.

Low-temperature exposure can determine sub-lethal effects on surviving *H. halys*, in particular in ENA adults, which showed an increase in longevity, but a reduction of their fecundity. When the winter temperature was higher than the average, *Nezara viridula* (L.) (Hemiptera: Pentatomidae) increased the number of eggs laid early in summer (Musolin et al. 2010; Takeda et al. 2010). Nevertheless, in previous experiments with diapausing *H. halys* adults exposed to low temperatures (up to -4.0 °C), which did not show short- or long-term effects on post-diapause survival, the adult fecundity appeared to increase during post-diapause (Lowenstein and Walton 2018). In general, studies on stink bug-related fecundity and lifespan after warming periods in winter often present complicated patterns and may result in some differences between studies. Moreover, further studies are required to clarify sub-lethal effect of low temperatures on this invasive species.

In conclusion, our results shed light on the impact of low temperature on *H. halys* mortality. These data can be used to forecast the survival rate after winter of this pest with implication in population abundance modeling. These data can also be used to predict the geographic distribution pattern of *H. hays* in a climate change scenario.

Acknowledgments

A sub-set of the data was included in the Master degree thesis of Luka Vanishvili at University of Padova.

References

Addo-Bediako, A., Chown, S.L., Gaston, K.J., 2000. Thermal tolerance, climatic variability and latitude. Proceedings of the Royal Society B: Biological Sciences 267: 739-745.

Andreadis S.S., Athanassiou C.G., 2017. A review of insect cold hardiness and its potential in stored product insect control. Crop Protection 91: 93-99.

Asahina E., 1970. Frost resistance in insects. Advances in Insect Physiology 6: l-49.

Badeck F.W., Bondeau A., Böttcher K., Doktor D., Lucht W., Schaber J., Sitch S., 2004. Responses of spring phenology to climate change. New Phytologist 162: 295-309.

Bergh J.C., Morrison III W.R., Joseph S.V., Leskey T.C., 2017. Characterizing spring emergence of adult *Halyomorpha halys* using experimental overwintering shelters and commercial pheromone traps. Entomologia Experimentalis et Applicata 162: 336-345.

Cambridge J., Payenski A., Hamilton G.C., 2015. The distribution of overwintering brown marmorated stink bugs (Hemiptera: Penatatomidae) in college dormitories. Florida Entomologist 98: 1257-1259.

Chown S.L., Jumbam K.R., Sørensen J.G., Terblanche J.S., 2009. Phenotypic variance, plasticity and heritability estimates of critical thermal limits depend on methodological context. Functional Ecology 23: 133-140.

Ciancio J., 2018. Overwintering biology of the brown marmorated stink bug, *Halyomorpha halys* (Hemiptera: Pentatomidae). MSc Thesis, The University of Western Ontario, Electronic Thesis and Dissertation Repository, 5813, https://ir.lib.uwo.ca/etd/5813.

Cira T.M., Venette R.C., Aigner J., Kuhar T., Mullins D.E., Gabbert S.E., Hutchison W.D., 2016. Cold tolerance of *Halyomorpha halys* (Hemiptera: Pentatomidae) across geographic and temporal scales. Environmental Entomology 45: 484-491.

Crozier L., Dwyer G., 2006. Combining population-dynamic and ecophysiological models to predict climate-induced insect range shifts. The American Naturalist 167: 853-866.

Danks H.V., 2000. Dehydration in dormant insects. Journal of Insect Physiology 46: 837-852.

Duthie C., 2012. Risk analysis of *Halyomorpha halys* (brown marmorated stinkbug) on all pathways. Ministry for primary industries. https://mpi.govt.nz/dmsdocument/2909-halyomorpha-halys-brown-marmorated-stink-bug-riskanalysis-november-2012. Accessed on 29 September 2019.

Forrest J.R., 2016. Complex responses of insect phenology to climate change. Current Opinion in Insect Science 17: 49-54.

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

Halbritter, D.A., Teets, N.M., Williams, C.M., & Daniels, J.C., 2018. Differences in winter cold hardiness reflect the geographic range disjunction of *Neophasia menapia* and *Neophasia terlooii* (Lepidoptera: Pieridae). Journal of Insect Physiology 107: 204-211.

Inkley D.B., 2012. Characteristics of home invasion by the brown marmorated stink bug (Hemiptera: Pentatomidae). Journal of Entomological Science 47: 125-130.

Inouye D.W., 2000. The ecological and evolutionary significance of frost in the context of climate change. Ecology Letters 3: 457-463.

Ju R.T., Xiao Y.Y., Li B., 2011. Rapid cold hardening increases cold and chilling tolerances more than acclimation in the adults of the sycamore lace bug, *Corythucha ciliata* (Say) (Hemiptera: Tingidae). Journal of Insect Physiology 57: 1577-1582.

Kiritani K., 2007. The impact of global warming and land-use change on the pest status of rice and fruit bugs (Heteroptera) in Japan. Global Change Biology 13: 1586-1595.

Koštál V., 2006. Eco-physiological phases of insect diapause. Journal of Insect Physiology 52: 113-127.

Kriticos D.J., Kean J.M., Phillips C.B., Senay S.D., Acosta H., Haye T., 2017. The potential global distribution of the brown marmorated stink bug, *Halyomorpha halys*, a critical threat to plant biosecurity. Journal of Pest Science 90: 1033-1043.

Leather S.R., Walters K.F.A., Bale J.S., 1993. The Ecology of Insect Overwintering. Cambridge University Press, UK, 255 pp.

Lee R.E., 1991. Principles of insect low temperature tolerance. In: Insects at low temperature, Lee R.E., Denlinger, D.L. (eds.). Chapman and Hall, New York, NY, pp. 17-46.

Lee D.-H., Leskey T.C., 2015. Flight behavior of foraging and overwintering brown marmorated stink bug, *Halyomorpha halys* (Hemiptera: Pentatomidae). Bulletin of the Entomological Research 105: 566-573.

Lee D.-H., Short B.D., Joseph S.V., Bergh J.C., Leskey T.C., 2013. Review of the biology, ecology, and management of *Halyomorpha halys* (Hemiptera: Pentatomidae) in China, Japan, and the Republic of Korea. Environmental Entomology 42: 627-641.

Lee R.E., Chen C.P.J., Denlinger D.L., 1987. A rapid cold-hardening process in insects. Science 238: 1415-1417.

Lee, R.E. 2010. A primer on insect cold-tolerance. In: Low temperature biology of insects, Denlinger D.L., Lee R.E. (eds.). Cambridge Univeristy Press, New York, NY, pp. 3-34.

Leskey T.C., Nielsen A.L., 2018. Impact of the invasive brown marmorated stink bug in North America and Europe: history, biology, ecology, and management. Annual Review of Entomology 63: 599-618.

Lowenstein D.M., Walton V.M., 2018. *Halyomorpha halys* (Hemiptera: Pentatomidae) winter survival, feeding activity, and reproduction rates based on episodic cold shock and winter temperature regimes. Journal of Economic Entomology 111: 1210-1218.

Musolin D.L., 2007. Insects in a warmer world: ecological, physiological and life-history responses of true bugs (Heteroptera) to climate change. Global Change Biology 13: 1565-1585.

Musolin D.L., Tougou D., Fujisaki K., 2010. Too hot to handle? Phenological and life-history responses to simulated climate change of the southern green stink bug *Nezara viridula* (Heteroptera: Pentatomidae). Global Change Biology 16: 73-87.

Nielsen A.L., Chen S., Fleischer S.J., 2016. Coupling developmental physiology, photoperiod, and temperature to model phenology and dynamics of an invasive heteropteran, *Halyomorpha halys*. Frontiers in Physiology 7: 165, doi:10.3389/fphys.2016.00165.

Niva C.C., Takeda M., 2003. Effects of photoperiod, temperature and melatonin on nymphal development, polyphenism and reproduction in *Halyomorpha halys* (Heteroptera: Pentatomidae). Zoological Science 20: 963-970.

Nixon L.J., Tabb A., Morrison W.R., Rice K.B., Brockerhoff E.G., Leskey T.C., van Koten C., Goldson S., Rostás M., 2019. Volatile release, mobility, and mortality of diapausing *Halyomorpha halys* during simulated shipping movements and temperature changes. Journal of Pest Science 92: 663-641.

Overgaard J., Kristensen T.N., Sørensen J.G., 2012. Validity of thermal ramping assays used to assess thermal tolerance in arthropods. PLoS ONE 7: e32758, doi:10.1371/journal.pone.0032758.

Rigby J.R., Porporato A., 2008. Spring frost risk in a changing climate. Geophysical Research Letters, 35, L12703, doi:10.1029/2008GL033955.

Robertson J.L., Savin N.E., Preisler H.K., Russell R.M., 2007. Bioassays with Arthropods, 2nd ed. CRC Press, Boca Raton, Florida, 2007, 224 pp.

Saulich A.H., Musolin D.L., 2007. Four Seasons: diversity of seasonal adaptations and ecological mechanisms controlling seasonal development in true bugs (Heteroptera) in the temperate climate. Proceedings of the Biological Institute of St. Petersburg State University), 53: 25-106 [in Russian with English summary].

Saulich A.K., Musolin D.L., 2012. Diapause in the seasonal cycle of stink bugs from the temperate zone. Entomological Review 92: 1-26.

Saunders D.S., 2002. Insect clocks – Third edition. Elsevier Science, Amsterdam, 576 pp.

Sinclair B.J., Coello Alvarado L.E., Ferguson L.V., 2015. An invitation to measure insect cold tolerance: methods, approaches, and workflow. Journal of Thermal Biology 53: 180-197.

Skillman V.P., 2017. Nutrient profile and nursery feeding damage of *Halyomorpha halys*. MSc thesis, Oregon State University.

