

Effects of Diapause on *Halyomorpha halys* (Hemiptera: Pentatomidae) Cold Tolerance

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Abstract

Diapause and cold tolerance can profoundly affect the distribution and activity of temperate insects. *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), an alien invasive species from Asia, enters a winter dormancy in response to environmental cues. We investigated the nature of this dormancy and its effects on *H. halys* cold tolerance, as measured by supercooling points, lower lethal temperatures, and overwintering field mortality. Dormancy was induced by rearing individuals in the laboratory or under field conditions. We confirmed *H. halys* dormancy to be a state of diapause and not quiescence, and the life stage sensitive to diapause-inducing cues is between the second and fifth instar. In the laboratory, supercooling points of diapausing adults reached significantly lower temperatures than nondiapausing adults, but only when given enough time after imaginal ecdysis. Supercooling points of diapausing adults in overwintering microhabitats also decreased over time. Diapause increased adult survival after acute cold exposure in the laboratory and prolonged cold exposure in the field. Following diapause induction in the laboratory, changes to temperature and photoperiod had no significant effect on lower lethal temperatures and changes to photoperiod had no effect on supercooling points. Additionally, induction of diapause in the laboratory did not result in significantly different cold tolerance than natural field induction of diapause. This work demonstrates that *H. halys* diapause confers greater cold tolerance than a nondiapausing state and likely improves the probability of successful overwintering in some temperate climates. Hence, knowledge of diapause status could be used to refine forecasts of *H. halys* overwintering field mortality.

Key words: brown marmorated stink bug, cold tolerance, dormancy, overwintering ecology, Pentatomidae

Halyomorpha halys (Stål) (Hemiptera: Pentatomidae) has become a serious pest since it spread from its native range in East Asia to North and South America and Europe (Hoebeke and Carter 2003, Wermelinger et al. 2008, Faúndez and Rider 2017). In invaded areas, many plants, including apples, corn, soybeans, and ornamentals, are at risk of *H. halys* damage (Rice et al. 2014, Bergmann et al. 2016). Additionally, *H. halys* can become a nuisance pest when adults seek overwintering sites in human dwellings (Watanabe et al. 1994, Hoebeke and Carter 2003, Inkleby 2012). In deciduous forests, dead standing trees with loose thick bark were found to be the preferred natural overwintering sites (Lee et al. 2014). Regardless of location, aggregating in protected sites to overwinter allows *H. halys* to cope with thermally unfavorable periods that occur in temperate climates (Cira et al. 2016). In addition, mean temperature in January and February was found to contribute to *H. halys* overwintering survival in the field (Kiritani 2007). Consequently, studying the overwintering ecology of *H. halys* is important for building *H. halys* phenology models (Nielsen et al.

2016) and creating sustainable *H. halys* management programs (Lee et al. 2014).

By winter, *H. halys* adults are said to be in diapause in temperate climates (Watanabe et al. 1978, Nielsen and Hamilton 2009, Nielsen et al. 2017). Diapause is a form of dormancy that often is not the result of adverse conditions, but arises in anticipation of adversity (Sehnal 1991). Environmental triggers are experienced by the brain, or in some cases the prothoracic gland (e.g., Numata and Hidaka 1984) and hormonal changes ensue (for review of diapause regulation see Denlinger 2002). These changes are not immediately reversible and persist beyond the adverse conditions (Danks 1987a). In contrast, quiescence, which is also a form of dormancy, results in a less extreme alteration of morphogenesis than diapause. Quiescence is an immediate response to adverse conditions and normal morphogenesis resumes as soon as favorable conditions return (Danks 1987a, Denlinger 1991, Košťál 2006). *H. halys* winter dormancy has not been explicitly evaluated to determine whether it is a true diapause or a quiescent state. Taylor et al. (2017) collected presumably dormant *H. halys* in autumn

from the field, then stored them at 9°C in complete darkness. After various lengths of time at 9°C individuals were maintained at 25°C to monitor mortality and reproduction. Oviposition did not resume within the time that nondormant individuals would be expected to begin laying eggs at 25°C 16:8 (L:D) h (Nielsen et al. 2008, Haye et al. 2014), however, Taylor et al. (2017) did not report the photoperiod that insects experienced at 25°C. Photoperiod is often the overriding cue controlling dormancy in temperate areas (Danks 1987b). Depending on the photoperiod in this experiment, insects may have remained in dormancy due to that photoperiod rather than morphogenetic changes from diapause. So, while this study offers some information about the nature of *H. halys* dormancy, it is incomplete.

Diapause is a dynamic process that can allow an insect to better cope with adverse conditions, such as cold and starvation, and synchronize development across a population (Denlinger 1991). However, diapause is not always linked to greater cold tolerance (Denlinger 1991). When diapause is induced in an insect, a suppressed developmental pathway is triggered, commonly manifesting as lowered metabolism and cessation of feeding and reproduction (Tauber et al. 1986). Laboratory colonies of *H. halys* maintained at 25°C and a 16:8 (L:D) h photoperiod continuously reproduce (Nielsen et al. 2008), a pattern that suggests these conditions do not induce dormancy and that *H. halys* dormancy is facultative, not obligatory. Previous work has investigated cues that trigger dormancy in *H. halys* in Japan (e.g., Watanabe et al. 1978; Watanabe 1979; Yanagi and Hagihara 1980; Niva and Takeda 2002, 2003), but the relationship between dormancy and *H. halys* cold tolerance is unknown.

