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Physiological Ecology

Cold Tolerance of *Halyomorpha halys* (Hemiptera: Pentatomidae) Across Geographic and Temporal Scales

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Abstract

The brown marmorated stink bug, *Halyomorpha halys* (Stål), is native to eastern Asia and is presently invading North America. Little is known about the exposure to and effects of winter temperatures in newly invaded regions on *H. halys*. The overwintering habitats that this species utilizes vary greatly in their thermal buffering capacity. They naturally overwinter in aggregations beneath loose bark on trees and in cliff outcroppings, but will also commonly aggregate in buildings. Effects of cold temperatures such as mortality and freezing have yet to be quantified in the invading population. We report that *H. halys* is chill intolerant (i.e., dies before reaching its freezing point), and that the degree of cold tolerance of populations in North America differs by season, sex, and acclimation location. The mean winter supercooling point ($\pm$ SEM) of individuals acclimated in Minnesota was $-17.06°C \pm 0.13$ and in Virginia was $-13.90°C \pm 0.09$. By using laboratory assays of lower lethal temperatures and ambient air temperature records, we accurately forecasted mortality for field experiments in Minnesota and Virginia. Temperature refugia provided by human-built structures are likely crucial for overwintering survival during atypically cold winters and possibly contribute to the northern geographic range expansion of this economically damaging insect in the temperate climates of North America.

Key words: cold hardiness, chill intolerance, supercooling point, invasive species

Halyomorpha halys (Stål), the brown marmorated stink bug, is native to East Asia and has become a severe invasive agricultural pest in the United States on over 33 crop host plants (Bergmann et al. 2015). Damage to Mid-Atlantic apples alone exceeded US$37 million in 2010 (U.S. Apple Association News Release 2011). This invading insect has been found in 42 US states since it was first detected in the United States in the mid-1990s (Northeastern IPM Center 2015) and is predicted to establish across the entire contiguous United States based on the geographic distribution of hosts and degree day models (Holtz and Kamminga 2010) and ecological niche models (Zhu et al. 2012). These inductive approaches to modeling, however, do not predict fluctuations in population levels nor do they define the impacts of environmental stresses on populations for ecological and economic risk assessment (Venette et al. 2010). Insects are poikilotherms; ambient temperature strongly affects their development and mortality. Cold often dictates the poleward limits of a species' distribution. To clarify the relationship between cold stress and mortality, we studied the overwintering strategies and lower lethal temperatures of this invasive species.

The capacity of *H. halys* to survive cold exposure (i.e., cold tolerance) is dependent on the temperatures individuals are exposed to

and the effects of those cold temperatures on an insects' physiology. An observational study in Japan found that winter mortality was reduced by 13.5% for every 1°C rise in mean January and February temperature above 4°C (Kiritani 2007). Our work expands on these observations by experimentally testing the effects of cold and assessing mortality and cold exposure of *H. halys* that are invading North America. As winter approaches, *H. halys* enters a facultative reproductive diapause (Watanabe et al. 1978) and begins to aggregate in protected areas (Watanabe et al. 1994). In natural settings, this species seeks shelter beneath loose bark on trees, but aggregations can also occur in human-made structures (Lee et al. 2014), which may offer greater thermal protection. We do not know the extent to which the North American population utilizes these overwintering habitats.

In order to examine the effects of cold temperatures on *H. halys*, we first determined *H. halys*' cold tolerance strategy. Three broad insect cold tolerance categories exist to describe the relationship between freezing and mortality. Freeze-tolerant insects are able to live after the formation of ice within their bodies, freeze-intolerant insects live up until the point at which they freeze, and chill-intolerant insects die before freezing occurs (Lee 2010). Most temperate insects in the Northern Hemisphere are freeze intolerant (Bale 1991).

To avoid freezing, various mechanisms are used, such as the production of cryoprotectants to depress the insect's supercooling point (i.e., the temperature at which body fluids begin to freeze). Overlaying supercooling points and mortality elucidates the relationship between freezing and mortality and thus an insects' cold tolerance strategy (Hanson et al. 2013).

To further examine the effects of cold temperatures on *H. halys*, we compared supercooling points across season, sex, acclimation location, and geographic origin. Acclimation, on both short (Lee et al. 1987, Lee and Denlinger 2010) and long (Salt 1961) time scales, can affect the cold tolerance of an insect. Short-term acclimation, such as rapid cold hardening, can quickly confer an increased cold tolerance (Lee et al. 1987), while long-term acclimation (i.e., acclimatization), such as from changing environmental temperature and photoperiod cues, leads to seasonal differences in cold tolerance (Salt 1961). The climate to which an individual is acclimated can also alter the degree of cold tolerance an insect can achieve (Bradshaw 2010). Extreme weather events have the potential to cause high mortality when insects are inadequately acclimated. During the winter of 2013–2014 much of North America experienced what was commonly referred to as the "polar vortex," i.e., low pressure and cold arctic air which escaped the typical circulation pattern that generally revolves around a low pressure system over the North Pole. The uncommonly low temperatures experienced during the polar vortex provided a rare opportunity to measure field mortality of *H. halys* under extreme conditions.

