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Cold Tolerance, Diapause and Winter Survival of the Brown Marmorated Stink Bug (*Halyomorpha halys*)

Naomi Sibayan
Central Washington University, naomi.sibayan@gmail.com

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COLD TOLERANCE, DIAPAUSE AND WINTER SURVIVAL OF THE BROWN MARMORATED STINK BUG (*HALYOMORPHA HALYS*)

A Thesis

Presented to

The Graduate Faculty

Central Washington University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

Biological Sciences

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by

Naomi Elizabeth Sibayan

March 2018

CENTRAL WASHINGTON UNIVERSITY

Graduate Studies

We hereby approve the thesis of

Naomi Elizabeth Sibayan

Candidate for the degree of Master of Science

APPROVED FOR THE GRADUATE FACULTY

______________ ______________________________
Dr. Jason Irwin, Committee Chair

______________ ______________________________
Dr. Alison Scoville

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Dr. Michael Bush

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Dean of Graduate Studies

ABSTRACT

COLD TOLERANCE, DIAPAUSE AND WINTER SURVIVAL OF THE BROWN MARMORATED STINK BUG (*HALYOMORPHA HALYS*)

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Naomi Elizabeth Sibayan

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The brown marmorated stink bug, *Halyomorpha halys*, is native to eastern Asia and is an invasive pest in the United States. Limited research has been done regarding the factors influencing this species' winter survival within invaded northern regions. Cold tolerance has been previously evaluated in the eastern United States, bot nut within western populations. Winter diapause. Along with any potential links between diapause and cold tolerance remain unstudied. I examined characteristics of cold tolerance, diapause and overwintering sites to evaluate the factors contributing to the winter survival and, ultimately, persistence of this pest within central Washington. This study measured seasonal changes in cold tolerance of *H. halys* by determining its cold tolerance strategy and supercooling ability via the supercooling point (SCP). Metabolic suppression (as measured by flow-through respirometry) indicated that diapause was induced by a critical photoperiod of 11h:13h (L:D), indicating a process potentially influenced by multiple environmental factors. Also at this time, females no longer had developed eggs in the ovaries, indicating reproductive arrest. Overwintering sites monitored with temperature dataloggers measured daily minimum temperatures at an outdoor enclosure and naturally-selected overwintering site. We verify that populations of *H. halys* are chill-intolerant (*i.e.* a portion of individuals die prior to freezing) with minimum cooling temperatures between -10 °C and -15 °C causing survival rates to drop in field and lab populations by 73% to 6% and 86% to 14%, respectively. Cold tolerance significantly differed by light-hour regime ($F_{(3)} = 4.55$, p-value = 0.0048), sex ($F_{(1)}$ = 9.49, p-value = 0.0026), and mass ($F_{(2)}$ = 4.62, p-value = 0.033). The mean supercooling point (± SEM) of individuals in central Washington was -12.6 ± 0.2 °C. Diapause was observed to be a prerequisite to achieving maximum levels of cold tolerance and must also involve a subsequent period of cold acclimation. Given their extent of cold tolerance, I conclude that under natural conditions, *H. halys* cannot effectively survive winters within Central Washington State, a conclusion supported by the 100% mortality in an outdoor population. To persist in this region, *H. halys* will likely need to rely on human-built thermally-insulated refugia.

ACKNOWLEDGMENTS

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INTRODUCTION

The brown marmorated stink bug, *Halyomorpha halys* (Stål 1855) (Heteroptera, Pentatomidae), is native to east Asia, and was first identified as an invasive insect species in the United States in 2001 in Allentown, Pennsylvania (Hoebeke & Carter 2003). Presently, *H. halys* has been detected in 42 states and the District of Columbia in the United States (Northeastern IPM Center 2018). This species is an increasingly serious pest for many agricultural crops, resulting in significant economic loss due to feeding injury. Damage exceeding $37 million on apple crops in the Mid-Atlantic region alone demonstrates the destructive potential of this pest (United States Apple Association 2010). Both nymphs and adults use their probosci to pierce plant surfaces, inject digestive enzymes and suck plant fluids causing severe plant tissue damage (Rice *et al.* 2014). This pest has a broad host range including annual row crops, orchard fruits, ornamental and non-crop plants (Leskey *et al.* 2012; Lee *et al.* 2013; Rice *et al.* 2014; Haye *et al.* 2015; Bergmann *et al.* 2016), and readily moves between hosts based on seasonal fruit availability (Chung *et al.* 1995, Funayama 2004). A broad host range and the tendency to shift host-plants augment the impacts of *H. halys*, thus extending its growing season and allowing it to outcompete native stink bugs (which typically have smaller seasonal outbreaks). In conjunction with its feeding habits, adult *H. halys* are highly mobile and capable of dispersing >2 km (Zhang *et al.* 1993), which poses challenges to managers attempting to effectively monitor and contain populations. Unlike other species, which are limited by the location or seasonality of a specific host species, the extensive host range and high mobility of *H. halys* make it unlikely that resource availability is a limiting factor in the geographic range of *H. halys*. Current management practices have consisted of season-long repeated insecticide treatments, resulting in disruptions of previous integrated pest management (IPM) programs (Leskey *et al.* 2012).

