

Journal of Applied Entomology (2022), Vol. 146, p. 319-327. <http://dx.doi.org/10.1111/jen.12955>

Behavioral responses of diapausing *Halyomorpha halys* (Hemiptera: Pentatomidae) to conspecific volatile organic compounds

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Abstract

The brown marmorated stink bug, *Halyomorpha halys* Stål (Hemiptera: Pentatomidae), is an invasive pest in America, Europe, and Asia and causes serious economic loss to crops, and nuisance problems during overwintering. Uninvaded southern hemisphere countries, such as New Zealand and Australia, see an increase in *H. halys* border interceptions during the northern hemisphere populations' overwintering period as large aggregations of *H. halys* can settle to diapause in items for export, e.g. vehicles, containers, etc.. Here, we explored aspects of diapausing *H. halys* behavior relative to release and perception of defensive odor compounds. First, to determine whether group size and agitation affect the release of defensive odors, diapausing *H. halys* were confined in glass tubes as individuals or in varying group sizes and mechanically agitated or remained stationary and the presence or absence of defensive odors was recorded. Using gas chromatography-mass spectrometry, we also established if exposure to individual defensive odor components (tridecane, (*E*)-2-decenal, 4-oxo-(*E*)-2-hexenal, and dodecane) induced individuals to release defensive compounds. Additionally, *H. halys* dispersal was measured in the laboratory following exposure to individual components of their defensive odor or their natural blend. We found that agitating individual bugs did not induce the release of defensive odors. The release of human-detectable odors was only found in groups of mechanically agitated *H. halys*, whereas, non-agitated bugs did not emit odor. Exposure to 4-oxo-(*E*)-2-hexenal was the only odor component that resulted in individual *H. halys* releasing defensive compounds. Diapausing *H. halys* exposure to the natural blend of defensive compounds resulted in increased horizontal distance moved and velocity, while

tridecane exposure increased distance moved, velocity, and angular velocity, and (*E*)-2-decenal exposure increased distance moved. Our behavioral and chemical data suggest that defensive compounds released by diapausing *H. halys* act as an alarm pheromone, particularly when adults are in aggregations.

Key words: brown marmorated stink bug, invasive species, horizontal mobility, insect behavior, Pentatomidae

Introduction

Native to China, Korea, Japan, and Taiwan, *Halyomorpha halys* Stål (Hemiptera: Pentatomidae) has been found in most states within the USA, a number of Canadian provinces, and is spreading throughout European countries (Leskey & Nielsen, 2018). *Halyomorpha halys* feeds on numerous crops including fruits, vegetables, field crops, and ornamentals. While economic damage has been reported within *H. halys*' native ranges (Lee et al., 2013a), serious and widespread damage has been reported in invaded regions such as the USA and Europe (Leskey & Nielsen, 2018). Extensive research has been conducted toward the management of this invasive species using insecticides (Kuhar & Kamminga, 2017), biological control (Dieckhoff et al., 2017), and IPM tactics (Leskey et al., 2020). However, economic impacts continue to be generated by this insect in the USA (Leskey & Nielsen, 2018) and parts of Europe (Bariselli et al., 2016; Costi et al., 2017). For those countries that have not been invaded, effective biosurveillance tools are of great importance. This is especially relevant in light of research confirming that initial populations within the USA originated from a single incursion from Beijing, China, with a predicated propagule size of two gravid females (Xu et al., 2014), indicating that a high level of sensitivity and precision is necessary for biosecurity programs. Moreover, uninvaded southern hemisphere countries such as New Zealand have experienced a noticeable increase in *H. halys* interceptions over recent years from northern hemisphere regions with invasive populations present, these are particularly prevalent during the insects' overwintering period (Duthie et al., 2012). Currently, visual inspection is the most common method used to examine imported goods and shipping containers for diapausing *H. halys* adults. As part of an effort to improve these inspections, volatiles released by diapausing and non-diapausing *H. halys* have been identified from mechanically agitated *H. halys* groups (Nixon et al., 2018). These included tridecane, (*E*)-2-decenal, 4-oxo-(*E*)-2-hexenal, and dodecane. Despite these efforts, the likelihood of using detection of these compounds as a biosurveillance method was determined to be unreliable as groups of diapausing adults were not found to release these compounds reliably based on typical movements experienced during simulated shipping voyages (Nixon et al., 2018; Nixon et al., 2019). However, diapausing *H. halys*, often found in aggregations (Chambers et al., 2019; Cullum et al., 2020), did reliably release defensive compounds when held in groups and vigorously mechanically agitated (Nixon et al., 2018). The behavioral responses of diapausing *H. halys* in relation to the compounds released during defense have not been closely studied. Here, we evaluated: 1) if diapausing *H. halys* were more likely to emit detectable defensive odors when in groups compared to individual bugs; 2) if exposure to individual defensive compound components resulted in release of defensive compounds

by conspecifics; and 3) if exposure to either the natural blend or individual defensive compounds affected dispersal behavior.