Field-acclimated *H. halys* from Minnesota were used to determine the species' cold tolerance strategy, and field-acclimated *H. halys* from Minnesota, Virginia, and West Virginia were used to investigate factors affecting supercooling points. As *H. halys* occurs in temperate climates, we hypothesized that *H. halys* would be freeze intolerant, that the temperatures at which *H. halys* freezes would vary according to season, sex, and acclimation location, but not geographic origin. We also assessed the degree to which our laboratory measurements could deductively forecast the winter field mortality in Minnesota and Virginia in two common types of overwintering habitats.

Materials and Methods

Insects

Virginia-Sourced and Minnesota-Acclimated Insects
Halyomorpha halys eggs were shipped overnight from Blacksburg, VA, to St. Paul, MN, on 2 July 2013. Eggs were maintained at 25°C with a photoperiod of 16:8 (L:D) h in a growth chamber until the insects molted into second instars. Second-instar nymphs were placed in mesh cages 38 by 38 by 61 cm^3 (BioQuip, Rancho Dominguez, CA) within a larger wire screen enclosure and provisioned with potted snap bean plants (*Phaseolus vulgaris* L. cv 'Romano Bush #14'), dried raw organic sunflower (*Helianthus annus* L.) seeds, and soybean (*Glycine max* (L.) Merr.) seeds as needed. These cages were maintained outdoors on the St. Paul campus of the University of Minnesota (44° 59′20.7″ N, 93° 11′10.6″ W, elevation 300 m). On 18 October 2013, insects were transferred into circular plastic dishes (18.5 cm diameter by 8 cm high; Pioneer Plastics, Inc., North Dixon, KY), with a 25- by 89-cm piece of cotton canvas provisioned with dry organic soybean seeds and placed into an unheated shed on the St. Paul campus of the University of Minnesota to mimic where and how the insects might otherwise aggregate.

Minnesota-Sourced and Minnesota-Acclimated Insects
Eggs, nymphs, and adults were maintained as above; however, eggs originated from a laboratory colony at the University of Minnesota which had been maintained since 2012. Additionally, adults were transferred from outdoors to either an unheated shed or a walk-in cooler on 30 October 2014. Mean walk-in cooler temperature ± standard error of the mean was 4.52°C ± 0.001, with constant darkness as a constant temperature control.

Virginia-Sourced and Virginia-Acclimated Insects
From June to October in 2012 and 2013, adult *H. halys* were collected from sweet corn and unmanaged trees located on a private farm, Garrett Farms, in Glenvar, VA. In October of each year, a sample of>2,000 adults was collected and placed in artificial overwintering habitats that consisted of eight plastic 18.9-liter buckets (Encore Plastics Corp., Byesville, OH) packed tightly with 12.7-mm-thick foam pipe insulation (Thermwell Products, Inc. Mahwah, NJ). A 10-cm-diameter ventilation hole was made into the side of each bucket, and covered with screen to prevent insect escape. Buckets were maintained outdoors in Blacksburg, VA (37° 12.417′ N, 80° 35.513′ W, elevation 616 m) throughout the winter. This approach allowed for access to a population of insects that were exposed to ambient winter temperatures.

West Virginia-Sourced and Virginia-Acclimated Insects
On 1 November 2012, overwintering adult *H. halys* were collected from a private facility in the panhandle of West Virginia and shipped overnight to Blacksburg, VA. They were maintained in the same artificial overwintering habitat as described above.

Cold Tolerance Strategy and Supercooling Point Testing Methods

Minnesota
Supercooling points and lower lethal temperatures were measured by using contact thermocouple thermometry where individual adults were placed in close proximity to coiled copper-constantan thermocouples (e.g., Hanson and Venette 2013) that were attached to a multichannel data logger (USB-TC, Measurement Computing, Norton, MA). Temperatures were recorded once per second and logged by using Tracer-DAQ software (Measurement Computing, Norton, MA). We identified the lowest temperature reached before the exotherm, or spontaneous release of heat indicative of a phase change from liquid to solid, to denote an individual's supercooling point.

In 2013, the insect and thermocouple were confined in a 20- or 35-ml syringe (Monoject syringes with leur lock tip) that was placed at the center of a 20- by 20- by 20-cm polystyrene cube and then into a −80°C freezer where the insects cooled at a realized rate of −0.82°C (± 0.008°C) per minute according to Carrillo et al. (2004). Supercooling points from 14 males and 14 females in fall (9–10 October 2013), and 10 males and 9 females in winter (7–9 December 2013) were measured. Additionally, on 7–9 December 2013, for the determination of cold tolerance strategy, 85 Virginia-sourced, Minnesota-acclimated adult *H. halys* were randomly assigned to one of the five temperature treatments (−20, −15, −10, −5°C, or 25°C), so $n = 17$ adults per temperature. Insects were cooled until they reached the desired temperature as tracked though the above thermocouple method, removed immediately, and allowed to warm to room temperature. After warming, insects were transferred to individual plastic cups provisioned with water and dry organic soybean seeds and monitored daily for mortality for four days. One day after treatment, all insects which were going to recover from chilling had done so and mortality measurements from one day after treatment were used for statistical analysis. Mortality

of the insect was defined as a lack of any movement after being gently prodded with a small paintbrush; moribund insects, defined as having the inability to right itself after ∼10 s or the inability to walk, were considered dead for the purposes of analysis.

In 2014, the insect and thermocouple were confined in an 18- by 150-mm (ODxL) Kimax glass test tube, stabilized with one sheet (11.18 by 21.34 cm) of Kimtech delicate task wipers, and a rubber test tube stopper with a 5-mm hole. This apparatus was placed in a refrigerated bath of silicon 180 oil (Thermo Fischer Scientific A40, Waltham, MA) at room temperature and chilled at a rate of −0.95°C (± 0.003°C) per minute. Eighty-five Minnesota-sourced, Minnesota-acclimated insects were chilled according to the above methods for cold tolerance strategy on 10 December 2014.