H. halys may have the ability to establish populations across the entirety of the contiguous United States based on degree-day models (Holtz & Kamminga 2010), ecological niche modeling (Zhu *et al.* 2012), and models involving phenology and population dynamics (Nielsen *et al.* 2016; Nielsen *et al.* 2017). While much research has been done on the damage caused by *H. halys* (*e.g.*, host-plant impacts and insect ontogeny) that exclusively occurs during the agricultural growing season (Rice *et al.* 2014), researchers have just begun to investigate the factors affecting the winter survival of this pest. Insects, being ectothermic, are strongly influenced by ambient air temperatures, which inevitably dictate their life histories (*e.g.*, development and mortality). As *H. halys* continues to move into more northern temperature regions, winter conditions will strongly affect its overall geographic distribution and population dynamics. The ability for a pest to survive these adverse conditions, relies heavily on multiple physiological factors. To better understand the abilities *H. halys* possesses for winter survival and its presence within the western United States, we studied the physiological factors of cold tolerance and diapause within Washington state.

Insects living within the Temperate Zone, occupy regions with seasonal, environmental variation, and must cope with periods of adverse conditions. The northern limits of other stink bug species have been linked to monthly winter minimum temperatures (Musolin 2007). An important adaptation for these insects has been the ability to gain effective physiological tolerance against cold, adverse conditions. This ability to survive is commonly referred to as cold tolerance (also “cold-hardiness”) and is a well-documented aspect of insect overwintering (Leather *et al.* 1993). Of the pentatomid species studied in relation to overwintering in the Temperate Zone, most species do so as adults (Saulich & Musolin 2012). For these species, there are three main cold-tolerance strategies: freeze tolerance, freeze intolerance, and chill intolerance

(Lee & Denlinger 2010). Freeze tolerance is the ability to tolerate ice forming within the body. Insects exhibiting freeze intolerance survive only to the point of freezing, and chill-intolerant insects face mortality before freezing occurs. Most temperate insects are freeze-intolerant within the Northern Hemisphere, and no known species of Heteroptera has been found to tolerate freezing (Bale 1991; Saulich & Musolin 2012). The point at which body fluids freeze can be altered via supercooling (SCP): reducing the temperature of ice crystallization (T_c) of the body fluid. This is typically accomplished through various mechanisms: polyol cryoprotectant accumulation (*e.g.* glycerol, sorbitol, etc.), voiding the gut of ice-nucleating agents, antifreeze proteins, and undergoing biochemical adjustments (Lee 1991, Leather *et al.* 1993, Koštál *et al.* 2004; Koštál *et al.* 2008). The determination of the SCP can be a good place to start when investigating cold tolerance, because it serves as the theoretical minimum temperature at which an insect can survive.

The seasonal cold tolerance of *H. halys* was investigated by Cira *et al.* (2016), where they measured the supercooling ability and determined the cold tolerance strategy of this pest in the eastern United States. Mean SCP measurements in Virginia (-13.48 ±0.4 °C) and Minnesota (-16.93 ±0.23 °C), showed that the region of cold-acclimation, rather than geographical origin strongly determined SCP ability. While SCP served to predict field mortality rates, it was determined that *H. halys* exhibits the cold tolerance strategy of chill intolerance, in which adults experienced high mortality rates above the temperature at which they froze. Because *H. halys* was first introduced to the United States on the eastern coast, the majority of research has focused on these eastern populations. However, by 2004 West Coast populations of *H. halys* became established, thus offering an opportunity to study geographic variation (Hoddle 2013;

Ingels & Varela 2014). In this study, I investigated the physiological responses to declining temperatures that have previously remained unstudied in the western United States.

In many temperate insects that overwinter, cold tolerance is known to evolve simultaneously with diapause, another adaptation that strongly contributes to insect survival during adverse conditions. Diapause is a period of hormone-regulated (endogenously-mediated) dormancy that allow insects to reduce energy consumption during periods of inactivity due to adverse environmental conditions (Mansingh 1971). For ease of understanding, I will follow the terminology of Koštál (2006), who defines the stages of diapause through an eco-physiological perspective. Diapause as a physiologically dynamic process, with factors that fluctuate by time, responsiveness, sensitivity, and intensity. The five phases of diapause include the induction, preparation, maintenance, termination, and finally post-diapause quiescence. The induction phase occurs during a “sensitive phase” (genetically mediated) where environmental cues are perceived and transduced into a physiological switch from direct development to diapause and begins when an environmental cue (*e.g.*, photoperiod, temperature, etc.) reaches a critical level. The preparation phase occurs in some insects when the induction phase is not immediately followed with diapause initiation, and involves a period of behavioral and physiological changes, that are necessary for later expression of diapause. Diapause initiation is the beginning phase of what is known as the period of “diapause development” where direct development (morphogenesis) ceases, and metabolic rate is significantly suppressed. The maintenance phase involves the persistence of endogenous developmental arrest, even when environmental conditions may be favorable for continued development, and metabolic rates remain relatively low and constant. The termination phase involves a reduction in diapause intensity, ultimately allowing for the potential resumption of development, which has the advantage of synchronizing a post-diapause reproductive population. Lastly, insects in post-diapause quiescence have the potential to resume “normal” developmental and metabolic processes in the presence of favorable conditions.