Skillman V.P., Wiman N.G., Lee J.C., 2018a. Monitoring nutrient status of Brown Marmorated Stink Bug adults and nymphs on Summer Holly. Insects 9: 120, doi:10.3390/insects9030120.

Skillman V.P., Wiman N.G., Lee J.C., 2018b. Nutrient declines in overwintering *Halyomorpha halys* populations. Entomologia Experimentalis et Applicata 166: 778-789.

Storey K.B., Storey J.M., 1987. Freeze tolerance: constraining forces, adaptive mechanisms. Canadian Journal of Zoology 66: 1122-1127.

Takeda K., Musolin D.L., Fujisaki K., 2010. Dissecting insect responses to climate warming: overwintering and post-diapause performance in the southern green stink bug, *Nezara viridula*, under simulated climate-change conditions. Physiological Entomology 35: 343-353.

Terblanche J.S., Hoffmann A.A., Mitchell K.A., Rako L., le Roux P.C., Chown S.L., 2011. Ecologically relevant measures of tolerance to potentially lethal temperatures. Journal of Experimental Biology 214: 3713-3725.

Tougeron K., 2019. Diapause research in insects: historical review and recent work perspectives. Entomologia Experimentalis et Applicata 167: 27-36.

Vandervoet T.F., Bellamy D.E., Anderson D., MacLellan R., 2019. Trapping for early detection of the brown marmorated stink bug, *Halyomorpha halys*, in New Zealand. New Zealand Plant Protection 72: 36-43.

Wilby R.L., Perry G.L.W., 2006. Climate change, biodiversity and the urban environment: a critical review based on London, UK. Progress in Physical Geography 30: 73-98.

Williams C.M., Henry H.A.L., Sinclair B.J., 2015. Cold truths: how winter drives responses of terrestrial organisms to climate change. Biological Review 90: 214-235.

Chapter 6

Current status of the egg parasitoids complex of *Halyomorpha halys* in northern Italy and early detection of the parasitoid *Trissolcus mitsukurii*

Manuscript submitted as: Update on the egg parasitoids complex of *Halyomorpha halys* in northern Italy with the earliest detection of the parasitoid *Trissolcus mitsukurii*

Davide Scaccini[1], Martina Falagiarda[2], Francesco Tortorici[3], Paola Tirello[1], Isabel Martinez-Sañudo[1], Yazmid Reyes Domínguez[2], Andreas Gallmetzer[2], Luciana Tavella[3], Pietro Zandigiacomo[4], Carlo Duso[1], Alberto Pozzebon[1]

[1]Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova, Italy;

[2]Laimburg Research Centre, Laimburg 6, Pfatten (Vadena), IT-39040 Auer (Ora), South Tyrol, Italy;

[3]Dipartimento di Scienze Agrarie, Forestali e Alimentari, University of Torino, Italy;

[4]Department of Agricultural, Food, Environmental and Animal Sciences (DI4A), University of Udine, Italy

Davide Scaccini contributed in study conceptualization, data collection and analysis as well as writing of the manuscript.

Abstract

Sustainable strategies such as classical or augmentative biological control are currently being evaluated for the long-term management of the invasive pest *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae). In this three-year study, carried out in northeastern Italy, we recorded parasitoids emerging from *H. halys* eggs from three Hymenoptera families: Scelionidae (*Trissolcus basalis* (Wollaston), *Trissolcus kozlovi* Rjachovskij, and *Trissolcus mitsukurii* (Ashmead)), Eupelmidae (*Anastatus bifasciatus* (Geoffroy)), and Pteromalidae (the hyperparasitoid *Acroclisoides solus* Grissell & Smith). The 'parasitoid impact' (i.e. number of parasitized eggs over the total number of field-collected eggs) was the highest for *T. mitsukurii*, followed by *A. bifasciatus*. Here we report the earliest known occurrence of *T. mitsukurii* in Europe, which emerged from *H. halys* egg masses in 2016. The phylogenetic tree for *T. mitsukurii* distinguished two clades, one covering samples from Italy, Japan and China, while the other from South Korea.

1. Introduction

The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), is a polyphagous pest native to eastern Asia, detected in North America in the 1990s (Hoebeke and Carter 2003), in Europe in the mid-2000s (Wermelinger et al. 2008), and in South America in 2017 (Faúndez and Rider 2017). Feeding on more than 170 host plants, *H. halys* causes serious damage on agricultural crops, ornamental plants, urban and forest trees (Nielsen and Hamilton 2009; Haye et al. 2015b; Leskey and Nielsen 2018; Moore et al. 2019). Moreover, *H. halys* can represent a nuisance problem in residential areas during fall and winter, when large numbers of adults invade the buildings searching for overwinter sites (Hoebeke and Carter 2003; Inkley 2012; Leskey et al. 2012). In Italy, *H. halys* was reported in Emilia Romagna region in 2012, in Piedmont region in 2013, in Veneto and in Friuli-Venezia Giulia regions in 2014, and two years later in Trentino-Alto Adige region (Maistrello et al. 2014, 2018; Bariselli et al. 2016; Unterthurner et al. 2017; Bosco et al. 2018). To date, the species is distributed in almost all the regions of the Peninsula (Cianferoni et al. 2018; Maistrello et al. 2018).

In the invaded areas, management strategies to control *H. halys* rely on the use of insecticides and exclusion nets (Lee et al. 2013; Candian et al. 2018). However, sustainable strategies such as classical or augmentative biological control may be necessary for a long-term management of the pest. In its native area, *H. halys* populations are exploited by several egg parasitoids belonging to the genera *Anastatus* (Hymenoptera: Eupelmidae), *Ooencyrtus* (Hymenoptera: Encyrtidae), *Telenomus* (Hymenoptera: Scelionidae) and *Trissolcus* (Hymenoptera: Scelionidae) (Lee et al. 2013; Abram et al. 2017). Among them, *Trissolcus japonicus* (Ashmead) and *Trissolcus mitsukurii* (Ashmead) were identified as the predominant egg parasitoids of *H. halys* in northern China and Japan, respectively (Arakawa and Namura 2002; Arakawa et al. 2004; Yang et al. 2009). Adventive populations of *T. japonicus* were found in North America in 2014 (Talamas et al. 2015a) and in Europe in 2018 (Stahl et al. 2018a). Furthermore, recent records of adventive populations of *T. mitsukurii* were reported in northern Italy, Lombardy and Friuli-Venezia Giulia regions (Sabbatini Peverieri et al. 2018; Moraglio et al. 2019). How these exotic parasitoids were introduced to Europe remains unclear, probably following the same invasion pathways of their host (Talamas et al. 2015a). Autochthonous species, such as *Anastatus bifasciatus* (Geoffroy), were also found parasitizing *H. halys* eggs in some regions of Italy, and it is one of the most common parasitoids of the *H. halys* in these habitats (Costi et al. 2019; Moraglio et al. 2019). Furthermore, the hyperparasitoid *Acroclisoides solus* Grissell & Smith (Hymenoptera: Pteromalidae) occasionally emerged from *H. halys* eggs in northern Italy (Moraglio et al. 2019; Sabbatini Peverieri et al. submitted).

The aim of this study was to assess the parasitoid complex of *H. halys* eggs in northeastern Italy and compare the impact of the different parasitoid species across the three years of the study. We also used molecular data of detected parasitoid species to assess phylogenetic relationship of target gene sequences obtained for *T. mitsukurii*, for which we have updated its distribution in Italy.

2. Materials and methods

2.1. Sampling sites and laboratory study

Eight sites were surveyed every 7–10 days, from June 2016 to October 2018, in Veneto region (Table 1). Additional observations were performed in the following periods in other sites located in northeastern Italy: from the beginning of September to October 2018 in Trentino-Alto Adige region, and in August 2018 in two sites in Friuli-Venezia Giulia region (Table 1). The selected sites were chosen for the relatively high *H. halys* population levels. Field surveys were conducted by searching for *H. halys* eggs on the vegetation; egg masses were hand-collected on the leaves or fruits and transferred to the laboratory. Field-collected egg masses were reared in a climatic chamber at 26 ± 1

°C, 65 ± 5% RH, and 16:8 L:D until parasitoid adult emergence. Emerged parasitoids were stored in 70% ethanol for morphological and molecular identification.

In the case of the Ora site (site 3), eggs from which no *H. halys* nymphs or parasitoids emerged were further assessed for other causes of mortality under a Leica stereo microscope (series MZ6) according to Morrison et al. (2016), and then ascribed to natural mortality (e.g., deformed and discolored eggs) and predation. For eggs where *H. halys* hatching or parasitoid emergence took place in the field before sampling, the category was assessed according to Jones et al. (2017) and eggs were classified as parasitized or hatched eggs. 'Exploitation efficiency' (number of parasitized eggs divided by the total number of eggs in the egg mass) was calculated for each site (Bin and Vinson 1991). On data collected from sites 3, 5, 8 and 9, the 'discovery efficiency' (percentage of egg masses with at least one parasitized egg for each site) and the 'parasitoid impact' (number of parasitized eggs divided by the total number of field-collected eggs) were calculated (Bin and Vinson 1991). Indices obtained from data of the latter sites were used to compare performances of the parasitoid species emerged from *H. halys* eggs. These indexes were analyzed using a Generalized Linear Model assuming a binomially distributed model with a logit link function with the GENMOD procedure of SAS (ver. 9.4). The effect of parasitoid species was tested using a Wald χ^2 test ($\alpha = 0.05$) followed by a Tukey's test ($\alpha = 0.05$) on the least square means. Only species observed in more than one site were included in the analyses.

Table 1. Collection sites of *Halyomorpha halys* egg masses.