Virginia

Starting in June of 2012 samples of 10 male and 10 female *H. halys* adults were tested at ∼2-mo intervals throughout the year to determine supercooling point. Supercooling points were determined by placing adult insects on an apparatus where a copper-constantan thermocouple (Omega Technologies, Stamford, CT) was placed on the ventral and dorsal side of each insect. Weighted aluminum blocks were used to apply pressure and ensure contact between the insect and the thermocouple. Additionally, a small amount (<0.1 ml) of thermal grease (zinc oxide) was placed on the tip of the thermocouple to assist with any gaps in contact. DaqView (MC Measurement Computing, Norton, MA) measured temperatures generated from a 50/50 water and ethanol mixture in a refrigerated water bath (Fisher Scientific Isotemp, Waltham, MA) in circulation with a cold plate (Stir-Kool Cold Plate [SK-31], Thermoelectrics Unlimited, Wimington, DE). This setup was able to cool the plate down to −28°C. The arena with insects was placed on the cold plate with a series of foam and aluminum blocks to reduce the temperature around 0.3°C per minute until the exotherm occurred (Bentz and Mullins 1999).

Field Temperature Observations

In Minnesota, air temperatures were recorded at 15-min intervals from 18 October 2013 to 5 March 2014 with an Hobo U12 4-External channel outdoor/industrial data logger (Onset Computing, Bourne, MA) and 30 October 2014 to 26 February 2015 via an U12 Temp/RH/2 External Channel Logger (Onset Computing, Bourne, MA). Temperature probes or loggers were placed next to insect cages in an unheated shed in Minnesota.

In Virginia, minimum daily air temperatures were collected from a NOAA weather station that was 3.75 km from where the insects were stored.

Statistics
Cold Tolerance Strategy

All statistics were run using R version 3.2.0 (R Core Team 2014) in RStudio version 0.98.1102 (RStudio 2014) and for all analyses an α value of 0.05 was used. Modified "survival curves," where temperature substituted for time, were created to describe the probability that *H. halys* acclimated in Minnesota and tested in winter would freeze or die at a particular temperature. Our analyses formally considered censoring (i.e., incomplete information about supercooling points or lethal temperatures) of individuals in the study. Insects that died were interval censored because death occurred between room temperature and the temperature at which the insects were removed. Insects that remained alive were considered right censored (i.e., they could survive the coldest temperature to which they were

exposed but would likely die if exposed to a colder temperature). Insects which did not freeze were right censored, as they would be expected to freeze at a temperature colder than when they were removed from chilling. The following R packages were used to estimate survival functions with censored data: *survival* (Therneau 2015) to create a survival object, and *interval* (Fay and Shaw 2010) to calculate the nonparametric maximum likelihood estimate for the distribution from interval censored data. Curves were fitted to the binomial data for mortality and cumulative supercooling points. A Kaplan–Meier–Turnbull nonparametric model was used for both years. Curves for freezing and mortality were compared within years via the *icfit* command from the interval package.

Supercooling Point Comparisons

Some of our supercooling points seemed unusually warm, which could occur if a water-bearing substance (e.g., feces) triggered exogenous ice formation as the insect was cooled. We removed any supercooling point which was more than two standard deviations warmer than the overall mean observed for the entire data set. Seven observations ranging from −3.27 to −2.16°C were removed, and the remaining data ($n = 188$ adults) were used for subsequent analyses.

Supercooling points for *H. halys* that were collected from West Virginia and Virginia and acclimated in Virginia were compared. A Shapiro–Wilk test for normality of residuals (W = 0.99; df = 11, 86; $P = 0.90$) and a Levene test for homogeneity of variance across groups ($F = 0.99$; df = 11, 86; $P = 0.46$) confirmed no violations of ANOVA assumptions, so a fully crossed three-factor analysis of variance was performed on untransformed data with location, season, and sex as main effects. This ANOVA revealed no significant main effects of location ($F = 2.40$; df = 1, 86; $P = 0.13$) nor interaction effects between season and location ($F = 2.32$; df = 2, 86; $P = 0.10$), sex and location ($F = 1.61$; df = 1, 86; $P = 0.21$), or the three-way interaction between season, sex, and location ($F = 0.41$; df = 2, 86; $P = 0.66$); therefore, data for individuals originally from West Virginia and Virginia and acclimated in Virginia were combined for analysis of season, sex, and acclimation location.

In all analyses, season was defined by the month the insect was tested and followed standard climatological definitions: Spring = April or May; summer = June, July, or August; fall = September, October, November; winter = December or February. Acclimation location was either Blacksburg, VA (37° 12.417′ N, 80° 35.513′ W, elevation 616 m) or St. Paul, MN (44° 59′18.9672″ N, −93° 10′51.06″ W, elevation 299 m). Where geographic origin was considered, location was determined by where eggs were laid in the field, either Blacksburg, VA (37° 12.417′ N, 80° 35.513′ W, elevation 616 m), or Harper's Ferry, WV (39° 19.31′ N, 77° 44.37′ W, elevation 489 m). Sex was determined by visual inspection of the genitalia on the posterior ventral surface of the insect's abdomen.