Materials and methods

Field sources of Halyomorpha halys

Cohorts of diapausing adult *H. halys* were collected from wooden overwintering shelters, as described by Bergh et al. (2017), that had been deployed prior to fall dispersal to potential overwintering sites (Table 1). After the dispersal period concluded, shelters were retrieved and maintained in a dark unheated shed at USDA-ARS Kearneysville, WV (39°21'18.69"N, 77°52'40.71"W) under ambient overwintering temperature conditions. Adults from these shelters were then used to represent different time periods throughout the diapause period which typically runs from late September to spring the following year (Bergh et al., 2017; Nielsen et al., 2008).

Table 1 Sources of diapausing *H. halys* used for experimentation including deployment and retrieval periods, locations, and geocoordinates.

Experimental cohort	Deployed	Retrieved	Location	Geocoordinates
Early Settling	September 2016	October 2016	USDA-ARS, Kearneysville, WV	39°21'18.69"N, 77°52'40.71"W
Fall	September 2016 and 2018	November 2016 November 2018	Martinsburg, WV	39°24' 50.11"N, 78°01'45.50"W
			Keedysville, MD	39°30'18.08"N, 77°44'35.57"W
			Keedysville WV	39°29'08.32"N, 77°46'02.04"W
			Shannondale, WV	39°12'28.76"N, 77°47'44.46"W
Winter	September 2015	November 2015	Inwood, WV	39°23'41.49"N, 78° 4'39.84"W
			Mount Weather, VA	39° 3'43.77"N, 77°53'29.65"W
			Boonsboro, MD	39°30'20.55"N, 77°44'34.95"W
			Gerrardstown, WV	39°24'22.19"N, 78° 5'54.68"W

Effect of group size and agitation

In order to test the hypothesis that increased group sizes leads to increased likelihood of defensive odor release within a specific cohort, individual adults, and groups of 2, 3, 5, and 10 adults were placed in 36 ml glass tubes and shaken vigorously for 1 min based on preliminary trials that revealed defensive compound release occurred within 15 – 30 s of agitation. A positive sample was based on olfactory detection by a human experimenter, as per Nixon et al. (2018). Because we

were interested in a yes/no answer, using the human nose as a sensor was considered as adequate to this question as it is sufficiently sensitive to the compounds given off by *H. halys*. *Halyomorpha halys* were evaluated at three points during the diapause period to ensure representative sampling of this biological state (1) post-settling (late September-late October), (2) fall (November), and (3) winter (February). Additionally, individual adults, and groups of, 2, 3, 5, and 10 adults from the fall cohort were also placed in 36 ml vials, left stationary for 1 min and then checked for odor release. A total of 50 replicates were completed for each treatment and time point. Data were analyzed using a binomial generalized linear model using a full factorial design with the group size (1, 2, 3, 5 and 10) and testing period (post-settling, fall and winter) as variables using RStudio Version 1.1.463 (2009-2018 RStudio ©, Inc.).

Defensive compound release

To assess whether defensive odor compounds released by *H. halys* resulted in other diapausing adults releasing defensive odors, *H. halys* were removed from overwintering shelters (21 – 29 November), placed in 36 ml glass tubes, and exposed to the individual defensive compounds. All trials were performed under laboratory conditions (25 ±2°C, RH 55 ±5%). Individual standards of tridecane, (*E*)-2-decenal, and dodecane (all >94%, Sigma-Aldrich, Australia), and 4-oxo-(*E*)-2-hexenal (synthetic material provided by Ashot Khimian, USDA-ARS, Beltsville, MD, USA) were prepared in dichloromethane (DCM). The amount of compound in each treatment corresponded to known emission rates per adult, as presented by Nixon et al. (2018), and were as follows: tridecane (40 µg), (*E*)-2-decenal (18 µg), 4-oxo-(*E*)-2-hexenal (16 µg), dodecane (2 µg), and clean DCM (5 µl) as a control. Compounds were pipetted directly into the glass tube immediately before insect introduction to minimize compound degradation before exposure; each droplet size was ≤5 µl to ensure it did not have direct contact with *H. halys*. A single adult was placed in each treated tube and left in place for 1 min, after which a headspace sample was collected from the tube. These samples were collected based upon methodology reported in Nixon et al. (2018) using a portable battery-operated air pump (PAS-500, Spectrex, CA, USA) drawing air through a volatile collection trap (VCT) containing 30 mg of Super-Q (Analytical Research Systems, FL, USA) at a rate of 400 ml/min for 10 min. Volatiles were extracted from VCTs using 250 µl of DCM with 20 ng/µl tetralin (Sigma-Aldrich, Australia) as an internal standard. A control blank for each treatment was taken using the same apparatus and extraction technique. Tubes were washed using soap and water, followed by acetone, hexane, and deionized water, and allowed to dry between trials.

Gas chromatography-mass spectrometry (GC-MS) analysis was performed on a Shimadzu GCMS2010 (Ultra) with an RTX-5MS column (30 m x 0.25 mm I.D.), with GCMSolutions software. Auto-sampling performed on PALS LHX-xt system. The GC-MS method used a high pressure 1 µl splitless injection at an injection temperature of 250°C. The GC was operated at a column flow of 0.6 ml/min. The temperature programme started at 40°C for 7 min, followed by temperature ramping of 6°C/min until a final temperature of 230°C was reached and held for 5 min. The spectra were obtained in electron-impact (EI) ionization mode at 70 eV. The mass spectrometer was run in total ion count mode, with a scanning range 25-550 m/z. Analytical standards used for insect exposure were also analyzed with GC-MS for sample identification.