Site number	Region	Province	Coordinates, altitude (m a.s.l.)	Survey period	Host plants	Habitat description
1	Friuli-Venezia Giulia	Pordenone	45.975556 N 12.451667 E, 98 m	August (2018)	*Ficus carica* (on leaves)	Organic orchard (*Ficus carica*)
2	Friuli-Venezia Giulia	Udine	46.032500 N 13.226944 E, 92 m	August (2018)	*Glycine max* (on leaves)	Experimental farm with soybean, other raw crops, vineyards and orchards
3	Trentino-Alto Adige	Bolzano	46.362028 N 11.298500 E, 224 m	September and October (2018)	*Acer* spp. (on leaves and fruits), *Ailanthus altissima* (on leaves) and linden (on leaves)	Urban area (parking zone) with maple trees (*Acer platanoides*, *Acer negundo* and *Acer pseudoplatanus*), ailanthus (*Ailanthus altissima*) and linden (*Tilia platyphyllos*)
4	Veneto	Padua	45.621319 N 11.719279 E, 43 m	June to November (2017-2018)	*Actinidia deliciosa* (on leaves and fruits)	Small organic orchard (*Actinidia deliciosa*), near urban area
5	Veneto	Padua	45.632120 N 11.799309 E, 40 m	June to November (2017-2018)	*Actinidia deliciosa* (on leaves and fruits)	Small organic orchard (*Actinidia deliciosa*), near urban area
6	Veneto	Padua	45.646779 N 11.740218 E, 50 m	August to October (2016), June to November (2017)	*Actinidia deliciosa* (on leaves and fruits)	Small organic orchard (*Actinidia deliciosa*), near urban area
7	Veneto	Padua	45.580714 N 11.787064 E, 27 m	August to October (2016), June to November (2017-2018)	*Actinidia deliciosa* (on leaves and fruits)	Organic orchard (*Actinidia deliciosa*), near urban area
8	Veneto	Treviso	45.715497 N 11.939604 E, 61 m	August to October (2016), June to November (2017-2018)	*Actinidia deliciosa*, *Prunus* spp., *Vitis vinifera* (on leaves)	Conventional farm with orchard (*Actinidia deliciosa*, *Malus domestica*, *Prunus avium*, *Prunus persica*, *Prunus* spp.), small vineyard and vegetables, with hedges
9	Veneto	Treviso	45.760649 N 12.007810 E, 94 m	June to November (2017-2018)	*Vitis vinifera* (on leaves)	Conventional farm with vineyard and orchard (*Actinidia deliciosa*, *Malus domestica, Prunus persica*)
10	Veneto	Treviso	45.795083 N 12.440934 E, 17 m	June to October (2017)	*Vitis vinifera* (on leaves)	Conventional farm with vineyard
11	Veneto	Vicenza	45.75157 N 11.68244 E, 100 m	August to October (2016-2017)	*Olea europaea* and *Vitis vinifera* (on leaves)	Conventional farm with *Olea europaea* and *Vitis vinifera*

2.2. Insect identification and molecular analysis

Ethanol-stored specimens were dried and glued on card points for morphological analyses. A Wild M3 stereomicroscope with magnification up to 200X and 2700k spotlight were used for morphological diagnosis. The Palaearctic genera of Scelionidae and *Trissolcus* species were respectively determined using keys from Kozlov and Kononova (1983) and Talamas et al. (2017). Moreover, *Trissolcus* specimens were compared with pictures of holotypes and paratypes in Hymenoptera Online (HOL) provided by Talamas et al. (2017). *Anastatus* species were identified using the key by Askew and Nieves-Aldrey (2004). *Acroclisoides* specimens were kindly identified by Dr. Mircea-Dan Mitroiu (Sabbatini Peverieri et al. submitted). The parasitoids used for morphological identification were deposited in the Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), Legnaro, Padua, Italy, in Dipartimento di Scienze Agrarie, Forestali e Alimentari (DISAFA), Grugliasco, Turin, Italy, and in Laimburg Research Center, Auer, Bolzano, Italy.

To confirm the results of the morphological identification for parasitoid species and to study phylogenetic relationship for the most common non-native parasitoid species, molecular analyses were conducted. Total DNAs from three *Trissolcus* adults and from seven egg masses collected in Trentino-Alto Adige were extracted by homogenizing each sample in 400 µL of CTAB buffer (CTAB 2.5%, Tris pH 8 100 mM, NaCl 1.4 M, EDTA 50 mM pH 8, PVP-40 1%, Proteinase K 10 mg/ml) in a microcentrifuge tube containing a 5 mm tungsten carbide bead (Qiagen). Samples were disrupted using a Retsch Mixer Mill MM 400, at 30 Hz for 3 min. After disruption, DNA was extracted using the DNeasy Plant Mini kit (Qiagen) following the instructions of the provider. Moreover, DNA of 14 parasitoid samples from Veneto and of three samples from Friuli-Venezia Giulia was extracted according to a previously described salting-out protocol (Patwary et al. 1994). A partial region of the cytochrome c oxidase subunit I (COI) gene was amplified with primers HCO2198 (5′GGTCAACAAATCATAAAGATATTGG3′) and LCO1490 (5′TAAACTTCAGGGTGACCAAAAAATCA3′) (Folmer et al. 1994). PCR amplification of samples analyzed in Trentino-Alto Adige region was performed in a final volume of 20 µL containing onefold of Phusion High-Fidelity PCR Master Mix with HF Buffer (New England BioLabs), 200 nM final concentration of each primer and 2 µL of DNA template extraction. Reactions of samples were performed on a Verity 96-well Thermal Cycler (Applied Biosystems) as follow: 30s at 98 °C followed by 35 cycles of 50s denaturation at 98 °C and 90s for annealing at 52 °C, 15s elongation at 72 °C. Amplifications of samples from Veneto and Friuli-Venezia Giulia regions were performed in 20 µL reactions (1x PCR Go Taq Flexi buffer – Promega, 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.5 µM for each primer, 0.5 U of Taq polymerase – Promega, 2 µL DNA template). Thermal cycling conditions were

5 min at 96 °C followed by 4 cycles of 96 °C for 1 min, 47 °C for 1 min, and 72 °C for 1 min, and other 35 cycles of 96 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, with a final extension of 72 °C for 5 min.

The produced amplicons were purified through a QIAquick PCR and Gel Cleanup Kit following the instructions of the provider (Qiagen). The purified amplicons were sequenced by LGC Genomics GmbH (Berlin, Germany). PCR products from Veneto and Friuli-Venezia Giulia samples were purified using exonuclease and antarctic phosphatase (GE Healthcare) and sequenced at the BMR Genomics Service (Padua, Italy).

Sequences were edited and aligned using MEGA X software (Kumar et al. 2018). To assess the identity of the sequences obtained, the integrated bioinformatics platform Barcode of Life Data System database (http://www.barcodinglife.org) was used, and a nucleotide Blast was performed using the Basic Local Alignment Search Tool (National Center for Biotechnology Information – NCBI). Similar sequences retrieved from both databases were added to our dataset. Additionally, sequences of the same target gene obtained in previous studies (Sabbatini Peverieri et al. 2018; Moraglio et al. 2019) were added to our dataset. To exclude the presence of stop codons in the coding sequences, all COI sequences were translated with Transeq (EMBOSS: http://www.ebi.ac.uk/Tools/emboss/transeq/index.html).

Phylogenetic relationships among sequences of non-native species were estimated with an approximate maximum-likelihood (ML) analysis, using MEGA X software. A GTR + I + G model was applied, with neighbor-joining starting tree and 100 bootstrap replications. A haplotype parsimony network of the final dataset with a probability cut-off at 90% was reconstructed following the TCS method in PopART (Leigh and Bryant 2015).

3. Results

In this study, 251 egg masses with more than 6,000 eggs were collected: 185 egg masses in Veneto, 63 in Trentino-Alto Adige and three in Friuli-Venezia Giulia (Table 2). Egg masses were mostly found on leaves and fruit of crop plants in Veneto, on maple leaves and fruits (93.7%), ailanthus and linden leaves in Trentino-Alto Adige, and on fig and soybean leaves in Friuli-Venezia Giulia (Table 1).

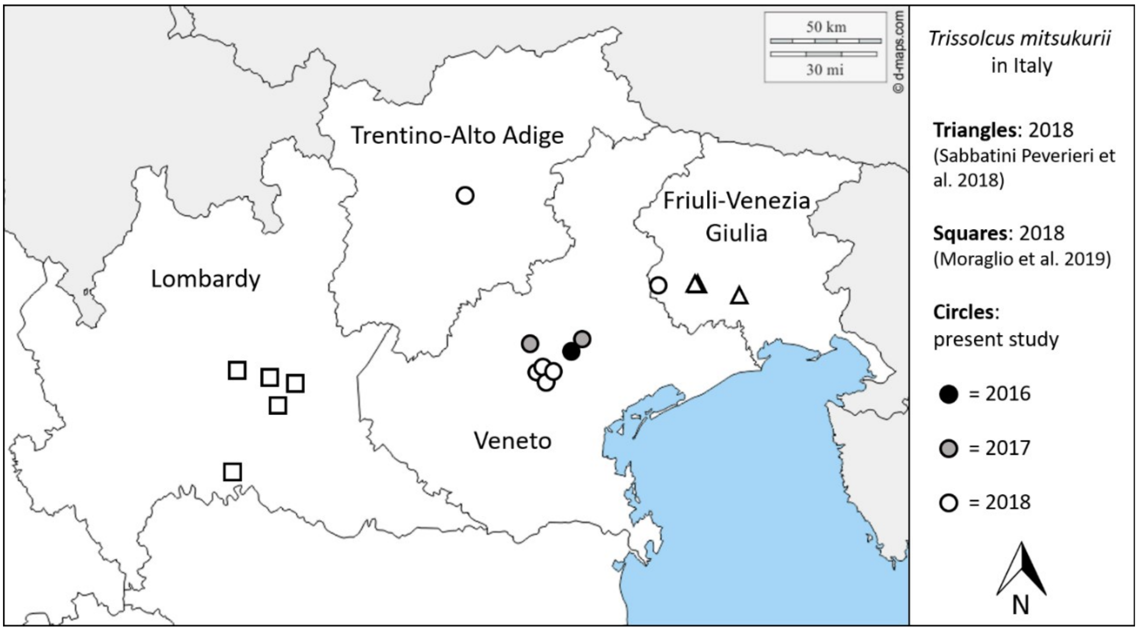
Five species of hymenopteran parasitoids emerged from 46 *H. halys* egg masses, and in five cases the egg mass was parasitized by more than one parasitoid species (Table 2). There were no sites with all five parasitoid species together, and in many cases only one species was found ('Relative abundance per site' in Table 2).