Season, Sex, and Acclimation Location Effect on Supercooling Points

To test the hypotheses that season, sex, and acclimation location affect cold hardiness, we ran a fully crossed three-factor ANOVA (season × sex × location) of supercooling points from Minnesota and Virginia adults from fall and winter. The data met assumptions of normality (Shapiro–Wilk W = 0.98; df = 9, 111; $P = 0.12$) but not homoscedasticity (Levene's test: $F = 2.03$; df = 9, 111; $P = 0.04$). A Box-Cox transformation ($y_\lambda = (y^\lambda - 1) / \lambda$) where $\lambda = 2.0$ was used and all analyses were done on transformed data. Sex and all interactions were not significant and were pooled for this analysis. Despite

the interaction of season and acclimation location being nonsignificant, we were still interested in comparing mean supercooling points between states and seasons to test the hypothesis that season matters to acclimation, and acclimation location matters to mean supercooling points. Tukey's HSD was used to determine the significance of each pairing of interest.

Geographic Origin Effect on Supercooling Points

A third hypothesis, that geographic origin matters to cold hardiness, was tested with a fully crossed three-factor analysis of variance (origin × season × sex) for adults that came from eggs laid either in West Virginia or Virginia but were all acclimated as adults in Virginia then tested in fall, winter, and spring. The data satisfied assumptions of homoscedasticity (Levene's test [$F = 1.35$; df = 11, 81; $P = 0.21$]) and normality (Shapiro–Wilk [$W = 0.99$; df = 11, 81; $P = 0.50$]). The interaction of season and sex was significant ($F = 3.37$; df = 2, 81; $P = 0.04$), so sexes were not pooled. Tukey's HSD was used to determine the significance of season by sex by acclimation location.

Predicted Field Mortality

Predicted field mortality in Minnesota was determined through the survival analysis as described above. Where only supercooling points have been measured and not mortality, an estimate of mortality can still be predicted from the cumulative distribution of supercooling points. With a chill-intolerant species, we can be confident that individuals that had frozen would be dead, even though mortality likely occurred before freezing began. Consequently, mortality estimates that are based on the cumulative relative frequency of supercooling points (i.e., the cumulative proportion of individuals that gave an exotherm when cooled to a specific temperature) would predict a species to be able to survive colder temperatures than in reality. This risk averse estimate is still useful; however, especially in cases when only supercooling points are known, because observed mortality is unlikely to be less than predicted mortality (unless insects are in a microhabitat which is not exposed to the recorded air temperatures). Based on the cumulative relative frequency of supercooling points for adult *H. halys* acclimated as adults in Virginia and tested in winter, we fit a three-factor Weibull curve which can be used to forecast field mortality in Virginia.

Results and Discussion

Cold Tolerance Strategy

Until now, the specific effects of cold temperatures on *H. halys* were unknown. Contrary to our original hypothesis that *H. halys* would be freeze intolerant, able to survive all temperatures up until the point of freezing, we found that *H. halys* is a chill-intolerant species; adults died at significantly warmer temperatures than they froze in 2013 ($Z = 2.50$, $P = 0.01$) and 2014 ($Z = 3.99$, $P < 0.001$; Fig. 1). In the course of our lower lethal temperature experiments, we found that no individual survived if it froze (Table 1), ruling out freeze tolerance as a strategy. In the groups of insects that were cooled to −5 or −10°C, no individuals froze but a portion of the insects died (Table 1), further supporting our assessment that *H. halys* is chill intolerant. Although the temperatures that caused *H. halys* to freeze or die in Minnesota varied between years, the cold tolerance strategy, i.e., chill intolerance, remained the same.
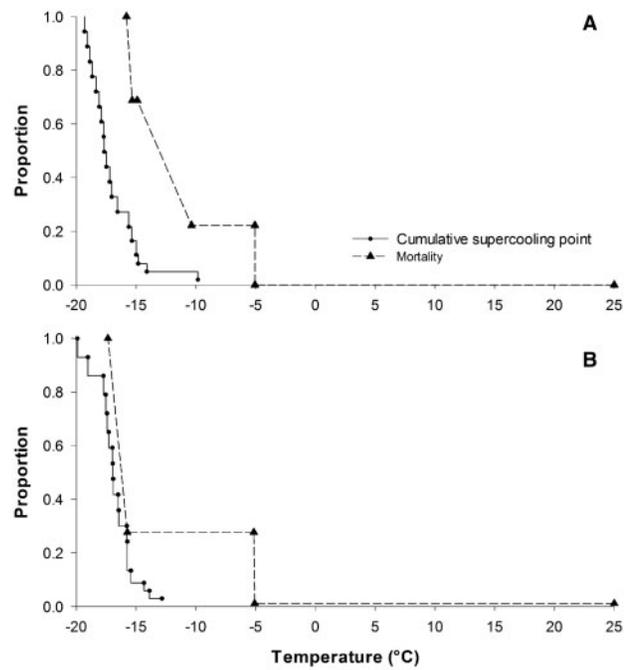

Fig. 1. Observed cumulative supercooling point distribution and mortality distribution for field-acclimated adult *H. halys* in Minnesota in (**A**) December 2013 and (**B**) December 2014. Extrapolation between observed data calculated with right and interval censored survival analysis.