Ten replicates of each treatment were performed, and resulting chromatograms were analyzed for the presence of the treatment compound, internal standard, and the three additional defensive compounds. Any chromatograms not containing the treatment compound and internal standard were

discarded. As tridecane was detected as a contaminant in most system blank controls, it was removed from analysis. Fisher's Exact Tests were performed to assess significant differences in compound presence between treatments.

Effect of defensive compounds on dispersal

Horizontal movement of individual adults exposed to components of defensive odors was tracked under laboratory conditions with a system previously used to monitor *H. halys* dispersal capacity (Lee et al., 2014a) and consequences of insecticide exposure (Morrison et al., 2017; Lee et al., 2013b). A video visualizer system (R(E)-350, Canon, Inc., Tokyo, Japan) was suspended above five Petri dish arenas with fluorescent backlights and used to track the movement of adults. The total distance moved (cm), mean velocity (cm/s), and mean angular velocity (deg/s) of individual *H. halys* adults were tracked for 10 mins using EthoVision software (version 3.1.16, Noldus Information Technology Inc., Leesburg, VA) (Noldus et al., 2002); for full method refer to Morrison et al. (2017). Petri dish arenas (diameter x height: 100 x 15 mm) were treated as described below prior to transferring individual adults directly from overwintering shelters (10 – 29 November) into them. Initially, the effect of the natural *H. halys* odor was tested; the treatment was prepared by enclosing 10 *H. halys* in the Petri dish and shaking vigorously for 1 min to trigger production of defensive odor, as demonstrated in the above group size and agitation experiment, these insects were then removed. To test the effect of individual components, individual standards of tridecane, (*E*)-2-decenal, and dodecane (all >94%, Sigma-Aldrich, Australia), and 4-oxo-(*E*)-2-hexenal were prepared as above. To test whether the dilution solvent, DCM, had any effect, one set of trials was run whereby the treatment comprised 5 μ l clean DCM. The amount of compound in each treatment corresponded to known emission rates per bugs as above. Compounds were pipetted directly onto the Petri dish; each droplet size was $\leq 5 \mu$ l to ensure it did not have direct contact with *H. halys*. To avoid cross contamination of compound treatments, a single treatment was run during each test day. Controls were also performed on each test day, which consisted of individual *H. halys* placed in Petri dishes without any additional compounds or agitation. The room was kept dark and maintained at temperatures between 23 – 26°C and 55 – 60% relative humidity for all replicates. For all treatments, a total of 50 treatment and 50 control replicates were performed. To assess if dispersal of *H. halys* was different for those exposed to individual defensive compounds or the natural blend and those that were unexposed, Welch's two-sample t-tests were performed for distance, velocity, and angular velocity data sets.

Results

Effect of group size and agitation

Notably, there were no defensive compounds detected from any samples of non-agitated control *H. halys* individuals or groups of any size (Figure 1). However, for agitated *H. halys*, group size had a significant effect on the proportion of samples with detectable defensive odors ($\chi^2 = 447.08$, d.f. = 4, $P < 0.001$), with the number of samples with detectable odors increasing with increasing group size and no detectable odor from samples of mechanically agitated individuals (Figure 1). Time period (post-settling, fall, and winter) did not have a significant effect on release of defensive compounds ($\chi^2 = 0.0002$, d.f. = 2, $P = 0.99$), and there was no significant interaction between time period and group size ($\chi^2 = 6.814$, d.f. = 8, $P = 0.56$).

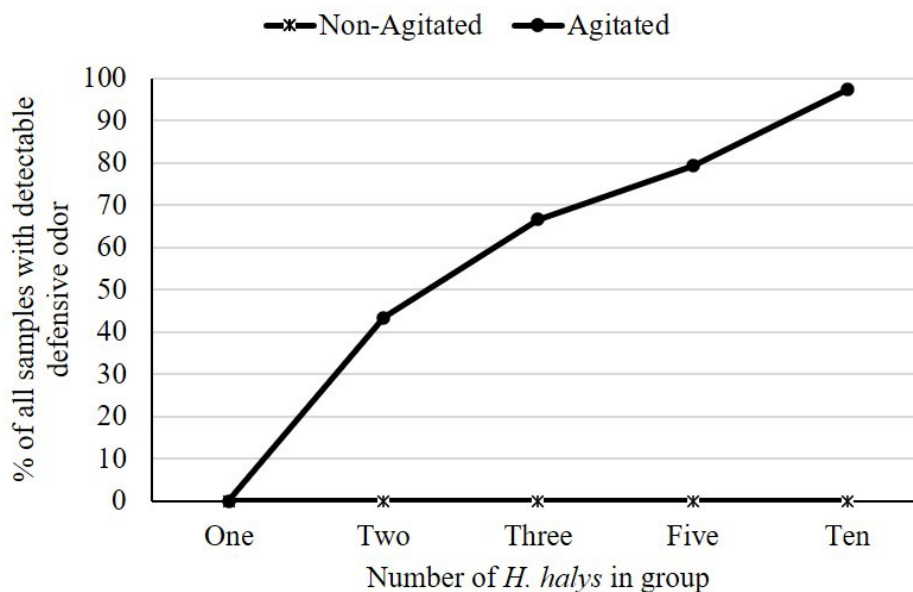


Figure 1 Odor release responses [%] of groups of 1, 2, 3, 5, and 10 *Halyomorpha halys* when exposed to mechanical agitation, in three biological states, with non-agitated control groups data shown.