Based on morphological analyses, *Trissolcus* individuals were identified as *Trissolcus basalis* (Wollaston), *T. kozlovi* Rjachovskij and *T. mitsukurii*. The COI sequence of the *T. kozlovi* specimens in this study had 100% sequence identity to those of Moraglio et al. (2019), while *T. mitsukurii* ones have a perfect match with data in Sabbatini Peverieri et al. (2018). Among *Trissolcus* species, *T. mitsukurii* was the most abundant and emerged from egg masses collected from June to October in 2016 and 2018, and from May to August in 2017 (Table 2). Both *T. basalis* and *T. kozlovi* were less frequently found parasitizing *H. halys* eggs (Table 2). Six *T. basalis* emerged from an egg mass collected in site 8 on 17 October 2016 and in site 2 on 15–17 August 2018, while two *T. kozlovi* individuals emerged from an egg mass in site 10 on 22 June 2017 (Table 2). Other Hymenoptera emerged from eggs were identified as *An. bifasciatus* and the hyperparasitoid *Ac. solus*. The first was found in six sites across Veneto and Trentino-Alto Adige regions, and *A. solus* individuals emerged from the egg mass in three sites, on 29 August 2017 (nine individuals: site 8; six individuals: site 9; Table 2) and on 27 September 2018 (14 individuals: site 3; Table 2).

During the three years of observation, exploitation efficiency varied from 34.6% to 100% for *T. mitsukurii*, from 23.1% to 100% for *A. solus*, and from 7.4% to 46.4% for *A. bifasciatus*, while for the other species it was always below 18.0% (Table 2). Parasitoid performances were compared among *A. solus*, *A. bifasciatus*, *T. basalis* and *T. mitsukurii*. The exploitation efficiency among species did not differ in 2016, while in 2017 *T. mitsukurii* showed an exploitation efficiency higher (70.2%) than *A. solus* (34.6%) and *A. bifasciatus* (10.7%; Table 3). In 2018, the exploitation efficiency was higher for *A. solus* in comparison to other species (Table 3). Between the latter, *T. mitsukurii* showed a higher exploitation efficiency than *A. bifasciatus* (Table 3). No differences were recorded among discovery efficiencies of parasitoids in 2016 and 2017. In 2018, *A. bifasciatus* showed a higher discovery efficiency than *A. solus*. The same parameter was similar between *T. mitsukurii* and the two other species (Table 3). Parasitoid impact varied among species in all years (Table 3), with *T. mitsukurii* showing always the highest value (Table 3). In 2018, the impact of *T. mitsukurii* was higher than *A. bifasciatus*, and these two species had a higher impact than *A. solus* (Table 3). In site 3, 327 eggs (out of 1520) were found with signs of sucking or chewing by predators. Deformed or discolored eggs were also observed; 939 *H. halys* eggs (61.8%) hatched (Falagiarda, unpubl.).

Concerning the distribution of *T. mitsukurii* in its non-native range, the parasitoid was recorded in seven sites in Veneto, and in one site in Trentino-Alto Adige and in Friuli-Venezia Giulia. In Veneto region, the first three records of this parasitoid occurred in 2016 (site 8) and 2017 (sites 9 and 11; Figure 1). The presence of *T. mitsukurii* emerging from *H. halys* eggs was recorded in these three sites also in the following year(s). To date, the distribution of *T. mitsukurii* in its non-native area covers many cultivated and natural areas in northern Italy, across four regions (Figure 1).

Figure 1. Current distribution of *Trissolcus mitsukurii* in its non-native area in northern Italy, with records separated by year. Adapted from www.d-maps.com. Data from Sabbatini Peverieri et al. (2018) and Moraglio et al. (2019) are included.



3.1. Molecular analysis

The results of the molecular analysis were consistent with the morphological identification of *T. basalis* and *T. mitsukurii*. A total of 20 *Trissolcus* specimens, 14 from Veneto, three from Trentino-Alto Adige and three from Friuli-Venezia Giulia were successfully amplified and sequenced. A fragment of 534 bp of COI was obtained for all sequences. A BLAST search of sequences from 18 adult insects showed significant alignment with the deposited *T. mitsukurii* sequence AB971831.1, giving an E value of 0.0, 100% of Query coverage and 99.32% sequence identity. Similarly, BOLD Systems databases showed a >99% similarity with *T. mitsukurii*.

The phylogenetic tree, inferred with sequences of this study, and sequences retrieved from GenBank database, distinguished between two highly supported clades. One of them grouped the *T. mitsukurii* samples from Italy, Japan and China, while the other clade contained specimens from South Korea (Figs. 2, 3). The TCS Network showed the presence of five haplotypes (Figure 2). The haplotype H5 grouped all sequences of this study and five sequences previously obtained from Italy (MK097188, MK097189 and MK097190 in Sabbatini Peverieri et al. 2018, and two sequences from Lombardy – kindly provided by S.T. Moraglio). The other four haplotypes corresponded to sequences from Asian samples. Two of them, H1 and H2, were separated from H5 by four and five mutational steps, respectively, and included samples from Japan and Yunnan province of China (Mita et al. 2015; Sabbatini Peverieri et al. 2018). The other two haplotypes, H4 and H3, differed from H5 by several mutational steps (12 and 13 respectively) and grouped sequences from South Korea (Sabbatini Peverieri et al. 2018).

Figure 2. Haplotypes network reconstructed with PopART, with the COI sequences obtained in this study and those present in GenBank. Each haplotype is represented by a circle, with the area of the circle proportional to its frequency. Each line represents a single mutation while crossing lines symbolize missing intermediate or unsampled haplotypes.

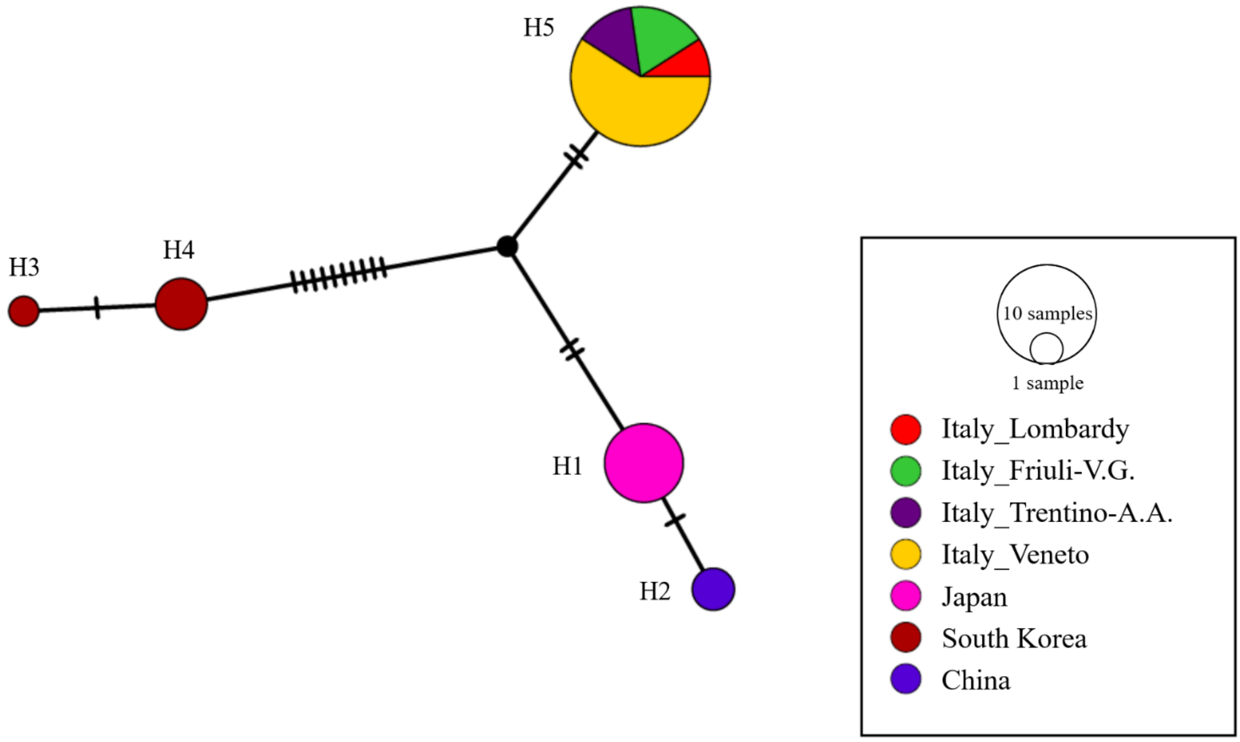


Figure 3. Maximum likelihood (ML) tree based on COI sequences of *T. mitsukurii*. Numbers on the nodes refer to ML bootstrap values.