Season, Sex, and Acclimation Location Effects on Supercooling Points

Halyomorpha halys mediates exposure to cold in a variety of ways. Previous research demonstrated that *H. halys* enters diapause, aggregates, and seeks shelter. Our work highlights an additional means by which *H. halys* reduces exposure to lethal temperatures, by acclimating seasonally and thus lowering the temperatures which would result in mortality. Seasonal collections of adults in Minnesota and Virginia showed that supercooling points changed with season (two-way ANOVA, $F = 63.03$; df = 1, 111; $P < 0.001$). Mean supercooling points (±SEM) were relatively high in even in winter. Pooling Minnesota and Virginia supercooling points, the means were −9.43 ± 0.42°C in summer, −15.40 ± 0.43°C in fall, and −16.11 ± 0.37°C in winter. Supercooling points of adults acclimated in Minnesota and Virginia were significantly lower in fall and winter than summer (Fig. 2) which supports a seasonal acclimation to cold temperatures that is typical of many temperate insects (Bale and Hayward 2010).

Other effects of cold changed based on acclimation location. For example, supercooling points significantly changed according to acclimation location (two-way ANOVA, $F = 28.74$; df = 1, 111; $P < 0.001$). Mean supercooling point (±SEM) across seasons in Virginia was −10.86 ± 0.40°C, and in Minnesota −16.93 ± 0.23°C. We found that *H. halys* acclimated in Minnesota became more cold tolerant earlier in the year as compared with those acclimated in Virginia, likely due to photoperiodic and temperature differences between latitudes (Fig. 2).

Our simultaneous comparison of the effects of season and acclimation location show that beginning in summer, we saw a steady reduction in supercooling points by season in Virginia (Fig. 2). In Minnesota, we saw no change in supercooling points from fall to winter. In fall, individuals acclimated as nymphs and adults in Virginia (−13.06 ± 0.14°C) had warmer mean supercooling points

Table 1. Proportion mortality ± SEM of adult *H. halys* acclimated outdoors in Minnesota in 2013 and 2014 and exposed to one of five temperatures. Numbers in parentheses indicate the total number of adults that were either chilled (unfrozen) or frozen upon reaching the target temperature

Temperature (°C)	2013		2014	
	Chilled	Frozen	Chilled	Frozen
−20	− (0)	1.00 ± 0.00 (17)	1.00 ± 0.00 (1)	1.00 ± 0.00 (16)
−15	0.64 ± 0.12 (14)	1.00 ± 0.00 (3)	0.13 ± 0.08 (16)	1.00 ± 0.00 (1)
−10	0.18 ± 0.09 (17)	− (0)	0.29 ± 0.11 (17)	− (0)
−5	0.18 ± 0.09 (17)	− (0)	0.24 ± 0.10 (17)	− (0)
25 (control)	0.00 ± 0.00 (17)	− (0)	0.00 ± 0.00 (17)	− (0)
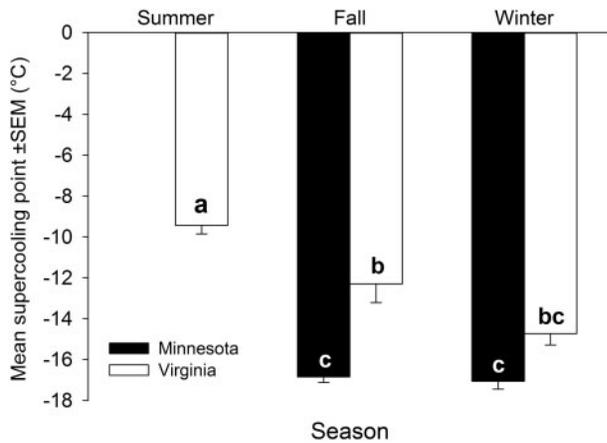
Fig. 2. Mean supercooling points of adult *H. halys* field-acclimated either in Virginia or Minnesota. Individuals remained outdoors, experiencing temperature and photoperiodic cues, until the time of testing. Error bars indicate standard error of the mean. Statistics were run on transformed data and bars with the same letter are not statistically different (*P* > 0.05; Tukey's HSD).

than in Minnesota (−16.85 ± 0.08°C). No statistical difference was found in winter between Virginia (−13.90 ± 0.09°C) and Minnesota (−17.06 ± 0.13°C). Sex did not significantly impact the supercooling point (two-way ANOVA, $F = 0.11$; df = 1, 111; $P = 0.73$), and no significant two- or three-way interactions occurred between location, season, and sex (Fig. 2).

Geographic Origin Effect on Supercooling Points

The supercooling points of adult *H. halys* collected in West Virginia and Virginia and held outside in Virginia were not different (two-way ANOVA, $F = 1.21$; df = 1, 81; $P = 0.27$). This supports our hypothesis that acclimation location, not geographic origin, has a stronger effect on the cold tolerance of *H. halys*. Supercooling points for individuals from both locations changed seasonally (two-way ANOVA, $F = 3.87$; df = 2, 81; $P = 0.02$), though this effect was not the same for both sexes (two-way ANOVA, interaction of sex and season: $F = 3.37$; df = 2, 81; $P = 0.04$). The sex of the insect did not have a consistent effect on supercooling point (two-way ANOVA, $F = 3.83$; df = 1, 81; $P = 0.05$; Fig. 3).