Defensive compound release

Here, only samples taken from adults exposed to 4-oxo-(*E*)-2-hexenal resulted in detectable defensive compounds, which were significantly different from the control treatment; of the seven validated chromatograms, five contained (*E*)-2- decenal (Fisher's Exact Test, $P=0.002$) and seven contained dodecane (Fisher's Exact Test, $P < 0.001$). There were no detections of the defensive compounds from adults following exposure to tridecane, (*E*)-2- decenal or dodecane.

Effect on dispersal

Exposure to the solvent DCM alone had no significant effect on *H. halys* horizontal mobility compared with controls (distance, $t = 0.40$, d.f. = 96.42, $P = 0.69$; velocity, $t = -1.11$, d.f. = 73.75, $P = 0.27$; angular velocity, $t = 0.57$, d.f. = 97.92, $P = 0.57$). Exposure to the natural odor treatment resulted in *H. halys* moving significantly greater distances ($t = 2.20$, d.f. = 87.88, $P = 0.03$) and at greater mean velocity ($t = 2.19$, d.f. = 86.99, $P = 0.03$) compared with the control, but this exposure did not significantly affect the mean angular velocity ($t = 0.69$, d.f. = 88.69, $P = 0.49$) (Figure 2). Among individual compounds, exposure to tridecane resulted in significantly greater total distance ($t = -3.51$, d.f. = 69.98, $P < 0.001$), mean velocity ($t = -4.06$, d.f. = 63.67, $P < 0.001$), and mean angular velocity ($t = -2.05$, d.f. = 97.13, $P = 0.04$) compared with the control (Figure 3), and exposure to (*E*)-2-decenal resulted in significantly greater distance moved ($t = 2.04$, d.f. = 87.67, $P = 0.05$) compared with the control (Figure 4).

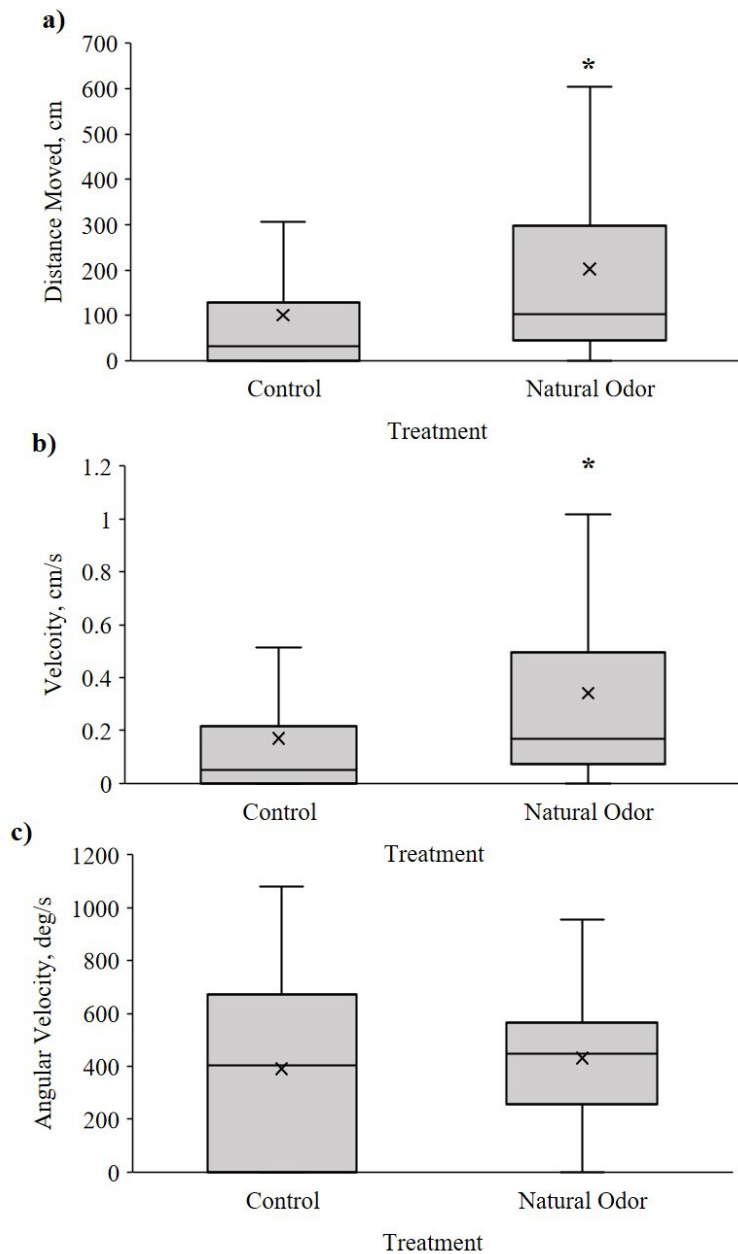


Figure 2 Effect of exposure to the naturally released defensive odor of *Halyomorpha halys* on the horizontal mobility of diapausing *H. halys*. Measurements include: a) total distance travelled in 15 min. b) mean velocity over 15 min. and c) mean angular velocity over 15 min.