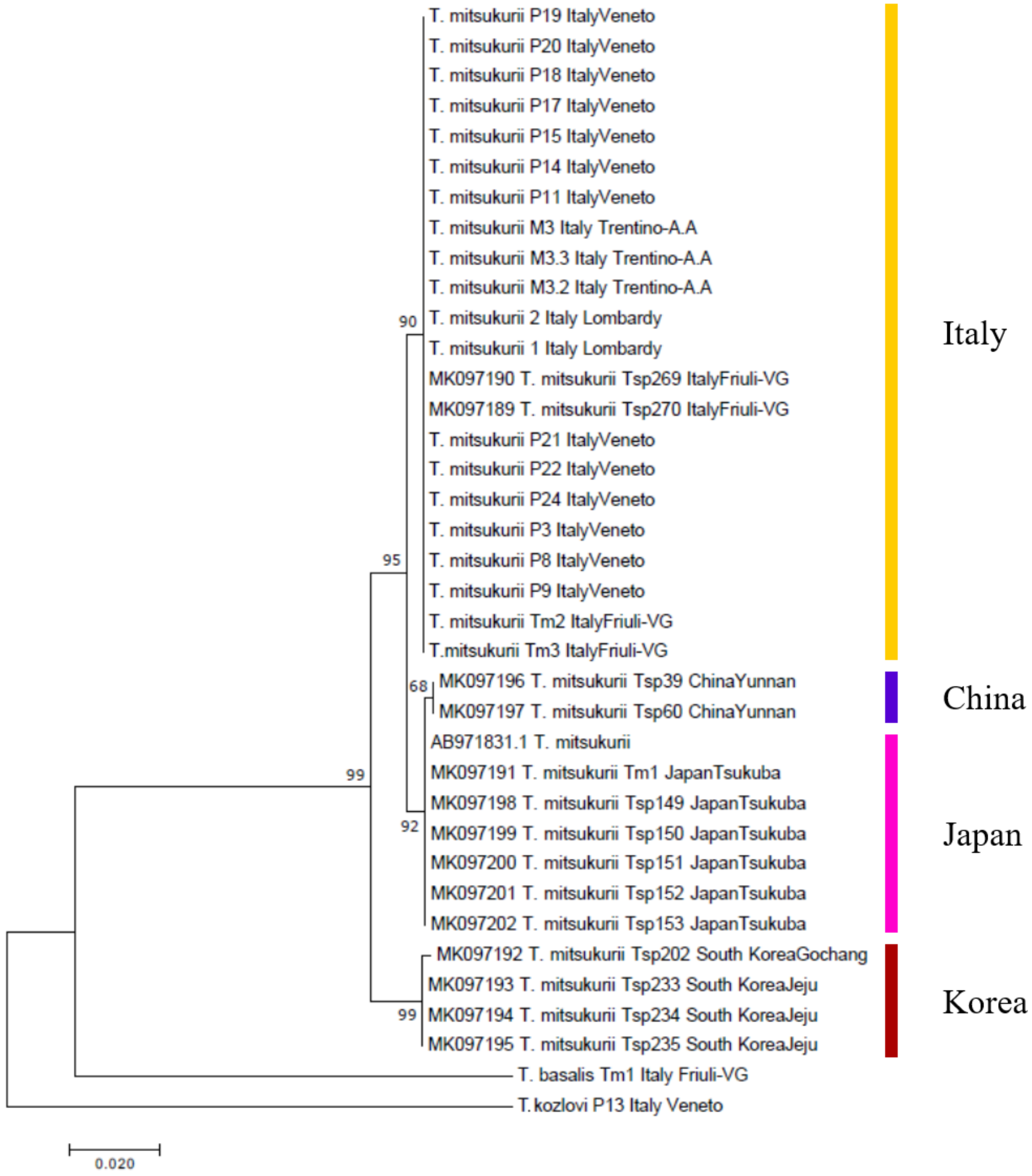


Table 2. Identity and abundance of *Halyomorpha halys* parasitoids and hyperparasitoids emerged from collected egg masses per site.

Site number	**Total N. egg masses**	**Total N. eggs**	**Mean (± st. err.) of eggs/egg mass**	**N. of parasitized egg masses**	**Parasitoid species**	**N. of parasitoid specimens**	**Relative abundance per site (%)**[a]	**Exploitation efficiency (%, mean ± st. err.) per egg mass (n of egg masses)**[b]	**N. of egg masses with more than a parasitoid species**
1	1	n.a.	n.a.	1	*Trissolcus mitsukurii*	24	100	n.a.	
2	2	55	27.5 (± 0.7)	1	*Trissolcus basalis*	1	100	3.57 (1)	
3[c]	63	1520	24.1 (± 0.2)	22	*Acroclisoides solus*	14	10.4	100 (1)	2 (*A. bifasciatus* and *T. mitsukurii*)
					Anastatus bifasciatus	66	49.3	31.1 ± 10.4 (11)	
					Trissolcus mitsukurii	54	40.3	43.5 ± 16.4 (5)	
4	10	271	27.1 (± 0.1)	1	*Trissolcus mitsukurii*	26	100	100 (1)	
5	9	241	26.8 (± 0.1)	2	*Anastatus bifasciatus*	8	25.8	28.6 (1)	
					Trissolcus mitsukurii	23	74.2	85.2 (1)	
6	22	n.a.	n.a.	3	*Anastatus bifasciatus*	18	40.0	29.6 (1)	1 (*A. bifasciatus* and *T. mitsukurii*)
					Trissolcus mitsukurii	27	60.0	n.a.	
7	32	838	26.2 (± 0.1)	2	*Anastatus bifasciatus*	13	59.1	46.4 (1)	
					Trissolcus mitsukurii	9	40.9	34.6 (1)	
8	82	n.a.	n.a	8	*Acroclisoides solus*	9	5.6	23.1 (1)	1 (*A. bifasciatus* and *T. mitsukurii*)
					Anastatus bifasciatus	6	3.7	10.7 (1)	
					Trissolcus basalis	5	3.1	17.9 (1)	
					Trissolcus mitsukurii	142	87.7	53.7 ± 12.0 (6)	
9	19	n.a.	n.a.	3	*Acroclisoides solus*	6	8.5	n.a.	1 (*Acroclisoides solus* and *T. mitsukurii*)
					Trissolcus mitsukurii	65	91.5	71.4 (1)	
10	5	137	27.4 (± 0.2)	1	*Trissolcus kozlovi*	2	100	7.1 (1)	
11	6	168	28.0 (± 0.1)	2	*Anastatus bifasciatus*	2	7.7	7.4 (1)	
					Trissolcus mitsukurii	24	92.3	82.8 (1)	

[a] As number of parasitoids for each species over the number of all parasitoids found in the site.

[b] As number of parasitized eggs by a species over the total number of eggs of the parasitized egg mass. n.a. indicate not available data on the number of eggs.

[c] In this site, some parasitoids emerged from egg masses were not identified.

Table 3. Exploitation efficiency, discovery efficiency, and parasitoid impact on *Halyomorpha halys* egg masses, by parasitoid and hyperparasitoid species observed in the three years.

Index	**Species**	**Year**											
		2016				**2017**				**2018**			
		Index	**χ^2**	**df**	**P**	**Index**	**χ^2**	**df**	**P**	**Index**	**χ^2**	**df**	**P**
Exploitation efficiency (%)	*T. basalis*	17.9 a	5.47	2	0.0648	n.a. -	31.54	2	<0.0001	n.a. -	29.01	2	<0.0001
	T. mitsukurii	46.6 a				70.2 a				52.7 b			
	An. bifasciatus	38.0 a				10.7 b				37.9 c			
	Ac. solus	n.a. -				34.6 b				100 a			
Discovery efficiency (%)	*T. basalis*	7.7 a	4.02	2	0.1343	n.a. -	1.14	2	0.5649	n.a. -	11.17	2	0.0038
	T. mitsukurii	30.8 a				17.3 a				13.2 ab			
	An. bifasciatus	7.4 a				4.8 a				16.1 a			
	Ac. solus	n.a. -				4.8 a				1.6 b			
Parasitoid impact (%)	*T. basalis*	1.5 b	61.68	2	<0.0001	n.a. -	76.06	2	<0.0001	n.a. -	74.66	2	<0.0001
	T. mitsukurii	15.1 a				9.6 a				7.7 a			
	An. bifasciatus	3.3 b				0.1 b				3.5 b			
	Ac. solus	n.a. -				0.3 b				0.9 c			

For each index, values followed by the same letter are not significantly different to the Tukey's test on the least square means ($\alpha = 0.05$).

4. Discussion

In its invaded range, *H. halys* is causing severe damage on crops. Current pest management strategies are mostly based on chemical control and often failed in controlling this pest. The implementation of biological control offers potential solutions for the management of this invasive pest (Leskey and Nielsen 2018) because the lack of effective natural enemies is the main explanation for high impact of exotic species in invaded territories (Elton 1958). Here, we investigated the egg parasitoid complex of *H. halys* during three years to delineate their potential to control pest populations. During this study, five different parasitic wasp species emerged from *H. halys* eggs: *A. solus*, *A. bifasciatus*, *T. basalis*, *T. kozlovi* and *T. mitsukurii*, and the last was the most common species.