Cold tolerance, or the capacity to survive low temperatures (Lee 1991), has previously been characterized for *H. halys* by Cira et al. (2016). They found that *H. halys* supercooling points, or the temperature at which body fluids begin to freeze, differed by sex, season, and the location individuals acclimatized. Individuals likely die before they freeze (Cira et al. 2016), so knowledge of the supercooling point provides insight on the limits to low-temperature survival. Lower lethal temperatures provide an estimate of the extent of mortality after acute exposure to a particular temperature. Supercooling points and lower lethal temperatures, both measured in the laboratory, are indicative of the extent of *H. halys* overwintering mortality in the field (Cira et al. 2016). For other insects, age can influence cold tolerance, but has often been overlooked (Bowler and Terblanche 2008), including for *H. halys*.

The objectives of this study were to determine if *H. halys* winter dormancy qualifies as diapause or quiescence and to evaluate the effects of dormancy and age on *H. halys* cold tolerance. We hypothesized that 1) *H. halys* in dormancy would have lower supercooling points and lower lethal temperatures than those not in dormancy, 2) the conditions (i.e., temperature and photoperiod) under which dormant adults were held and their age could affect acclimation and cold tolerance, and 3) dormancy would increase overwintering survival. We investigated these questions with the purpose of discerning whether inducing dormancy in the laboratory resulted in significantly different cold tolerance than inducing dormancy naturally in the field. It is necessary to understand differences between laboratory and field reared *H. halys* in cold tolerance experiments to accurately extrapolate laboratory data to field scenarios and improve accuracy of winter mortality forecasts based on laboratory studies.

Materials and Methods

Insects

H. halys were from a laboratory colony reared at 25°C and a 16:8 (L:D) h photoperiod at the University of Minnesota (see Cira et al.

2016 for origins and rearing methods) or from a natural field population collected in Wyoming, MN. Under these laboratory rearing conditions, individuals develop and reproduce without entering dormancy. The population in Wyoming, MN reflects the first known reproducing population of *H. halys* in Minnesota where nymphal exuvia were found in 2013 (Koch 2014) and nymphs and adults have been found every year since (T.M.C., personal observation).

Effects of Dormancy, Photoperiod, and Time on Supercooling Points

In February 2014, second instar *H. halys* from the laboratory colony were randomly assigned to one of three rearing regimes (n = number of nymphs from each treatment that survived to adulthood for testing): 25°C and 16:8 (L:D) h photoperiod (n = 64); 20°C and 12:12 (L:D) h photoperiod (n = 26); or 20°C and 8:16 (L:D) h photoperiod (n = 37), and otherwise reared in the same manner as the laboratory colony. Development was monitored so that as adults eclosed they were separated into cohorts of similarly aged individuals (imaginal ecdysis ≤ 7 d apart) while being maintained under the same rearing conditions. Immediately after collecting the last cohort from each rearing condition, after 2 wk for the 20°C regime and 3 wk for the 25°C regime, supercooling points of all adults were measured. This approach allowed us to test individuals from different cohorts (i.e., ages) of the same rearing treatment at the same time. As expected, development rates differed between the two rearing temperatures (Nielsen et al. 2008, Haye et al. 2014), so individuals from different regimes could not be tested simultaneously.

Supercooling points were measured using contact thermocouple thermometry; 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). Each insect and thermocouple was confined within an 18 × 150-mm (OD×L) Kimax glass test tube, stabilized with one sheet (11.18 × 21.34 cm) of Kimtech delicate task wipers, and a rubber test tube stopper with a 5-mm hole. Batches of 16 of these apparatuses at a time were placed in a refrigerated bath of circulating silicon 180 oil (Thermo Fischer Scientific A40, Waltham, MA) at room temperature and cooled at a realized rate of $0.95 \pm 0.003^\circ\text{C}$ (SEM) per minute. Batches of 16 insects were cooled simultaneously. Temperatures were recorded once per second and logged using Tracer-DAQ software (Measurement Computing, Norton, MA). When an exotherm (i.e., spontaneous release of heat indicative of a phase change from liquid to solid) was observed, the lowest temperature reached before the exotherm was recorded as the supercooling point of an individual (Lee 1991).

After supercooling points were measured, dormancy was verified by dissecting females under 8× magnification. Ovary development was characterized as per Watanabe et al. (1978), and we considered ovaries from the IV stage of development (i.e., at least one fully formed oocyte) onward to be developed and indicative of a nondormant state. However, the converse relationship was not true (i.e., immature ovaries alone were not fully indicative of dormancy) because *H. halys* have been found to have a preoviposition period of 118 (Haye et al. 2014) and 148 (Nielsen et al. 2008) accumulated degree days (ADD) using base temperatures 13.0 and 14.2°C, respectively. To ensure that females would have had enough physiological time for ovaries to develop if they were not dormant, we calculated the number of ADD using a lower developmental threshold of 14.2°C, for each cohort from each rearing condition by using the formula: (rearing temperature – 14.2°C) × (number of days from imaginal ecdysis) according to Nielsen et al. (2008).

Individuals in a cohort were assigned the same number of ADDs by taking the mid-point between the lowest and highest ADDs from the cohort. In the 25°C and 16:8 (L:D) h photoperiod a portion of all females from each of the three age cohorts had developed ovaries and were not dormant. Conversely, no females from the two 20°C regimes had developed ovaries and were dormant (data not shown).