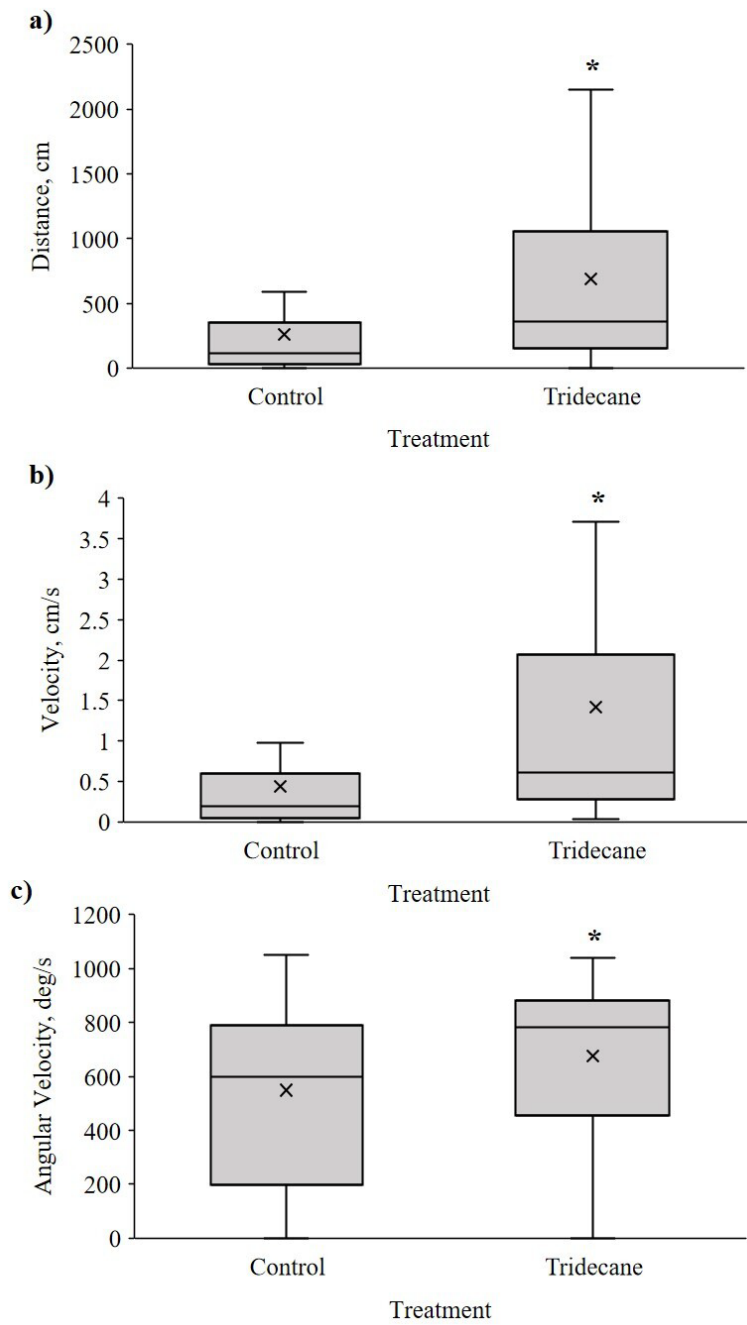


Figure 3 Effect of exposure to tridecane on the horizontal mobility of diapausing *Halyomorpha halys*. Measurements include: a) total distance travelled in 15 min. b) mean velocity over 15 min. and c) mean angular velocity over 15 min.