Trissolcus mitsukurii is an Asian species reported to develop in *H. halys* eggs (Arakawa and Namura 2002; Talamas et al. 2017). Previous literature reported its presence in Italy from 2018 (Sabbatini Peverieri et al. 2018; Moraglio et al. 2019), and this study provides important information to track the distribution of this species. Indeed, the first three records of this parasitoid occurred in 2016 (site 8) and 2017 (sites 9 and 11; Figure 1). The data here reported show that this parasitoid species has been present in Europe since 2016, two years before the previously published records (Sabbatini Peverieri et al. 2018; Moraglio et al. 2019). These data are of importance for tracking the interaction between the invasive pest and its Asian parasitoid understating their population dynamics. Additionally, the distribution area of this parasitoid is wider than previously quoted, and includes all regions of northern Italy (except Aosta Valley), covering the area across Friuli-Venezia Giulia, Veneto, Trentino-Alto Adige, Lombardy and Piedmont regions (Sabbatini Peverieri et al. 2018; Moraglio et al. 2019; Tortorici and Tavella, unpubl.). All samples of *T. mitsukurii* found in Veneto share the same haplotype of the samples from Trentino-Alto Adige, Lombardy, and Friuli-Venezia Giulia (Sabbatini Peverieri et al. 2018), suggesting a single introduction event of the parasitoid, which possibly followed the invasion pathways of its invasive host (Talamas et al. 2015a). However, multiple introductions of individuals with the same COI haplotype cannot be excluded. Samples of *T. mitsukurii* retrieved in Italy are genetically close to samples from Japan and China, suggesting that this may be their origin, even though some missing haplotypes separate them. Possile introduction(s) from Japan also occurred, following the movements of the host as also suggested in Chapter 2. Further analyses using other molecular markers and expanding collection sites in their native area will help to improve the knowledge on the genetic variability of this parasitoid and delineate its patterns of invasion in Europe. This kind of study has already been performed with *H. halys* (e.g, Gariepy et al.

2014a; 2015; Cesari et al. 2015; 2018; Morrison et al. 2017; Kapantaidaki et al. 2019), showing high genetic variability in invaded areas and suggesting multiple introduction events.

Trissolcus mitsukurii emerged from *H. halys* eggs collected in orchards (organic and conventional), vineyards, and ornamental plants in urban areas, suggesting no habitat limitations for this species. Its potential to track *H. halys* in different habitats is advantageous because *H. halys* is highly polyphagous (Leskey and Nielsen 2018). The superior performances of *T. mitsukurii* in parasitizing *H. halys* eggs is not surprising given that it coevolved with its host. *Trissolcus mitsukurii* represents an example of incidental introduction of an exotic parasitoids of *H. halys* since no classical biological control program has been performed in Europe. Another example is represented by the Asian *T. japonicus*, which was found emerging from *H. halys* eggs in Europe and North America (Talamas et al. 2015a; Dieckhoff et al. 2017; Stahl et al. 2018a; Sabbatini Peverieri et al. 2018), where the species may have an impact on both the stink bug population and the parasitoids community (Konopka et al. 2017). Both these species are consider key parasitoids of *H. halys* in its native area (Yang et al. 2009; Arakawa and Namura 2002; Arakawa et al. 2004; Lee et al. 2013), and their occurrence where *H. halys* is now considered a major pest offers interesting perspectives for its control. No *T. mitsukurii* emerged from collected egg mases of other stink bug as *Nezara viridula* L. (AP, DS, MF and PT unpubl.). Although we found *T. mitsukurii* only from eggs of *H. halys*, it is known to attack many Pentatomid species in Asia, including *Biprorulus bibax* Breddin, *Cuspicona privata* Walker, *Dolycoris baccarum* (L.), *Gonopsis affinis* (Uhler), *Lagynotomus assimulans* (Distant), *Nezara antennata* Scott, *Nezara viridula*, and *Piezodorus rubrofasciatus* (Fabricius) (Watanabe 1951; Ryu and Hirashima 1984; Johnson 1991). Some of these stink bugs associated with *T. mitsukurii* are very common in northern Italy (Faraci and Rizzotti Vlach 1995, Fischnaller and Messner 2018) and parasitism by *T. mitsukurii* is likely occurring on them. For *T. japonicus* the fundamental host range in Europe has been already studied in different countries (Charles et al. 2019; Haye et al. 2019; Lara et al. 2019), and similarly studies are necessary to understand the potential impact of *T. mitsukurii* on non-target species.

Among the other species that emerged from *H. halys* eggs, *A. bifasciatus* was the second most frequently found. This is not surprising, since this species has been reported to be a common egg parasitoid of *H. halys* in Europe (Haye et al. 2015a; Roversi et al. 2016; Costi et al. 2019; Moraglio et al. 2019). This species exhibits a wide host range that includes 30 known host species belonging to the orders Hemiptera and Lepidoptera (Noyes 2018; Stahl et al. 2018b), and it use has been proposed for augmentative biocontrol against *H. halys* (Stahl et al. 2018b; 2019a,b). However, the parasitism rates of *A. bifasciatus* in field conditions are generally low (Haye et al. 2015a; Roversi et al. 2016; Costi et al. 2019; Stahl et al. 2019b; Moraglio et al. 2019), and data here reported show a lower impact of this parasitoid compared to *T. mitsukurii*. It should be noted that previous research (Konopka et al. 2017) suggested that *A. bifasciatus* and exotic parasitoids can act synergistically in the control of *H. halys*.

In this study, other *Trissolcus* species emerged from *H. halys* eggs. In particular, *T. kozlovi* was obtained from a *H. halys* egg mass in summer 2017. This species is morphologically and genetically similar to *T. japonicus* (Talamas et al. 2017; Moraglio et al. 2019), and was first recorded in Italy emerging from eggs of *H. halys* and other stink bugs in 2016 (Moraglio et al. 2019). The acceptance of *H. halys* eggs is here confirmed, but its occurrence in the field was limited and showed a low exploitation efficiency. In two sites, *T. basalis* emerged from *H. halys* egg masses. Despite the low exploitation efficiency of the species, the ability of *T. basalis* to develop on *H. halys* eggs has to be taken into account because of its wide distribution in Europe and USA (Talamas et al. 2015b; 2017), and it has been reported to parasitize live *H. halys* eggs in the southeastern USA (Balusu et al. 2019).

The hyperparasitoid *A. solus* was found to emerge from *H. halys* eggs collected in sites in northeastern Italy. *Acroclisoides solus* was recently found to develop on *H. halys* eggs, associated with other parasitoid species in northern Italy (Moraglio et al. 2019). Pteromalidae belonging to the genus *Acroclisoides* were recorded in different studies acting as hyperparasitoids of Scelionidae (Clarke and Seymour 1992; Grissell and Smith 2006; Gariepy et al. 2014b). For instance, Gariepy et al. (2014b) recorded *A. solus* from an egg mass of the pentatomid *Chinavia hilaris* (Say) (= *Acrosternum hilare*), which was found to be primarily parasitized by *Trissolcus* sp. Similarly, *Acroclisoides* sp. emerged from egg masses of *N. viridula* primarily parasitized by *T. basalis* in Australia (Clarke and Seymour 1992). Therefore, in the present study the high level of exploitation efficiency have to be taken into account considering the parasitoid species that primarily parasitized *H. halys* eggs, thus possibly affecting more the parasitoid species than the host. Little is known about the influence of this hyperparasitoid on the primary parasitoids of pentatomids and on its biology, but its occurrence should be considered in the definition of long-term biocontrol strategies, as showing potential impacts on the primary parasitoid species.

Ecological interactions between these parasitic wasps and their host in the field need to be studied for the selection of effective species as biocontrol agents against *H. halys*. The studies performed on the egg parasitoid complex of *H. halys* in northern Italy provides promising results for the control of a pest that is having a dramatic impact on Italian agriculture. Data on species distribution and genetic variability suggest that *T. mitsukurii* has spread from a limited area in Veneto region in 2016 to different sites across the whole northern part of Italy in 2018. However, its distribution appears spottier than evenly distributed, since there are sites in Veneto region where only a limited number of native parasitoids are recorded. Biological control is likely to have an impact on *H. halys* populations, but further studies are needed to assess the host range of *T. mitsukurii* in Europe and which factors influence the distribution of this exotic parasitoid.

References

Abram P.K., Hoelmer K.A., Acebes-Doria A., Andrews H., Beers E.H., Bergh J.C., Bessin R., Biddinger D., Botch P., Buffington M.L., Cornelius M.L., Costi E., Delfosse E.S., Dieckhoff C., Dobson R., Donais Z., Grieshop M., Hamilton G., Haye T., Hedstrom C., Herlihy M.V., Hoddle M.S., Hooks C.R.R., Jentsch P., Joshi N.K., Kuhar T.P., Lara J., Lee J.C., Legrand A., Leskey T.C., Lowenstein D., Maistrello L., Mathews C.R., Milnes J.M., Morrison W.R., Nielsen A.L., Ogburn E.C., Pickett C.H., Poley K., Pote J., Radl J., Shrewsbury P.M., Talamas E., Tavella L., Walgenbach J.F., Waterworth R., Weber D.C., Welty C., Wiman N.G., 2017. Indigenous arthropod natural enemies of the invasive brown marmorated stink bug in North America and Europe. Journal of Pest Science 90:1009-1020.

Arakawa R., Namura Y., 2002. Effects of temperature on development of three *Trissolcus* spp. (Hymenoptera: Scelionidae), egg parasitoids of the brown marmorated stink bug, *Halyomorpha halys* (Hemiptera: Pentatomidae). Entomological Science 5: 215-218.

Arakawa R., Miura M., Fujita M., 2004. Effects of host species on the body size, fecundity, and longevity of *Trissolcus mitsukurii* (Hymenoptera: Scelionidae), a solitary egg parasitoid of stink bugs. Applied Entomology and Zoology 39: 177-181.

Askew R.R., Nieves-Aldrey J.L., 2004. Further observations on Eupelminae (Hymenoptera, Chalcidoidea, Eupelmidae) in the Iberian Peninsula and Canary Islands, including descriptions of new species. Graellsia 60: 27-39.

Bariselli M., Bugiani R., Maistrello L., 2016. Distribution and damage caused by *Halyomorpha halys* in Italy. Bulletin OEPP/EPPO Bulletin 46:332–334

Balusu R.R., Cottrell T.E., Talamas E.J., Toews M.D., Blaauw B.R., Sial A.A., Buntin D.G., Vinson E.L., Fadamiro H.Y., Tillman G.P., 2019. New record of *Trissolcus solocis* (Hymenoptera: Scelionidae) parasitising *Halyomorpha halys* (Hemiptera: Pentatomidae) in the United States of America. Biodiversity Data Journal 7: e30124, doi:10.3897/BDJ.7.e30124.