We also wanted to compare supercooling points of *H. halys* reared in the field (and likely to be dormant) with those reared in the laboratory for this portion of the study. Second instar *H. halys* from the laboratory colony were placed in mesh cages (38 × 38 × 61 cm BioQuip, Rancho Dominguez, CA) within a larger wire screen enclosure outdoors on the St. Paul campus of the University of Minnesota (44.988266 N, 93.180824 W) in July 2013 until October 2013 (see Cira et al. 2016 for origins and rearing methods). ADD from imaginal ecdysis for field insects was calculated using the single sine-wave method (Synder 2005) where $T_{low} = 14.2^{\circ}\text{C}$ from the date of first imaginal ecdysis to the date of testing. Daily temperatures were taken from a St. Paul weather station (Meteorological ID: USC00218450). Supercooling points were measured as follows: the insect and thermocouple were confined in a 20- or 35-ml syringe (Monoject syringes with leur lock tip), per Hanson and Venette (2013), that was placed at the center of a 20 × 20 × 20 cm polystyrene cube calibrated to cool at approximately $-1^{\circ}\text{C}/\text{min}$ in a -80°C freezer according to Carrillo et al. (2004). Batches of seven insects were cooled at the same time. The realized cooling rate of a subset of insects was measured to be $-0.82 \pm 0.008^{\circ}\text{C}$ per minute. Ovary development was measured as described previously.

Statistical analysis first focused on the effects of photoperiod, ADD, and their interaction on supercooling points for individuals reared at 20°C. All analyses were conducted in R version 3.4.0 (R Core Team 2017) and RStudio Desktop version 1.0.136 (RStudio Team 2016). After a Box-Cox transformation ($y_{\lambda} = [(y^{\lambda} - 1)/\lambda]$; $\lambda = 1.7979$; R package, *command(s)*: MASS, *boxcox*; Ripley and Venables 2002) supercooling points did not violate assumptions of normality (Shapiro-Wilk: $W = 0.97$; $P = 0.15$) or heteroscedasticity (Breusch-Pagan: $\chi^2 = 0.30$; $P = 0.58$; car, *ncvTest*; Fox and Weisberg 2011). An ANOVA performed on transformed data found photoperiod ($F = 2.39$; $df = 1, 59$; $P = 0.12$) and the interaction of photoperiod and ADD ($F = 0.001$; $df = 1, 59$; $P = 0.97$) did not significantly affect supercooling points, but ADD did have an effect ($F = 23.63$; $df = 1, 59$; $P < 0.0001$). Therefore, data for individuals reared at 20°C with the same number of ADD, were combined across photoperiods for future analysis.

A linear mixed effects cell means model (lmerTest, lmer; Kuznetsova et al. 2016) was used to test the effects of six treatments on supercooling points: three ADD from the 25°C laboratory regime, two ADD from the combined 20°C laboratory regimes, and one ADD from the field rearing regime. Batch number (i.e., the set of insects cooled at the same time) was included in the model as a random effect. As explained previously, not all treatments were represented in each batch, but 3–6 batches, or replicated measurements, of individual treatments occurred. Supercooling points were squared and transformed values did not violate assumptions of normality ($P > 0.01$) (Shapiro-Wilk test: $W = 0.99$; $P = 0.67$; RVAideMemoire, *plotresid*; Hervé 2016) or heteroscedasticity (Levene Test: $F = 2.61$; $df = 5, 149$; $P = 0.03$; car, *leveneTest*; Fox and Weisberg 2011). Tukey's HSD (multcomp, *cld*, *glht*; Hothorn et al. 2008) was used to determine significant differences ($\alpha = 0.05$) among treatments.

Effects of Dormancy and Time on Supercooling Points, Mass, and Overwintering Survival

To establish a baseline of comparison for laboratory measurements, we caught individuals in the field that were likely dormant

and preparing to overwinter. Wild insects were gathered from the exterior of a residence in Wyoming, MN. Before testing insects were maintained outdoors in circular, ventilated plastic dishes (18.5 cm diameter × 8 cm; Pioneer Plastics, Inc., North Dixon, KY) with a 25 × 89 cm piece of cotton canvas and dry organic soybean seeds. Mortality, supercooling points, mass, and ovary development were measured on 18 October 2014, for insects collected between 16 October and 18 October 2014, and on 6 November 2014, for insects collected 19 October to 5 November 2014.

Laboratory insects were reared as follows: second instars from the laboratory colony were reared in the field where dormancy was naturally-induced, or in the laboratory under standard non-dormancy-inducing conditions. Adults that had matured in the field or the laboratory were placed into circular plastic dishes (as described above) in October 2014 and randomly assigned to one of two overwintering habitats: 1) an unheated shed in St. Paul, MN (44.98908 N, 93.18628 W) with constant darkness mimicking a cold overwintering microhabitat (dormant $n = 145$, nondormant $n = 60$), or 2) a walk-in cooler at a mean $4.5^{\circ}\text{C} \pm 0.001$ (SEM) with constant darkness (dormant $n = 79$, nondormant $n = 58$) to mimic a cool but not cold overwintering microhabitat. Temperature in each location was measured with a Hobo U12 4-External channel outdoor/industrial data logger or a Hobo U12 Temp/RH/2 External Channel Logger (Onset Computing, Bourne, MA). Mortality, supercooling points, mass, and ovary development were measured from groups of 19–85 adults from the field and 19–20 adults from the laboratory. Individuals were pulled from each location monthly from December 2014 to March 2015. Mortality of adults was assessed by placing individuals in the walk-in cooler (4.5°C) for ~10 min, and gently prodding them with a soft-bristle paintbrush. Individuals that did not move were considered dead. At this temperature movement of legs and antennae was possible and declamation was less likely than at warmer temperatures. Supercooling points, mass, and ovaries were only measured from living insects in this study. Dormancy status was assessed by dissection ~24 h after supercooling points were measured, as described above.