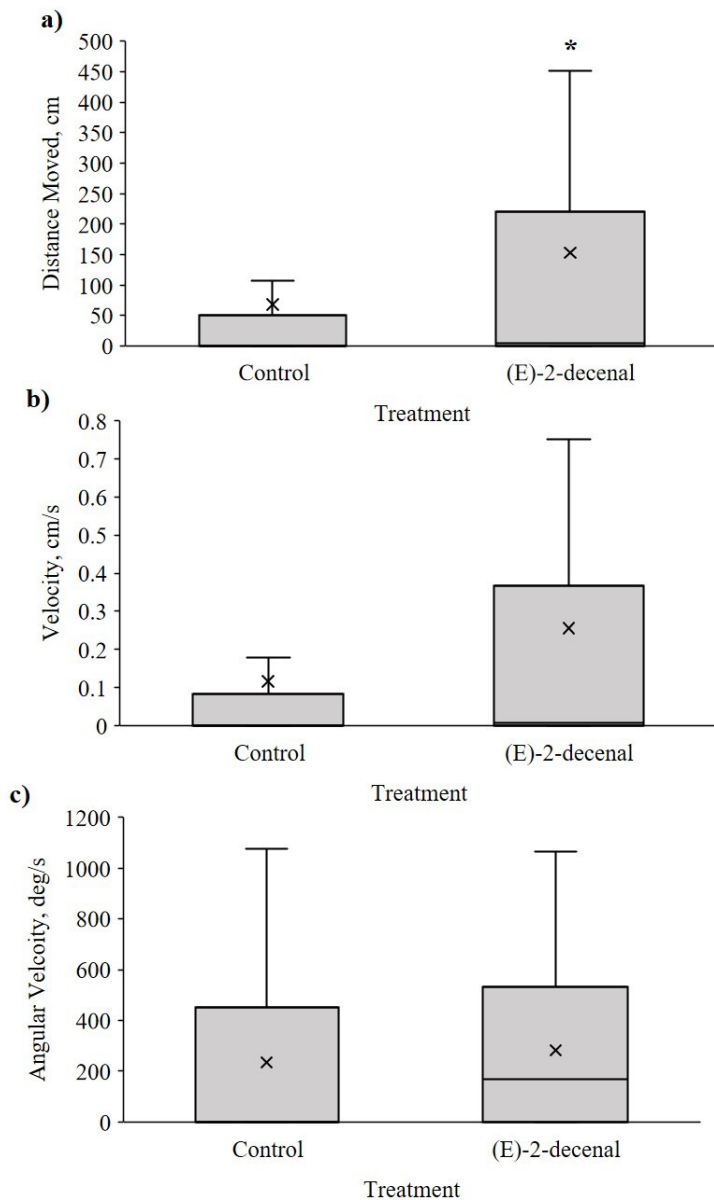


Figure 4 Effect of exposure to (E)-2-decenal on the horizontal mobility of diapausing *Halyomorpha halys*. Measurements include: a) total distance travelled in 15 min. b) mean velocity over 15 min. and c) mean angular velocity over 15 min.

Exposure to 4-oxo-(E)-hexenal did not have a significant effect on horizontal mobility (distance, $t = 1.59$, d.f. = 85.85, $P = 0.12$; velocity, $t = 1.54$, d.f. = 85.49, $P = 0.13$; angular velocity, $t = 1.13$, d.f. = 96.96, $P = 0.26$) (Figure 5), nor did dodecane (distance, $t = -0.97$, d.f. = 91.32, $P = 0.33$; velocity, $t = -1.02$, d.f. = 91.58, $P = 0.31$; angular velocity, $t = -2.01$, d.f. = 97.95, $P = 0.05$) (Figure 6).

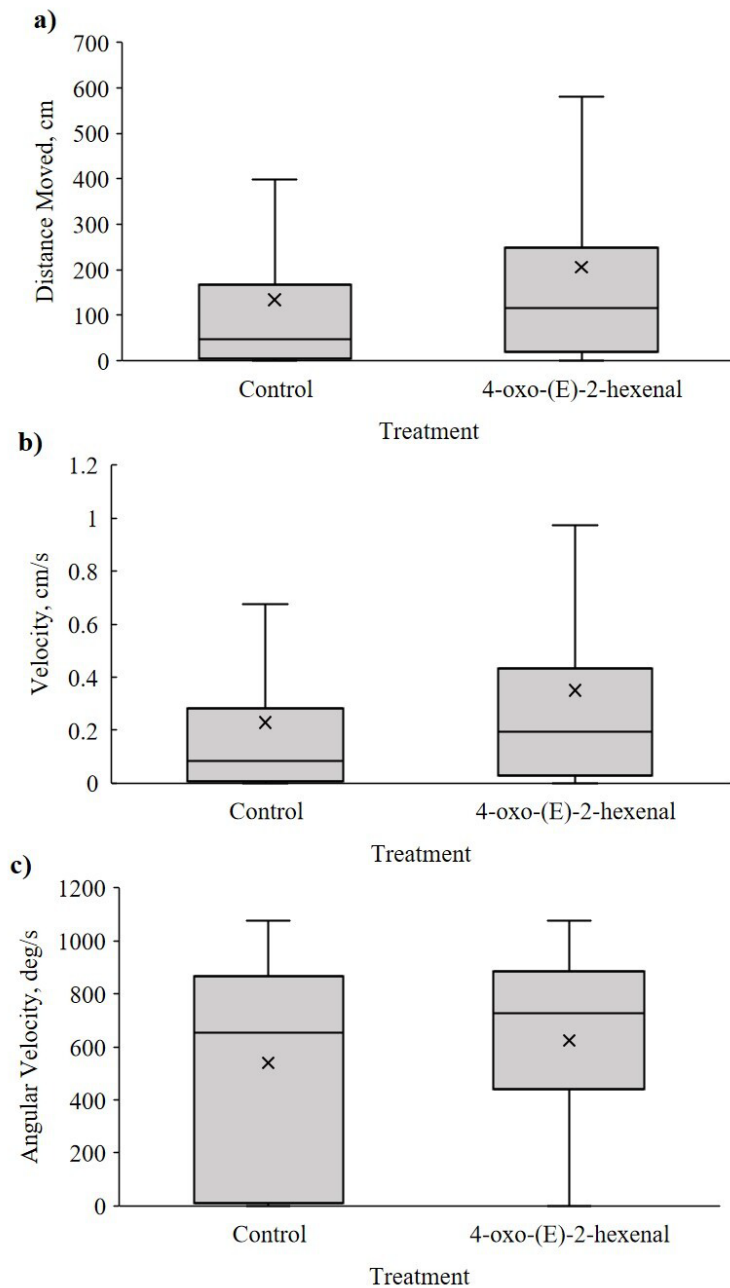


Figure 5 Effect of exposure to 4-oxo- (*E*)-2-hexenal on the horizontal mobility of diapausing *Halyomorpha halys*. Measurements include: a) total distance travelled in 15 min. b) mean velocity over 15 min. and c) mean angular velocity over 15 min.