Bin F., Vinson S.B., 1991. Efficacy assessment in egg parasitoids (Hymenoptera); proposal for a unified terminology. In Trichogramma and other parasitoids, Wajnberg E., Vinson S.B. (eds.). 3rd International Symposium on Le Colloques de l'INRA, vol. 56, pp 175-179.

Bosco L., Moraglio S.T., Tavella L., 2018. *Halyomorpha halys*, a serious threat for hazelnut in newly invaded areas. Journal of Pest Science 91: 661-670.

Candian V., Pansa M.G., Briano R., Peano C., Tedeschi R., Tavella L., 2018. Exclusion nets: a promising tool to prevent *Halyomorpha halys* from damaging nectarines and apples in NW Italy. Bulletin of Insectology 71: 21-30.

Cesari M., Maistrello L., Ganzerli F., Dioli P., Rebecchi L., Guidetti R., 2015. A pest alien invasion in progress: potential pathways of origin of the brown marmorated stink bug *Halyomorpha halys* populations in Italy. Journal of Pest Science 88: 1-7, doi:10.1007/s10340-014-0634-y.

Cesari M., Maistrello L., Piemontese L., Bonini R., Dioli P., Lee W., Park C.-G., Partsinevelos G.K., Rebecchi L., Guidetti R., 2018. Genetic diversity of the brown marmorated stink bug *Halyomorpha halys* in the invaded territories of Europe and its patterns of diffusion in Italy. Biological Invasions 20: 1073-1092.

Charles J.G., Avila G.A., Hoelmer K.A., Hunt S., Gardner-Gee R., MacDonald F., Davis V., 2019. Experimental assessment of the biosafety of *Trissolcus japonicus* in New Zealand, prior to the anticipated arrival of the invasive pest *Halyomorpha halys*. BioControl 64: 367-379.

Cianferoni F., Graziani F., Dioli P., Ceccolini F., 2018. Review of the occurrence of *Halyomorpha halys* (Hemiptera: Heteroptera: Pentatomidae) in Italy, with an update of its European and World distribution. Biologia 73: 599-607.

Clarke A.R., Seymour J.E., 1992. Two species of *Acroclisoides* Girault and Dodd (Hymenoptera: Pteromalidae) parasitic on *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae), a parasitoid of *Nezara viridula* (L.) (Hemiptera: Pentatomidae). Australian Journal of Entomology 31: 299-300.

Costi E., Haye T., Maistrello L., 2019. Surveying native egg parasitoids and predators of the invasive *Halyomorpha halys* in Northern Italy. Journal of Applied Entomology 143: 299-307.

Dieckhoff C., Tatman K.M., Hoelmer K.A., 2017. Natural biological control of *Halyomorpha halys* by native egg parasitoids: a multi-year survey in northern Delaware. Journal of Pest Science 90: 1143-1158.

Elton C.S., 1958. The ecology of invasion by plants and animals. Melthuen, London, 181 pp.

Faraci F., Rizzotti Vlach M., 1995. Heteroptera. In Checklist delle specie della fauna italiana, Minelli A., Ruffo S., La Posta S. (eds.). Fascicolo 41. Calderini, Bologna.

Faúndez E.I., Rider D.A., 2017. The brown marmorated stink bug *Halyomorpha halys* (Stål, 1855) (Het.: Pentatomidae) in Chile. Arquivos Entomolóxicos 17: 305-307.

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

Fischnaller S., Messner M., 2018. Wanzen im Südtiroler Apfelanbau. Obstbau Weinbau 54: 24-28.

Gariepy T.D., Haye T., Fraser H., Zhang J., 2014a. Occurrence, genetic diversity, and potential pathways of entry of *Halyomorpha halys* in newly invaded areas of Canada and Switzerland. Journal of Pest Science 87: 17-28.

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

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

Grissell E.E., Smith D.R., 2006. First report of *Acroclisoides* Girault and Dodd (Hymenoptera: Pteromalidae) in the western Hemisphere, with description of a new species. Proceedings of the Entomological Society of Washington 108: 923-929.

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

Haye T., Gariepy T., Hoelmer K., Rossi J.P., Streito J.C., Tassus X., Desneux N., 2015b. Range expansion of the invasive brown marmorated stinkbug, *Halyomorpha halys*: an increasing threat to field, fruit and vegetable crops worldwide. Journal of Pest Science 88: 665-673.

Haye T., Moraglio S.T., Stahl J., Visentin S., Gregorio T., Tavella L., 2019. Fundamental host range of *Trissolcus japonicus* in Europe. Journal of Pest Science, doi:10.1007/s10340-019-01127-3.

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

Inkley D.B., 2012. Characteristics of home invasion by the brown marmorated stink bug (Hemiptera: Pentatomidae). Journal of Entomological Science 47: 125-130.

Johnson N.F., 1991. Revision of Australasian *Trissolcus* species (Hymenoptera: Scelionidae). Invertebrate Taxonomy 5: 211-239.

Jones A.L., Jennings D.E., Hooks C.R., Shrewsbury P.M., 2017. Field surveys of egg mortality and indigenous egg parasitoids of the brown marmorated stink bug, *Halyomorpha halys*, in ornamental nurseries in the mid-Atlantic region of the USA. Journal of Pest Science 90: 1159-1168.

Kapantaidaki D.E., Evangelou V.I., Morrison W.R. III, Leskey T.C., Brodeur J., Milonas P., 2019. *Halyomorpha halys* (Hemiptera: Pentatomidae) genetic diversity in North America and Europe. Insects 10: 174, doi:10.3390/insects10060174.

Konopka J.K., Haye T., Gariepy T.D., McNeil J.N., 2017. Possible coexistence of native and exotic parasitoids and their impact on control of *Halyomorpha halys*. Journal of Pest Science 90: 1119-1125.

Kozlov M.A., Kononova S.V., 1983. Telenominae of the fauna of the USSR (Hymenoptera, Scelionidae, Telenominae). Nauka – Leningradskoe Otdelenie, Leningrad [in Russian].

Kumar S., Stecher G., Li M., Knyaz C., Tamura K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution 35: 1547-1549.

Lara J.R., Pickett C.H., Kamiyama M.T., Figueroa M., Romo M., Cabanas C., Bazurto V., Strode V., Briseno K., Lewis M., Oliva J., Hernandez G., Hoddle M.S., 2019. Physiological host range of *Trissolcus japonicus* in relation to *Halyomorpha halys* and other pentatomids from California. BioControl, doi:10.1007/s10526-019-09950-4.

Lee D.-H., Short B.D., Joseph S.V., Bergh J.C., Leskey T.C., 2013. Review of the biology, ecology, and management of *Halyomorpha halys* (Hemiptera: Pentatomidae) in China, Japan, and the Republic of Korea. Environmental Entomology 42: 627-641.

Leigh J.W., Bryant D., 2015. POPART: full-feature software for haplotype network construction. Methods in Ecology and Evolution 6: 1110-1116.

Leskey T.C., Hamilton G.C., Nielsen A.L., Polk D.F., Rodriguez-Saona C., Bergh J.C. et al., 2012. Pest status of the brown marmorated stink bug, *Halyomorpha halys* in the USA. Outlooks on Pest Management 23(5): 218-226.

Leskey T.C., Nielsen A.L., 2018. Impact of the invasive brown marmorated stink bug in North America and Europe: history, biology, ecology, and management. Annual Review of Entomology 63: 599-618.

Maistrello L., Dioli P., Vaccari G., Nannini R., Bortolotti P., Caruso S., Costi E., Montermini A., Casoli L., Bariselli M., 2014. Primi rinvenimenti in Italia della cimice esotica *Halyomorpha halys*, una nuova minaccia per la frutticoltura. Atti Giornate Fitopatologiche CLUEB 2014: 283-288.

Maistrello L., Dioli P., Dutto M., Volani S., Pasquali S., Gilioli G., 2018. Tracking the spread of sneaking aliens by integrating crowdsourcing and spatial modelling: the Italian invasion of *Halyomorpha halys*. BioScience 68: 979-989.

Mita T., Nishimoto H., Shimizu N., Mizutani N., 2015. Occurrence of *Trissolcus basalis* (Hymenoptera, Platygastridae), an egg parasitoid of *Nezara viridula* (Hemiptera, Pentatomidae), in Japan. Applied Entomology and Zoology 50: 27-31.

Mohammadpour M., Ziaaddini M., Jalali M.A., Hashemirad H., Mohammadi-Khoramabadi A., 2016. Egg parasitoids of the pistachio green stink bug, *Brachynema germari* (Hemiptera: Pentatomidae) in Kerman province, Iran. Zoology and Ecology 26: 28-34.

Moore L.C., Tirello P., Scaccini D., Toews M.D., Duso C., Pozzebon A., 2019. Characterizing damage potential of the brown marmorated stink bug (Hemiptera: Pentatomidae) in cherry orchards. Entomologia Generalis in press.

Moraglio S.T., Tortorici F., Pansa M.G., Castelli G., Pontini M., Scovero S., Visentin S., Tavella L., 2019. A 3-year survey on parasitism of *Halyomorpha halys* by egg parasitoids in northern Italy. Journal of Pest Science, doi:10.1007/s10340-019-01136-2.

Morrison W.R. III, Mathews C.R., Leskey T.C., 2016. Frequency, efficiency, and physical characteristics of predation by generalist predators of brown marmorated stink bug (Hemiptera: Pentatomidae) eggs. Biological Control 97: 120-130.