To test the effect of time on adult mass of dormant individuals in the walk-in cooler, separate ANOVA models were made for each sex because females are known to weigh more than males (Lee and Leskey 2015). Female mass did not violate assumptions of normality (Shapiro-Wilk: $W = 0.98$; $P = 0.58$) or heteroscedasticity (Breusch-Pagan: $\chi^2 = 0.17$; $P = 0.68$). After a Box-Cox transformation with $\lambda = -0.909$, male mass did not violate assumptions of normality (Shapiro-Wilk: $W = 0.98$; $P = 0.38$) or heteroscedasticity (Breusch-Pagan: $\chi^2 = 1.48$; $P = 0.22$). Each ANOVA was followed by Tukey's HSD tests. To compare the extent of mortality of dormant and nondormant adults in a particular overwintering location each month, Fisher's exact tests were used. No adequate transformation was found to correct for heteroscedasticity of supercooling points, so a Kruskal-Wallis rank sum test was used to test the effect of month on supercooling points of dormant adults held in the walk-in cooler, followed by Dunn's test with a Holm's multiple comparisons adjusted α ($\alpha = 0.05$) (dunn.test, *dunn.test*; Dinno 2017). High levels of mortality of dormant adults in the unheated shed and nondormant adults in the shed and walk-in cooler prevented us from including these treatments in analyses of supercooling points or mass.

Nature of Winter Dormancy and Effects of Maintenance Conditions and Time on Lower Lethal Temperatures of Dormant *H. halys*

Second instars from the laboratory colony were placed at 20°C with a photoperiod of 12:12 (L:D) h to imaginal ecdysis to induce

dormancy. Newly eclosed adults were transferred individually every Monday, Wednesday, and Friday to a lidded plastic cup (Translucent 473 ml, Consolidated Plastics Stow, OH) and randomly assigned to one of three adult maintenance conditions (25°C and 16:8 (L:D) h, 20°C and 12:12 (L:D) h, or 10°C and 12:12 (L:D) h) for 7, 14, or 21 d. The first set of maintenance conditions is known to support ovarian development in adults (Nielsen et al. 2008). Both 14 and 21 d in this rearing regime surpasses the required number of degree days for ovaries to develop (Nielsen et al. 2008, Haye et al. 2014). Thus, the reproductive status of individuals in these treatments will indicate whether *H. halys*' dormancy is reversible, indicative of quiescence, or persists despite favorable conditions, indicative of diapause. Individuals ($n = 31$ –36 for each maintenance condition $\times$ time combination) were provisioned with three dry organic soybean seeds and a cotton ball soaked in water. Soybean seeds were removed and replaced every 3–7 d. Cotton balls were re-wetted as needed. All testing occurred between June 2014 and July 2015 in a completely random design.

To assess lower lethal temperatures an individual was randomly assigned to one of 16 exposure temperatures (every 1°C from -5 to -20°C , inclusive) or a room temperature control, handled in the same way except for exposure to cold temperatures as per Rosenberger et al. (2017). After remaining at their adult maintenance conditions for the assigned length of time, insects were cooled in a refrigerated bath, as described above, until they reached the assigned exposure temperature. They were immediately removed from the refrigerated bath to warm to room temperature, transferred to individual plastic cups, provisioned with soybean seeds and water, and placed at 25°C 16:8 (L:D) h. After 24 h, they were checked for survival. Mortality was defined as described above.

A generalized linear model with a binomial logit link function was used to test the effect of exposure temperature, conditions dormant adults experienced (i.e., maintenance conditions after imaginal ecdysis), length of time at maintenance conditions, and all interactions on proportion mortality. Model parameters were determined through modified backward elimination (i.e., step-wise removal of nonsignificant terms [$P > 0.05$] starting with the term with the highest P -value).

Based on the results of the modified backward elimination, all maintenance conditions and lengths of time at maintenance conditions from this experiment were pooled and compared to lower lethal temperatures after field-induction of dormancy previously collected in December of 2013 and 2014 (reported and analyzed in: Cira et al. 2016) and nondormant adults collected in the following manner. In December 2013, 85 nondormant adults (43 females, 42 males) were taken from the laboratory colony, randomly assigned to one of five exposure temperatures (-20 , -15 , -10 , -5°C or a room temperature control) and cooled in the refrigerated bath as described previously. A generalized linear model with a binomial logit link function followed by a Tukey's HSD test was used to test the effect of exposure temperature, dormancy status, and their interaction.