Females from the eastern United States exhibited less seasonal change than males, which suggests that once acclimated in fall, females do not continue to acclimate, but males do. West Virginia males in the fall had the warmest mean supercooling points (−7.93 ± 1.93°C) and Virginia males in winter had the coldest (−15.72 ± 0.56°C). Mean supercooling points for all other season,
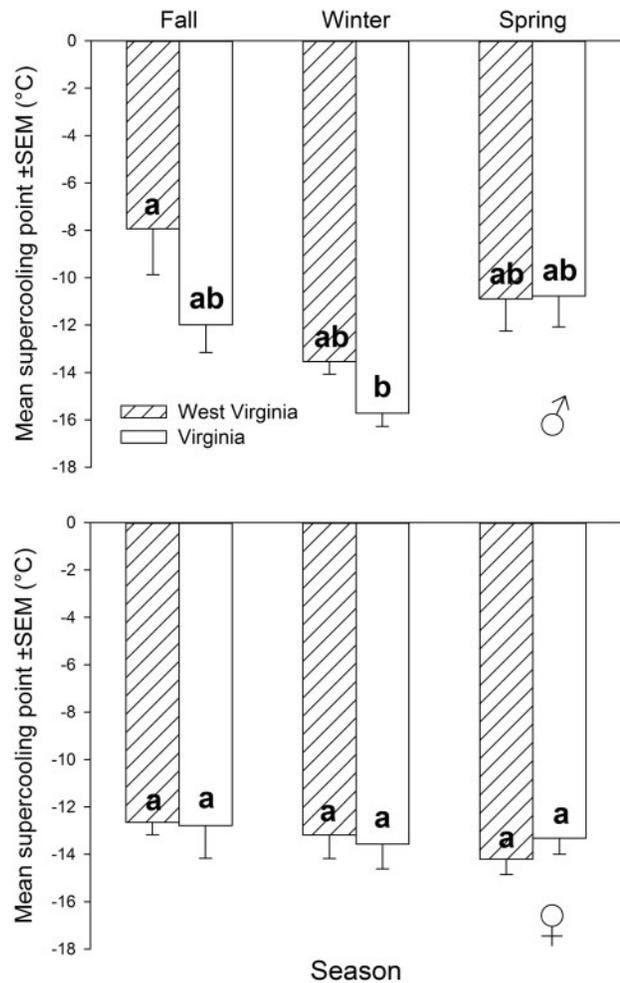

Fig. 3. Mean supercooling points of *H. halys* adults originating from either West Virginia or Virginia and field-acclimated as adults in Virginia (Top panel) male, (Bottom panel) female. Individuals remained outdoors, experiencing temperature and photoperiodic cues, until the time of testing. Error bars indicate standard error of the mean. Across both panels bars with the same letter are not statistically different (*P* > 0.05; Tukey's HSD).

sex, and geographic location combinations were not significantly different from each other (Fig. 3).

No interactions occurred between geographic origin and season ($F = 1.12$; df = 2, 81; $P = 0.33$), origin and sex ($F = 1.84$; df = 1, 81; $P = 0.16$), or between origin, season, and sex ($F = 0.37$; df = 2, 81; $P = 0.69$). In each season, the mean SCPs between West Virginia and Virginia were statistically equivalent (Fig. 3).

Predicted and Observed Field Mortality of *H. halys*

Supercooling points are relatively easy to measure and give clear results, so they are often a starting point for cold hardiness experiments when specimens are limited (Morey et al. 2012). The cumulative frequency distribution of supercooling points can also provide estimates of mortality for a chill-intolerant or freeze-intolerant species after acute exposure to a specified temperature. It should be noted, however, that these forecasts do not apply to chronic exposures to cold nor do they account for other causes of winter mortality such as starvation or desiccation. For chill-intolerant species, we know that the estimate will be biased, consistently forecasting less mortality (i.e., greater survivorship) than will be observed (Fig. 4). Despite this, forecasts from a cumulative supercooling point curve can still prove to be useful when making conservative estimates of winter mortality (Table 2).

Using field temperature data, laboratory-determined cumulative frequency distribution of supercooling points from *H. halys* in Virginia (Fig. 1), and laboratory mortality measurements from *H. halys* in Minnesota (Fig. 4), we accurately predicted field mortality in Minnesota and Virginia before and after low temperature
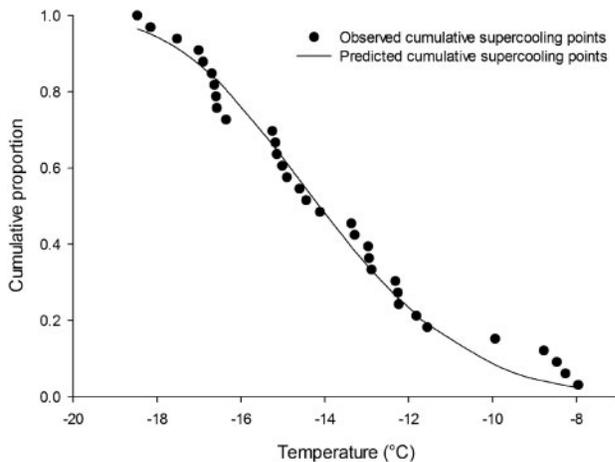

Fig. 4. Predicted and observed cumulative supercooling point distribution for field-acclimated adult *H. halys* in Virginia in winter. Predicted curve calculated with a Weibull distribution.