Morrison W.R. III, Milonas P., Kapantaidaki D.E., Cesari M., Di Bella E., Guidetti R., Haye T., Maistrello L., Moraglio S.T., Piemontese L., Pozzebon A., Ruocco G., Short B.D., Tavella L., Vétek G., Leskey T.C., 2017. Attraction of *Halyomorpha halys* (Hemiptera: Pentatomidae) haplotypes in North America and Europe to baited traps. Scientific Reports 7: 16941, doi:10.1038/s41598-017-17233-0.

Nielsen A.L., Hamilton G.C., 2009. Life history of the invasive species *Halyomorpha halys* (Hemiptera: Pentatomidae) in northeastern United States. Annals of the Entomological Society of America 102: 608-616.

Noyes J.S., 2018. Universal Chalcidoidea Database. In Natural History Museum, London. https://www.nhm.ac.uk/our-science/data/chalcidoids/database/. Accessed on 29 September 2019.

Patwary M.U., Kenchington E.L., Bird C.J., Zouros E., 1994. The use of random amplified polymorphic DNA markers in genetic studies of the sea scallop *Placopecten magellanicus* (Gmelin, 1791). Journal of Shellfish Research 13: 547-553.

Ryu J., Hirashima Y., 1984. Taxonomic studies on the genus *Trissolcus* Ashmead of Japan and Korea (Hymenoptera, Scelionidae). Journal of the Faculty of Agriculture, Kyushu University 29: 35-58.

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

Sabbatini Peverieri G., Talamas E., Bon M.C., Marianelli L., Bernardinelli I., Malossini G., Benvenuto L., Roversi P.F., Hoelmer K.A., 2018. Two Asian egg parasitoids of *Halyomorpha halys* (Stål) (Hemiptera, Pentatomidae) emerge in northern Italy: *Trissolcus mitsukurii* (Ashmead) and *Trissolcus japonicus* (Ashmead) (Hymenoptera, Scelionidae). Journal of Hymenoptera Research 67: 37-53.

Stahl J., Totorici F., Pontini M., Bon M.C., Hoelmer K., Marazzi C., Tavella L., Haye T., 2018a. First discovery of adventive populations of *Trissolcus japonicus* in Europe. Journal of Pest Science 92: 371-379.

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

Stahl J.M., Babendreier D., Haye T., 2019a. Life history of *Anastatus bifasciatus*, a potential biological control agent of the brown marmorated stink bug in Europe. Biological Control 129: 178-186.

Stahl J.M., Babendreier D., Marazzi C., Caruso S., Costi E., Maistrello L., Haye T., 2019b. Can *Anastatus bifasciatus* be used for augmentative biological control of the brown marmorated stink bug in fruit orchards? Insects 10: 108, doi:10.3390/insects10040108.

Talamas E.J., Herlihy M.V., Dieckhoff C., Hoelmer K.A., Buffington M., Bon M.C., Weber D.C., 2015a. *Trissolcus japonicus* (Ashmead) (Hymenoptera, Scelionidae) emerges in North America. Journal of Hymenoptera Research 43: 119-128.

Talamas E.J., Johnson N.F., Buffington M.L., 2015b. Key to Nearctic species of *Trissolcus* Ashmead (Hymenoptera, Scelionidae), natural enemies of native and invasive stink bugs (Hemiptera, Pentatomidae). Journal of Hymenoptera Research 43: 45-111.

Talamas E.J., Buffington M.L., Hoelmer K., 2017. Revision of Palearctic *Trissolcus* Ashmead (Hymenoptera, Scelionidae). Journal of Hymenoptera Research 56: 3-185.

Unterthurner M., Zelger A., Fischnaller S., Walcher M., Haye T., 2017. Die Marmorierte Baumwanze im Visier. Obstbau Weinbau 53: 5-11.

Watanabe C., 1951. On five scelionid egg-parasites of some pentatomid and coreid bugs from Shikoku, Japan (Hymenoptera: Proctotrupoidea). Transactions of the Shikoku Entomological Society 2: 17-26.

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

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

Conclusions

Invasive species cause ecologic and economic impacts in areas of invasion (Kenis and Branco 2010; Vaes-Petignat and Nentwig 2014). In new areas, *H. halys* is known to cause damage on crops and nuisance problems for the human population, and management practices are necessary to reduce the economic losses caused by this pest (Leskey and Nielsen 2018).

In Europe, *H. halys* distribution resulted from multiple and ongoing introduction events (Cesari et al. 2018), and studying the invasion pathways of this pest may be a useful task to avoid further colonization of new territories. In Chapter 2, we elucidate the pathways of expansion of the pest across the invaded area, and in particular in Italy, coupling the genetic structure study of *H. halys* population with those of its primary symbiont, *P. carbekii*. Some populations from north-eastern Italy showed the highest values of genetic diversity, and possible introductions from Japan, as well as China and North America, are suggested considering the affinity with symbiont haplotypes. These findings improve the understanding of the potential origin of multiple accidental introductions in Italy, possibly helping to optimize management strategies against *H. halys*.

The impact of this pest on crops is notable, and the damage is mainly due to the feeding activity of the pest (Leskey and Nielsen 2018). Despite the high polyphagia, a few studies showed the impact of *H. halys* on grape, an economically important species in America and in many European countries. In Chapter 3, we described the seasonal dynamics of *H. halys* in multi-cultivar vineyards, and we evaluated the damage on grape clusters by *H. halys*. Pest abundance was higher on cultivars like Cabernet Franc, Merlot and, with a less extent, Pinot gris than on the others, and insect population was found to fluctuate across time and space. More *H. halys* individuals were found on red cultivars than on white ones, and on varieties that ripen relatively late in the season and on the border of the patches. *Halyomorpha halys* infestations also caused damage on berries, with a different cultivar and plant phenological stages susceptibility. Damages were in general higher with high infestations, but these population levels were not commonly found in the studied vineyards. Moreover, *Botrytis cinerea* occurrence was correlated to *H. halys* presence, which seemed to facilitate pathogen infestations. We determined from these data that the most important economic damage given the current population levels is that feeding damage may increase a pathway for pathogen entry. This effect on grapevines should be considered for its management.

Suitable areas for *H. halys* invasion cover most of the horticultural temperate areas in Europe and in the Southern Hemisphere (Kriticos et al. 2017), and temperature ranges are important for traits influencing population dynamics of *H. halys* (Haye et al. 2014; Cira et al. 2016; 2018; Lowenstein and Walton 2018). In Chapters 4 and 5, we studied lethal and sub-lethal effects of high- and low-temperature exposures of *H. halys* adults, considering the state of diapause and evaluating their nutritional status. The exiting diapause adults were more sensitive to low- and high-temperature exposures than entering ones. While the nutritional status of tested *H. halys* did not affect high-temperature tolerance, low-temperature exposures resulted in higher mortality levels for adults with low nutritional levels. Low-temperature exposures increased longevity, but reduced fecundity of entering diapause *H. halys* females. Thus, winter and spring low temperatures may influence the survival rate and population development of *H. halys*, as a spring frost can potentially reduce insect numbers of the overwintered population. On the other hand, the mortality-curves obtained from high-temperature exposures showed that survival of adults can be reduced by heat-wave exceeding 37.3 °C for 1 hour. Additionally data obtained by high-temperature exposures experiment can be used to define cost-effective heat treatments aimed at the *H. halys* control, like for goods shipped overseas.

Finally, natural enemies can affect *H. halys* population dynamics. Classical or augmentative biological control should be considered as possible tactics in long-term management of this pest. In Chapter 6, we recorded *H. halys* egg parasitoids from a three-year study conducted in north-eastern Italy. This study showed that a complex of species is related to *H. halys* in this area. Also species previously recorded only from Asia were found, and in particular *T. mitsukurii* have shown higher impact on host eggs than the other species found. Furthermore, we reported the earliest known occurrence of *T. mitsukurii* in Europe, which was in 2016, and its genetic variability suggested a relation between Italian samples and Japanese and Chinese ones, which were separated from the others from South Korea. The study performed on the egg parasitoid complex of *H. halys* in northern Italy provides promising results for the control of *H. halys*, and further studies are needed to assess the host range of *T. mitsukurii* in Europe and the factors influencing its distribution.

References

Cira T.M., Venette R.C., Aigner J., Kuhar T., Mullins D.E., Gabbert S.E., Hutchison W.D., 2016. Cold tolerance of *Halyomorpha halys* (Hemiptera: Pentatomidae) across geographic and temporal scales. Environmental Entomology 45: 484-491.

Cira T.M., Koch R.L., Burkness E.C., Hutchison W.D., Venette R.C., 2018. Effects of diapause on *Halyomorpha halys* (Hemiptera: Pentatomidae) cold tolerance. Environmental Entomology 47: 997-1004.

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

Kenis M., Branco M., 2010. Chapter 5: Impact of alien terrestrial arthropods in Europe. In: Alien terrestrial arthropods of Europe, Roques A., Kenis M., Lees D., Lopez-Vaamonde C., Rabitsch W., Raspulus J.-Y., Roy D. (eds.). BioRisk 4(1): 51-71.

Kriticos D.J., Kean J.M., Phillips C.B., Senay S.D., Acosta H., Haye T., 2017. The potential global distribution of the brown marmorated stink bug, *Halyomorpha halys*, a critical threat to plant biosecurity. Journal of Pest Science 90: 1033-1043.

Leskey T.C., Nielsen A.L., 2018. Impact of the invasive brown marmorated stink bug in North America and Europe: history, biology, ecology, and management. Annual Review of Entomology 63: 599-618.

Lowenstein D.M., Walton V.M., 2018. *Halyomorpha halys* (Hemiptera: Pentatomidae) winter survival, feeding activity, and reproduction rates based on episodic cold shock and winter temperature regimes. Journal of Economic Entomology 111: 1210-1218.

Vaes-Petignat S., Nentwig W., 2014. Environmental and economic impact of alien terrestrial arthropods in Europe. NeoBiota 22: 23-42.