Results

Effects of Dormancy, Photoperiod, and Degree Days on Supercooling Points

Treatment had a significant effect on supercooling points ($F = 18.67$; $df = 5$; 149.03; $P < 0.0001$). Supercooling points of dormant individuals were significantly lower than nondormant individuals, but only in the two longest ADD treatments (Fig. 1). Supercooling points of individuals with field induction of dormancy were statistically equivalent to individuals with laboratory-induced dormancy, but only in the longest laboratory ADD treatment (Fig. 1).

The mean supercooling point $\pm$ SEM for dormant adults at 20°C maintained at 8:16 (L:D) h was -16.5 ± 0.5 and was not significantly different ($F_{1,61} = 1.75$; $P = 0.19$) from supercooling points of adults maintained at 12:12 (L:D) h (-15.3 ± 0.7).

Effects of Dormancy and Long-Term Cold Exposure on Overwintering Survival

At a constant low, but not freezing temperature (4.5°C) in a walk-in cooler, significantly higher adult mortality was seen for nondormant adults compared with dormant adults in December, January, and February ($P < 0.0001$) (Fig. 2A). No differences in mortality were observed between nondormant and dormant adults that were in an unheated shed, though by December complete mortality was observed

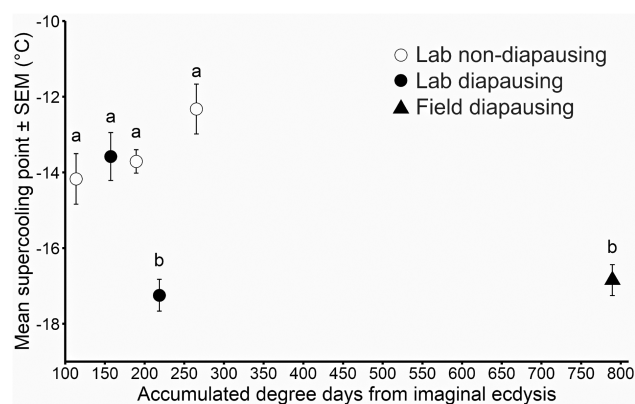


Fig. 1. Supercooling points of diapausing and non-diapausing adult *H. halys* reared in the laboratory or field. ADD were calculated based on a lower developmental threshold for total development of 14.2°C (Nielsen et al. 2008). Different letters indicate significant differences among treatments ($P < 0.05$).

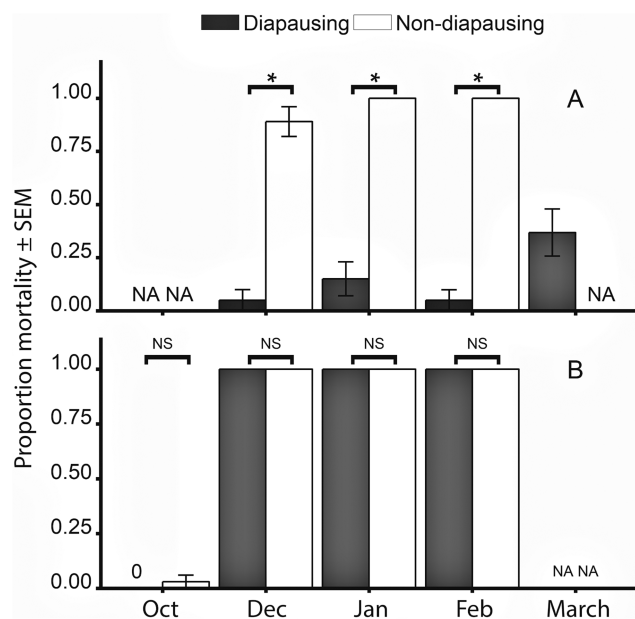


Fig. 2. Overwintering mortality of diapausing and non-diapausing adult *H. halys* in (A) a walk-in cooler (mean temperature $\pm$ SEM; $4.52 \pm 0.001^\circ\text{C}$, complete darkness), or (B) an unheated shed (fluctuating temperatures, complete darkness) in the winter of 2014–2015 in St. Paul, MN. Asterisks indicate significant differences of proportions within a month ($P < 0.05$). NA indicates no individuals were tested and 0 indicates no mortality was observed.

for both groups (Fig. 2B). Minimum, maximum, and median temperatures measured in the unheated shed were -0.20 , 13.40 , and 6.76°C from 30 October 2014 to 6 November 2014 and -12.09 , 8.94 , and -3.36°C from 6 November 2014 to 10 December 2014. No dormant females were found with developed ovaries in any month of testing and the proportion $\pm$ SEM of females with developed ovaries in nondormant treatments was 0.80 ± 0.13 , 0.70 ± 0.14 , 0.80 ± 0.13 in the unheated shed and 0.80 ± 0.13 , 0.70 ± 0.14 , 0.56 ± 0.17 in the walk-in cooler from December, January, and February, respectively.

Effects of Dormancy and Long-Term Cold Exposure on Supercooling Points and Mass

The month of testing ($\chi^2 = 33.28$; $\text{df} = 5$; $P < 0.0001$) significantly affected supercooling points of dormant individuals in the field. Supercooling points in October were significantly higher than all other months (Table 1). Month of testing significantly affected female mass ($F = 4.69$; $\text{df} = 5, 56$; $P = 0.001$), and male mass ($F = 6.53$; $\text{df} = 5, 51$; $P < 0.0001$) though there was not a consistent increasing or decreasing trend for either over time (Table 1). No developed ovaries were found at any date of testing in these field-induced dormant females.