events (Table 2). In December 2014, the expected mortality for overwintering *H. halys* in an unheated shed in Minnesota was greater numerically than our observed mortality; however, observed mortality at this time falls within the 95% confidence interval for expected mortality (0–44%). Our laboratory measurements provided an accurate way to forecast winter field mortality of unheated *H. halys* aggregations in Minnesota and Virginia; however, *H. halys* utilizes an array of overwintering habitats. The tendency of *H. halys* to aggregate in thermally protected areas, such as human-made structures, means the unheated microclimates into which our experimental insects were placed are not representative of the whole population. Following the extreme low temperatures of the polar vortex of 2014, *H. halys* were still present in the summer of 2014 (Herbert 2014). This suggests that while lethal temperatures were reached across much of the United States, exposure to those temperatures did not occur across the population. Refugia from cold temperatures for *H. halys* are likely contributing to the northern geographic range expansion and ability to survive winter temperatures in North America. Further data should be gathered on a range of overwintering microclimate environments to determine the exposure of *H. halys* to cold temperatures and to better forecast overall overwintering mortality.

In 2014, a subset of insects were maintained in a walk-in cooler as a control group. The temperature in the walk-in cooler was below *H. halys* lower developmental threshold (14.17°C) (Nielsen et al. 2008) and never reached a point at which we expected mortality; however, in December and January, we observed 5 and 15% mortality, which was greater than the 0% (0–4%; 95% CI) that was expected when the coldest temperature these insects experienced was 4.3°C (Table 2). This indicates that other factors, possibly desiccation or starvation, are contributing to overwintering mortality in addition to cold temperatures. Future directions could include questions about multiple stressors and their effects on overwintering mortality. Additional factors such as nutrition (Gash and Bale 1985), rate of cooling (Baust and Rojas 1985), life stage (Lee 1991), and temperature fluctuations (Salt 1961) can also contribute to cold tolerance and could be investigated in future studies.

Diapause can enhance insect cold tolerance but does not always necessarily do so (Denlinger 1991). *Halyomorpha halys* is known to go into a reproductive diapause before overwintering (Niva and

Table 2. Observed and expected adult *H. halys* winter mortality based on minimum temperatures reached in Minnesota and Virginia in the winters of 2013–2014 and 2014–2015.

		2013		2014	
		MN unheated shed	VA unheated 19-liter bucket	MN unheated shed	MN walk-in cooler
Dec.	Min. temp. reached (°C)	5.5	15.0	−12.09	4.30
	Expected mort. (95% CI)[a]	0.0% (0–4%)	0.0%	27.7% (0–44%)	0.0% (0–4%)
	Observed mort.[b]	0.01 %	N/A	0.0%	5.0%
	Sample size	86	N/A	85	20
Jan.	Min. temp. reached (°C)	−22.6	−20.7	−17.86	4.35
	Expected mort. (95% CI)[a]	99% (53–100%)	99%	99% (17–100%)	0.0% (0–4%)
	Observed mort.[c]	100%	97.6%	100%	15.0%
	Sample size	92	2,654[d]	20	20

[a] Based on the minimum temperature reached before testing dates from each location and the mortality curves from Fig. 1 for Minnesota (MN) and Fig. 3 for Virginia (VA).

[b] Mortality determined on 6 December 2013 and 10 December 2014 in Minnesota.

[c] Mortality determined on 6 January 2014 and 10 January 2015 in Minnesota and 12 February 2014 in Virginia.

[d] This large sample size was possible in Virginia where *H. halys* is abundant.

Takeda 2003, Nielsen and Hamilton 2009). However, nothing is known about the relationship between diapause and cold hardiness in *H. halys*. A limitation of our studies was that no dissections were done to positively determine if individuals were in diapause when being tested. Nevertheless, because *H. halys* is predicted to be univoltine in Mid-Atlantic states, we believe that, by the time winter testing occurred, insects would have received the cues needed to enter diapause (Nielsen and Hamilton 2009). More work is needed to understand how and when *H. halys* enters diapause and what effects, if any, diapause has on *H. halys* overwintering capabilities.

Our work provides new insight into the exposure and effects of cold temperatures on *H. halys*. Studying cold stress on this species has the potential to illuminate new modes of management. *Halyomorpha halys* has become a severe pest in parts of North America. This exotic invasive species causes severe economic damage to many crops and seeks shelter, sometimes in homes, to overwinter, making it an agricultural pest a well as a structural and nuisance pest. A lack of exposure to lethal temperatures when *H. halys* overwinters in thermally buffered areas not only affects the potential geographic range of *H. halys* but also enlarges management problems and solutions beyond typical agricultural settings. More studies are needed to: quantify the proportion of *H. halys* in specific types of overwintering sites, model the relationship between those microhabitats and the reported air temperature, and investigate other stressors leading up to and through winter that may contribute to mortality.

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References Cited

Bale, J. S. 1991. Insects at low temperature: A predictable relationship? Funct. Ecol. 5: 291–298.

Bale, J. S., and S. A. L. Hayward. 2010. Insect overwintering in a changing climate. J. Exp. Biol. 213: 980–994.

Baust, J. G., and R. R. Rojas. 1985. Review—Insect cold hardiness: Facts and fancy. J. Insect Physiol. 31: 755–759.

Bentz, B. J., and D. E. Mullins. 1999. Ecology of mountain pine beetle (Coleoptera: Scolytidae) cold hardening in the Intermountain West. Environ. Entomol. 28: 577–587.