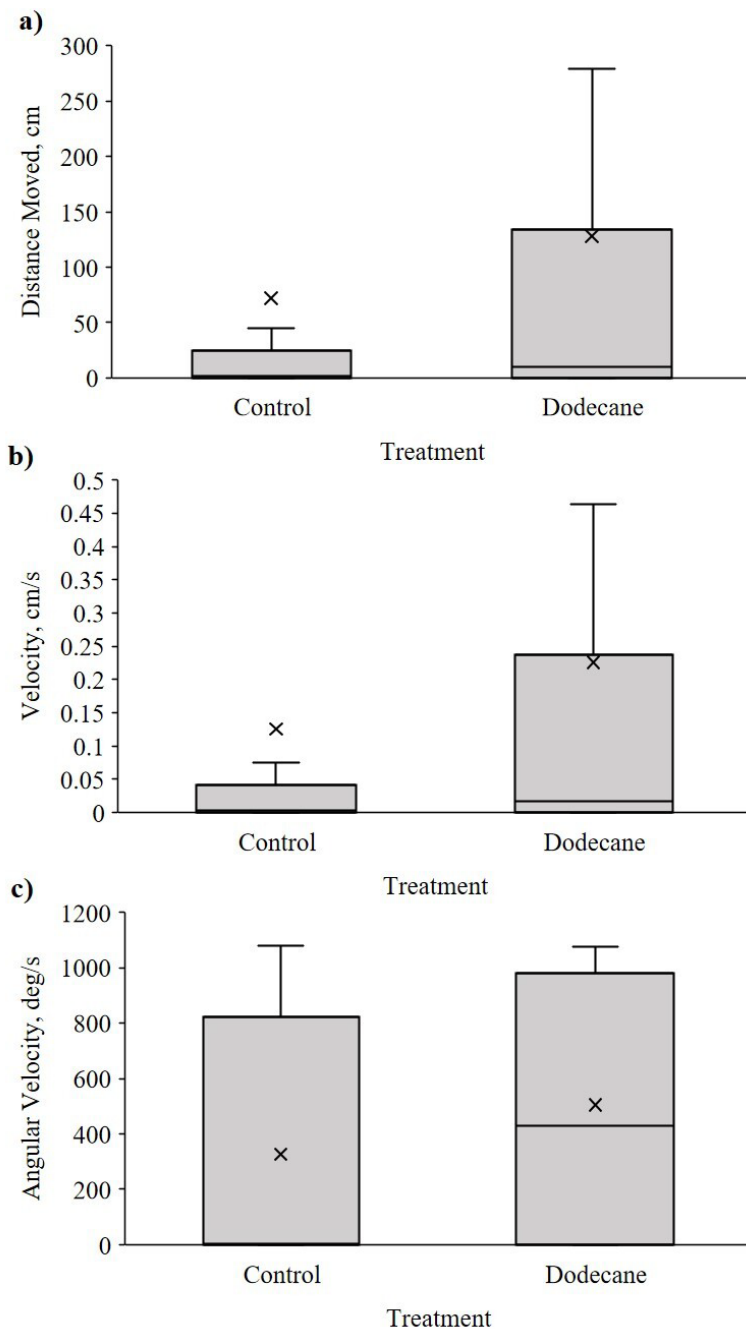


Figure 6 Effect of exposure to dodecane on the horizontal mobility of diapausing *Halyomorpha halys*. Measurements include: a) total distance travelled in 15 min. b) mean velocity over 15 min. and c) mean angular velocity over 15 min.

Discussion

Here, we demonstrated that defensive odor release from diapausing *H. halys* that were mechanically agitated occurred more frequently when in a group compared with individually held adults that were mechanically agitated. Groups or individually held diapausing adult *H. halys* that were not mechanically agitated did not release detectable odor (Figure 1). While we do not know how many individuals in a mechanically group actually released odor, as group size increased, so did the

number of samples with detectable defensive odor. Additionally, lack of detectable odor from samples of non-agitated groups and individuals indicates that defensive odor release from diapausing adults is likely related to group presence and their disturbance. Indeed, *Halyomorpha halys* forms large quiescent aggregations to overwinter and prefer to settle into tight, concealed cavities (Chambers et al., 2019, 2020; Cullum et al., 2020); chosen sites can be in human-made structures (Inkley, 2012), as well as natural areas beneath the bark of dead, standing trees (Lee et al., 2014b). The aggregative behavior exhibited by diapausing adults likely acts as a means for sharing an appropriate overwintering location and defensive strategy (Toyama et al., 2006). Aggregations of non-aposematic insects like *H. halys* may be used by individual members to decrease the overall likelihood of attack by predators, and defensive compounds released by them could serve to deter attacking predators and decrease the likelihood of future revisits to the aggregation (Alcock, 1970). Therefore, the release of defensive odors by *H. halys* group samples, as observed here, likely provides a defensive mechanism for overwintering *H. halys* to deploy against potential predators, especially as adults often remain in these aggregations for longer than six months (Bergh et al., 2017). Indeed, a logical next step may be to perform bioassays of defensive odor release by groups of and individual *H. halys* when threatened by a predator to determine the frequency of release and the number of individuals within a group actually releasing defensive odors.