Nature of Winter Dormancy and Effects of Maintenance Conditions and Time on Lower Lethal Temperatures of Dormant *H. halys*

In all three maintenance conditions, for all three lengths of time, we found no females with developed ovaries. The irreversibility of female reproductive activity within 14 and 21 d in optimum conditions indicates that *H. halys* dormancy is a true diapause as opposed to quiescence and will hereafter be referred to as such. Changes to diapausing adults' maintenance conditions, and length of time at those conditions, did not influence the proportion of females with developed ovaries in the subset that was dissected. No females from the colder (10°C 12:12 (L:D) h, $n = 33$), constant (20°C 12:12 (L:D) h, $n = 31$), or warmer (25°C 16:8 (L:D) h, $n = 30$) maintenance conditions had developed ovaries or laid any eggs. All individuals in this experiment were in diapause for the entirety of the experiment.

Exposure temperature was the only significant predictor of mortality ($\chi^2 = 116.63$; $\text{df} = 1$; $P < 0.0001$). As exposure temperature decreased, mortality increased (Fig. 3A–C). Mortality was not affected by maintenance condition ($\chi^2 = 1.09$; $\text{df} = 2$; $P = 0.58$), length of time at maintenance condition ($\chi^2 = 1.42$; $\text{df} = 2$; $P = 0.49$), their interaction ($\chi^2 = 2.46$; $\text{df} = 4$; $P = 0.65$), the interaction of exposure temperature and maintenance condition ($\chi^2 = 2.16$; $\text{df} = 2$; $P = 0.34$), the interaction of exposure temperature and length of

time at maintenance condition ($\chi^2 = 1.03$; $\text{df} = 2$; $P = 0.60$) or the three-way interaction of all main effects ($\chi^2 = 5.07$; $\text{df} = 6$; $P = 0.53$) (Fig. 3A–C). For each maintenance condition and length of time, nearly all control individuals that were held at room temperature ($\sim 23^{\circ}\text{C}$), instead of being chilled, survived handling (proportion survival $\pm$ SEM, 0.99 ± 0.01 [$n = 83$]).

When comparing lower lethal temperatures for field-induced diapause, laboratory-induced diapause, and nondiapausing adults, mortality rates were significantly affected by exposure temperature ($\chi^2 = 186.24$; $\text{df} = 1$; $P < 0.0001$) and diapause status ($\chi^2 = 35.02$; $\text{df} = 2$; $P < 0.0001$), but not their interaction ($\chi^2 = 2.57$; $\text{df} = 2$; $P = 0.28$). Laboratory- and field-induction of diapause resulted in higher survival at significantly lower temperatures than nondiapausing adults (Fig. 4). Predicted mortality was modeled as: $1 / (e^{-b_1 x_1 - b_0} + 1)$ for field-induction of diapause, $1 / (e^{-b_1 x_1 - b_0 + b_2} + 1)$ for laboratory-induction of diapause, and $1 / (e^{-b_1 x_1 - b_0 + b_3} + 1)$ for nondiapausing adults where $b_0 = -4.2996099$, $b_1 = -0.3223392$, $b_2 = -0.4452276$, $b_3 = 1.6829432$, x_1 = temperature in the range of -20 to 5°C . As exposure temperature decreases mortality increases, and mortality occurs at warmer temperatures for nondiapausing *H. halys*.

Discussion

While previous literature refers to *H. halys* winter dormancy as diapause, empirical evidence of this categorization was lacking. Our results definitively distinguish *H. halys* winter dormancy as diapause as opposed to quiescence. This difference is not merely semantic, the biological effects of diapause on growth and development of individuals and populations is far greater than the effects of quiescence. The form and function of diapausing individuals can be drastically different than nondiapausing individuals (Danks 1987c). In the case of *H. halys*, this could have implications for phytosanitary concerns. For example, if aggregations of diapausing *H. halys* are inadvertently shipped to less adverse conditions, for instance from the northern hemisphere to the southern, they will not immediately start reproducing upon reaching this new environment. Yet, diapausing individuals will be better able to survive phytosanitary cold treatments than nondiapausing *H. halys*.

An increased capacity to supercool enhances arthropod cold tolerance (Tauber et al. 1986). It has previously been shown that *H. halys* supercooling points change based on sex, season, and acclimation location (Cira et al. 2016). In the present study, we found that supercooling points of field (Table 1) and laboratory (Fig. 1) diapausing *H. halys* decrease over time, eventually to a significantly

Table 1. Supercooling points (SCP) and mass of diapausing adult *H. halys* reared in the field

Date tested ^a	Median SCP ($^{\circ}\text{C}$): first and third quartile (n)	Mean mass (mg) $\pm$ SEM (n)	
		Adult female	Adult male
18 Oct. 2014	-8.66 : -12.75 , -8.25 (19)a	188.1 ± 10.2 (10)a	129.4 ± 6.0 (9)ab
6 Nov. 2014	-15.29 : -16.68 , -14.06 (34)b	188.0 ± 5.1 (21)a	128.2 ± 4.3 (13)ab
10 Dec. 2014	-16.23 : -17.22 , -15.81 (19)b	166.0 ± 13.9 (9)ab	142.1 ± 9.0 (10)a
10 Jan. 2015	-16.48 : -17.21 , -14.20 (17)b	135.3 ± 8.7 (9)b	106.2 ± 5.0 (7)c
11 Feb. 2015	-16.50 : -16.97 , -15.61 (19)b	170.7 ± 6.5 (9)ab	108.7 ± 2.7 (10)bc
10 Mar. 2015	-17.09 : -17.59 , -16.39 (12)b	178.3 ± 13.1 (4)ab	109.9 ± 4.9 (8)bc

Different letters within a column indicate significant differences among medians or means ($P < 0.05$). No females were found with developed ovaries at any point during this experiment.