Bergmann, E., K. M. Bernhard, G. Bernon, M. Bickerton, S. Gill, C. Gonzales, G. C. Hamilton, C. Hedstrom, K. Kamminga, C. Koplinka-Loehr, et al. 2015. Host plants of the brown marmorated stink bug. (http://www.stopbmsb.org/where-is-bmsb/host-plants last accessed on 29 December 2015).

Bradshaw, W. E. 2010. Insects at not so low temperatures: Climate change in the temperate zone and its biotic consequences, pp. 242–275. *In* D. L. Denlinger and R. E. Lee (eds.), Low temperature biology of insects. Cambridge Univeristy Press, New York, NY.

Carrillo, M. A., N. Kaliyan, C. A. Cannon, R. V. Morey, and W. F. Wilcke. 2004. A simple method to adjust cooling rates for supercooling point determination. CryoLetters 25: 155–160.

Denlinger, D. L. 1991. Relationship between cold hardiness and diapause, pp. 174–198. In R. E. Lee and D. L. Denlinger (eds.), Insects at low temperature. Cambridge Univeristy Press, New York, NY.

Fay, M. P., and P. A. Shaw. 2010. Exact and asymptotic weighted logrank tests for interval censored data: the interval R package. J. Stat. Softw. 36: 1–34.

Gash, A. F., and J. S. Bale. 1985. Host plant influences on supercooling ability of the black-bean aphid. CryoLetters 6: 297–304.

Hanson, A. A., and R. C. Venette. 2013. Thermocouple design for measuring temperatures of small insects. CryoLetters 34: 261–266.

Hanson, A. A., R. C. Venette, and J. P. Lelito. 2013. Cold tolerance of Chinese emerald ash borer parasitoids: *Spathius agrili* Yang (Hymenoptera: Braconidae), *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae), and *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae). Biol. Control 67: 516–529.

Herbert, D. A. 2014. Virginia Ag Pest and Crop Advisory. (https://blogs.ext.vt.edu/ag-pest-advisory/bmsb-and-kudzu-bug-update-sept-4-2014/ last accessed on 29 December 2015).

Holtz, T., and K. Kamminga. 2010. Qualitative analysis of the pest risk potential of the brown marmorated stink bug (BMSB), *Halyomorpha halys* (Stål), in the United States. Plant Epidemiology and Risk Analysis Laboratory of the Center for Plant Health Science and Technology (USDA-APHIS). (https://www.michigan.gov/documents/mda/BMSB_Pest_Risk_Potential_-_USDA_APHIS_Nov_2011_344862_7.pdf last accessed on 29 December 2015).

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

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

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

Lee, R. E., and D. L. Denlinger. 2010. Rapid cold-hardening: Ecological significance and underpinning mechanims, pp. 35–58. *In* D. L. Denlinger and R. E. Lee (eds.), Low temperature biology of insects. Cambridge University Press, New York, NY.

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

Lee, D.-H., J. P. Cullum, J. L. Anderson, J. L. Daugherty, L. M. Beckett, and T. C. Leskey. 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.

Morey, A. C., W. D. Hutchison, R. C. Venette, and E. C. Burkness. 2012. Cold hardiness of *Helicoverpa zea* (Lepidoptera: Noctuidae) pupae. Environ. Entomol. 41: 172–179.

Nielsen, A. L., and G. C. Hamilton. 2009. Life history of the invasive species *Halyomorpha halys* (Hemiptera: Pentatomidae) in northeastern United States. Ann. Entomol. Soc. Am. 102: 608–616.

Nielsen, A. L., G. C. Hamilton, and D. Matadha. 2008. Developmental rate estimation and life table analysis for *Halyomorpha halys* (Hemiptera: Pentatomidae). Environ. Entomol. 37: 348–355.

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

Northeastern IPM Center. 2015. State-by-State. (http://www.stopbmsb.org/where-is-bmsb/state-by-state last accessed on 29 December 2015).

R Core Team. 2014. R: A language and environment for statistical computing. Vienna, Austria. (http://www.r-project.org/ last accessed on 29 December 2015).

RStudio. 2014. Integrated development environment for R, Version 0.98.1102. (http://www.rstudio.org/ last accessed on 29 December 2015).

Salt, R. W. 1961. Principles of insect cold-hardiness. Annu. Rev. Entomol. 6: 55–74.

Therneau, T. M. 2015. A package for survival analysis in S, Version 2.38. (http://cran.r-project.org/package=survival last accessed on 29 December 2015).

U.S. Apple Association News Release. 2011. Losses to Mid-Atlantic apple growers at \$37 million from brown marmorated stink bug. (http://www.usapple.org/index.php?option=com_content&view=article&id=160:bmsb-loss-midatlantic&catid=8:media-category last accessed on 29 December 2015).

Venette, R. C., D. J. Kriticos, R. D. Magarey, F. H. Koch, R. H. A. Baker, S. P. Worner, N. N. Gómez Raboteaux, D. W. McKenney, E. J. Dobesberger, D. Yemshanov, et al. 2010. Pest risk maps for invasive alien species: A roadmap for improvement. Bioscience 60: 349–362.

Watanabe, M., K. Kamimura, and Y. Koizumi. 1978. The annual life cycle of *Halyomorpha mista* and ovarian development process. Toyama J. Rur. Med. 9: 95–99.

Watanabe, M., R. Arakawa, Y. Shinagawa, and T. Okazawa. 1994. Anti-invading methods against brown marmorated stink bug, *Halyomorpha mista*, in houses. Anim. Health 45: 25–31.

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