Upon our evaluation of behavioral responses of individual *H. halys* when exposed to components of the defensive odor (tridecane, (*E*)-2-decenal, 4-oxo-(*E*)-2-hexenal, and dodecane) we found that exposure to 4-oxo-(*E*)-2-hexenal was the only individual component that resulted in individual *H. halys* releasing defensive compounds, while exposure to the natural blend of defensive compounds resulted in increased horizontal distance moved and velocity. The individual components that resulted in increased dispersal behavior were tridecane and (*E*)-2-decenal. These assays were performed with *H. halys* contained to small, enclosed spaces, as would be the case for naturally diapausing insects which show a preference for settling in tight cavities (Chambers et al., 2019, 2020). (*E*)-2-decenal, is considered to be an alarm pheromone or allomone for other stink bug species (Weber et al., 2017). Alarm pheromones are defined as chemicals produced and released by individuals of one species that warns other conspecific individuals of danger, often leading to dispersal away from or aggression towards a threat (Napper & Pickett, 2008). Moreover, increased dispersal and signal amplification are behaviors generally associated with alarm pheromones (Wyatt, 2014). For example, aphid species (Hemiptera: Aphididae) respond to the release of (*E*)- β -farnesene, a common component of their alarm pheromone with a variety of species-dependent behaviors, including, but not limited to, walking away from the release site of the alarm pheromone (Pickett & Griffiths, 1980; Vandermoten et al., 2012; Verheggen et al., 2010). Exposure to the major component of the defensive odor, tridecane (Nixon et al., 2018), resulted in adult *H. halys* moving significantly greater distances and at greater velocity and angular velocity. Changes in dispersal behavior were also noted when adults were exposed to the natural blend of defensive compounds as well as to (*E*)-2-decenal. Although tridecane and (*E*)-2-alkenals have known anti-predator properties amongst pentatomids, (Eliyahu et al., 2012; Gregorovičová & Černíková, 2015; Krall et al., 1999; Waterhouse et al., 1961; Zhong et al., 2017), the changes in dispersal behaviors exhibited by *H. halys* exposed to defensive compounds in our study suggest a secondary function which includes increased dispersal away from a potential threat (Aldrich, 1988; Kou et al., 1989). A

previous study (Chambers et al., 2019) found that adult *H. halys* avoided fresh conspecific corpses but had no reaction to desiccated corpses, likely due to the remnant odors from the metathoracic glands. Interestingly, exposure to 4-oxo-(*E*)-2-hexenal did not affect mobility behavior but did result in exposed individuals releasing their own defensive odor.

Defensive compounds released by *H. halys* groups to a perceived threat (based on mechanical agitation) may also be considered a form of aggression toward would-be predators as these compounds are considered repellent. Indeed, components of the defensive odor released by *H. halys*, tridecane and (*E*)-2-alkenal, have been shown to have anti-predator properties among pentatomids, thereby acting as defensive compounds (Eliyahu et al., 2012; Gregorovičová & Černíková, 2015; Krall et al., 1999; Waterhouse et al., 1961; Zhong et al., 2017).

Our findings suggest that *H. halys* defensive compounds serve as an alarm pheromone for diapausing *H. halys*. Interestingly, a study using non-diapausing *H. halys* found that exposure of adult males to tridecane resulted in reduced release of the male-produced aggregation pheromone, (3*S*,6*S*,7*R*,10*S*)-10,11-epoxy-1-bisabolen-3-ol and (3*R*,6*S*,7*R*,10*S*)-10,11-epoxy-1-bisabolen-3-ol (Harris et al., 2015), indicating that components of the defensive odors may affect other *H. halys* behaviors during the active growing season when feeding and reproduction take place. These results suggest that defensive compounds may play additional roles for *H. halys* throughout the growing season, and therefore would be worthwhile to explore how these compounds affect the behavior of both nymphal and non-diapausing adult *H. halys* as well.

Acknowledgments

Contributions by L.J.N., E.G.B., and M.R. were supported by the New Zealand government via MBIE core funding to Plant and Food Research and Scion (contract C04X1104), respectively, and the Better Border Biosecurity Collaboration (www.b3nz.org). Contributions by K.R. and W.R.M. were supported by USDA NIFA SCRI CAP Grants (# 2011-51181-30937 and 2016-51181-25409). Thanks to Keirston Rinehart, Alyssa Lucas, and Torri Hancock (USDA-ARS) for their excellent technical assistance. Mention of trade names or commercial products in this publication is solely for the purpose of providing scientific information and does not constitute recommendation or endorsement by the United States Department of Agriculture. USDA is an equal opportunity provider and employer.

Conflict of Interest Statement

The authors declare no conflict of interest.

Authors' Contributions

All authors conceived, facilitated, and designed the research. L.J.N. conducted the experiments. A.K. contributed materials. L.J.N. analyzed the data and conducted statistical analyses. L.J.N. and T.C.L. wrote the manuscript. M.R., E.G.B., and T.C.L. secured funding. All authors read, edited, and approved the manuscript.

Data Availability Statement

Data that support the findings of this study are available from an online repository accessed via the link below.

https://figshare.com/projects/Behavioral_responses_of_diapausing_Halyomorpha_halys_to_conspectific_volatile_organic_compounds/114291

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