^aIndividuals tested in October and November were collected as adults in the field in Wyoming, MN. In all other months, individuals were reared in the field then maintained in a cold room ($4.52^{\circ}\text{C} \pm 0.001$, complete darkness) from October until the testing date.

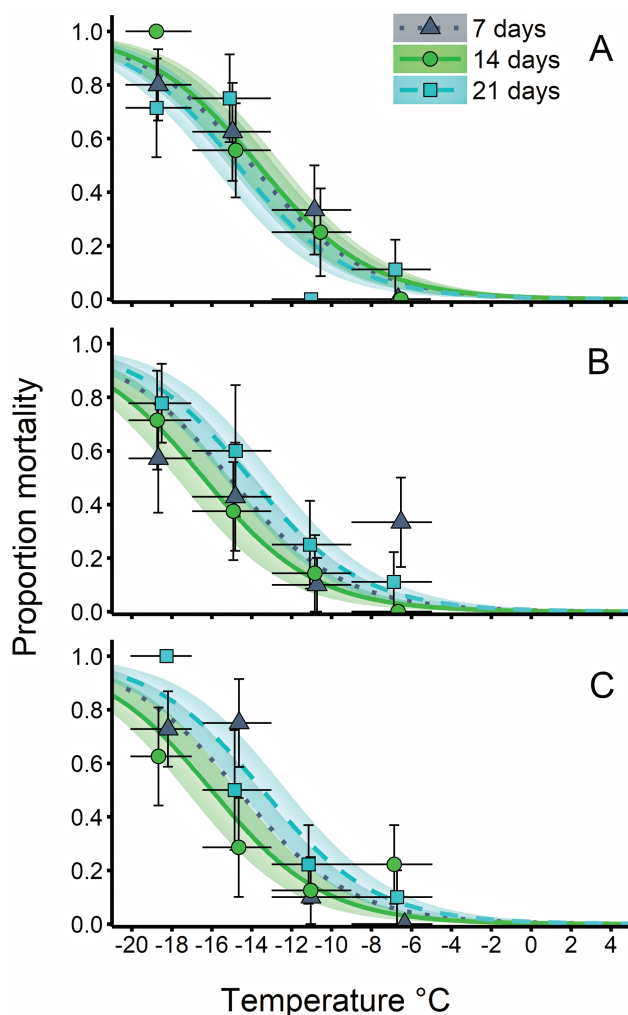


Fig. 3. Lower lethal temperatures of diapausing adult *H. halys*. Individuals were reared at 20°C 12:12 (L:D) h until imaginal ecdysis, then were randomly assigned to one of three adult rearing conditions: (A) 10°C 12:12 (L:D) h, (B) 20°C 12:12 (L:D) h, and (C) 25°C 16:8 (L:D) h and tested at one of three adult ages (7, 14, or 21 d from imaginal ecdysis). Each point represents (A) 7–10 individuals, (B) 5–10 individuals, and (C) 6–11 individuals. Vertical bars represent the SEM for mortality, horizontal bars represent the range of binned temperatures, and shading indicates the 95% confidence interval around the predicted line. There were no significant differences in mortality among maintenance conditions, lengths of time at maintenance conditions, or their interaction.

lower temperature than nondiapausing *H. halys* (Fig. 1). This is an interesting, though not surprising finding. Supercooling points of other diapausing insects have been shown to continue to decrease over time (e.g., Hodkova and Hodek 1997, Bemani et al. 2012). In this experiment we did not attempt to mimic all field conditions such as fluctuating temperature, which ultimately affects ADD, but also could affect supercooling points (e.g., Colinet et al. 2006). Photoperiod did not significantly affect supercooling points of diapausing individuals. Therefore, diapause and age of diapausing adults can be considered additional factors affecting *H. halys* supercooling points. Diapause has been found to lower supercooling points in other insect species (e.g., Hodkova and Hodek 1997, Šlachta et al. 2002, Morey et al. 2012).

Diapause allowed individuals reared both in the field and the laboratory to survive significantly lower temperatures than nondiapausing individuals (Fig. 4). Lower lethal temperatures of diapausing

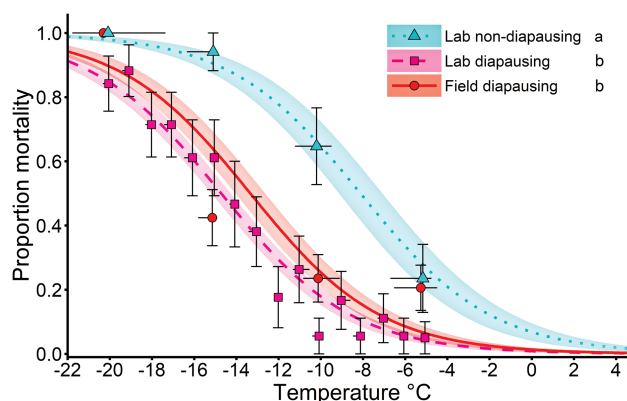


Fig. 4. Lower lethal temperatures of diapausing and nondiapausing adult *H. halys*. Each laboratory diapausing point represents 15–21 individuals, each field diapausing point represents 34 individuals, and each laboratory non-diapausing point represents 17 individuals. Vertical bars represent the SEM for mortality, horizontal bars represent the range of binned temperatures, and shading indicates the 95% confidence interval around the predicted line. Different letters indicate significant differences among treatments ($P < 0.05$).

individuals did not change based on maintenance conditions or the length of time held at maintenance conditions (Fig. 3). Meaning that when diapausing adults were maintained at their optimum temperature (25°C) (Nielsen et al. 2008) and long-day photoperiod for up to 3 wk, they did not lose their ability to tolerate cold and diapause was not broken. Additionally, being placed at a colder temperature than they were reared at did not enhance their ability to tolerate cold. This indicates that this metric of cold tolerance is stable within the first ~300 ADD as an adult. The lack of change to lower lethal temperatures due to external cues would provide a critical adaptive advantage when adults are confronted with fluctuating autumn temperatures, preventing de-acclimation with spurious warming temperatures.

Diapause affects both supercooling points (Fig. 1) and lower lethal temperatures (Fig. 4), but neither metric was significantly affected by maintenance conditions, and time affected only supercooling points. Over the same range of ADDs, supercooling points of diapausing individuals significantly decreased (Fig. 1) while lower lethal temperatures did not significantly change (Fig. 4). While supercooling points may be easy to measure and roughly reflect *H. halys* cold tolerance, they continue to change while an individual is in diapause and thus are not a reliable indicator of diapause status. We are confident in our categorization of diapausing and non-diapausing individuals, because all females were dissected to assess ovary development.

Mortality after long-term exposure to low, but not freezing temperatures, in a simulated overwintering microhabitat showed that diapause significantly increased survival (Fig. 2A). Previous work found only four reproductively active (i.e., vitellogenic) *H. halys* females in overwintering habitats across the United States, though the authors state they should not have been categorized as overwintering individuals due to the date of collection (Nielsen et al. 2017). This corroborates our results that, nondiapausing *H. halys* are unable to successfully overwinter (Fig. 2A). In our study, in the microhabitat less thermally buffered from ambient temperatures, all individuals, regardless of diapause status, died before the date of testing in December 2014 (Fig. 2B). While our work was not designed to determine the precise cause(s) of mortality, factors such as the intensity, duration, and fluctuation of cold temperatures can affect insect overwintering survival (e.g., Payne 1927, Colinet et al. 2006),

in addition to other nonmutually exclusive factors such as desiccation and predation. In the unheated shed, the minimum temperature reached before complete mortality was found was -12.09°C (i.e., intensity) and by that date, insects experienced 640 h below 0°C (i.e., duration). The mean difference $\pm$ SEM between the daily minimum and maximum temperatures until that date was $3.98 \pm 0.22^{\circ}\text{C}$ (i.e., fluctuation). This illustrates that while diapause increases winter survival, it cannot prevent death under all conditions.

In accordance with Nielsen et al. (2017), we found that once female *H. halys* became reproductively active (i.e., a nondiapausing state) they did not revert to a previtellogenic state when placed in overwintering microhabitats. Our work indicates that the life stage sensitive to diapause-inducing cues is between the second and fifth instar. Thus, individuals that do not receive diapause-inducing trigger(s) as nymphs will not persist through the winter when temperatures reach -4°C or lower for extended periods of time. Intraspecific lower temperature tolerance has been found to vary by latitude for some species (e.g., Hoffmann et al. 2002, Klok and Chown 2003) but not all (e.g., reviewed by Hoffmann et al. 2003, Kimura 2004). Thus, additional work to assess and compare cold tolerance of *H. halys* populations from a range of latitudes could make broader forecasts of overwintering mortality more robust. Additionally, work to determine the exact threshold of diapause-inducing cues, which specific cues (e.g., temperature, light, or an interaction of the two) are the predominant trigger(s) to induce diapause, and which life stage(s) is sensitive to cues will further improve population models and forecasts.

In summary, for the metrics we evaluated, diapause enhanced *H. halys* cold tolerance. Whether diapause was induced in the field or the laboratory, it did not have a significant effect on the achieved level of cold tolerance. The ability to produce diapausing adult *H. halys* in the laboratory with equivalent cold tolerance as field-reared individuals facilitates large-scale, year-round, cold tolerance experiments with greater uniformity and control of tested individuals. Additionally, this equivalency in cold tolerance is promising for extrapolating results of laboratory studies to field settings. Supercooling points of diapausing individuals decreased over time, while lower lethal temperatures were unaffected by time, and neither metric was affected by the maintenance conditions we tested. As *H. halys* supercooling points are not indicative of mortality (Cira et al. 2016) and lower lethal temperatures were unchanged in diapausing adults, the lower lethal temperature metric provided a more stable and reliable predictor of diapause status. Diapause significantly increased overwintering survival in suitable overwintering microhabitats, which provides important information about the fate of nondiapausing adults in the autumn. These results can contribute to predictions of overwintering mortality, and development of novel management strategies harnessing cold to induce mortality.

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