The behavioral and physiological changes of diapause differ between species but can be manifest and measured as suppressed metabolic rate and arrested development (*e.g.* reproductive status) (Tauber *et al.* 1986). Diapause is often necessary for the synchronization of reproductive individuals once sufficient resources are again available (Musolin 2007; Mansingh 1971) and can occur in a species either through an obligate (a fixed component of ontogeny) or facultative (responsive to environmental token stimuli) process (Koštál 2006). Many environmental cues can regulate diapause induction and termination, with photoperiod and temperature being the most common. As days get shorter, north-temperate insects cease development, suppress their metabolism, and often improve cold tolerance. The timing of diapause development is critical, especially in species where cold tolerance and diapause are linked, as in some pentatomid species (Šlachta *et al.* 2002). Entrance into diapause too early cuts short the season that allows direct development, whereas entrance too late can leave the insects susceptible to fatal cold temperatures early in the fall, and can present the most immediate impediment to range expansion in the temperate zone (Bradshaw *et al.* 2000, 2001). Determining how insects can overcome the physiologic challenges associated with extreme weather conditions is a critical, particularly in pest scenarios. Effectively understanding the dynamics of a population throughout its entire life cycle, including the seasonal periods where no direct environmental interaction occurs but the population persists, aids in the forecasting of future outbreaks, and predictions of long-term impacts.

Within their native range *H. halys* is known to undergo facultative diapause (Watanabe 1978): diapause is photoperiod-dependent with temperature playing a synergistic role in the early phases (*e.g.* induction and preparation) (Saulich & Musolin 2012;Watanabe 1979; Niva & Takeda 2002; Niva & Takeda 2003). The critical photoperiod (daylength that induces diapause, also referred to as photoperiodic threshold) for diapause termination in the spring has been extensively studied, and has an estimated daylength between 13.5h and 12.7h (Watanabe 1979; Yanagi & Hagihara 1980; Nielsen *et al.* 2016; Nielsen *et al.* 2017). However, prior research has also cautiously assumed that this critical photoperiod serves the same function for diapause induction in the fall, even as there currently lacks sufficient quantitative support for this notion. An accurate estimation of the critical photoperiod in the fall is necessary to predict the potential for northern range expansion of this species, particularly if the photoperiod bears the risk of becoming maladaptive in colder regions (Musolin 2007). Like other stink bugs (*e.g.*, *Nezara viridula*; Musolin & Numata 2003a, 2003b), photoperiod is the primary cue for diapause induction in *H. halys* (Niva & Takeda 2003), with late instars (4th & 5th) and adults being sensitive to this environmental stimulus. The northward expansion of *H. halys* may be limited in colder climates, including the Pacific Northwest, where cold weather arrives before diapause is induced via shorter photoperiods.

Cold tolerance and diapause occur simultaneously in temperate insects, which complicates our ability to understand one independently of the other. Additionally, while there may exist certain endocrinological links between the two, one cannot assume the basis of one on account of the other (Denlinger 1991). The cold tolerance of this species has been investigated in the eastern United States, but the relationship between that and diapause has remained unresolved. One strategy to distinguish the relationship between these two factors, is to compare the physiological responses of a species to decreases in ambient temperature (through natural seasonal acclimatization and artificial acclimation) in both diapausing and non-diapausing populations (Šlachta *et al.* 2002). Through this comparative approach, one can evaluate: (1) the regulatory mechanisms responsible for the physiological changes in overwintering insects and (2) of how each physiological response is reflected in the insect's acquired level of cold hardiness.

In this study, we present data that improves our understanding of (1) the characteristics of diapause throughout the onset and duration of the winter season, (2) the seasonal changes in cold tolerance of overwintering adults in the western United States, and (3) the potential links between the onset of diapause and cold tolerance in adults via changes in the physiological parameters of *Halyomorpha halys.* Determining how *H. halys* overcomes physiologic challenges associated with adverse winter conditions is critical in forecasting potential outbreaks, the potential for range expansion to the north, and predicting their long-term impact within a region, especially during this period of warming temperatures.

METHODS

Collection and Monitoring of Live Specimens

Adult and late-instar (3^{rd} – 5^{th}) nymphs were collected within Washington state in Yakima and Walla Walla, WA, at two weeks to monthly intervals from April to October. Individuals were collected from host-plant vegetation and human structures using insect nets, or on drop cloths following beating of vegetation with wooden sticks. Individuals were stored within BugDorm Rearing and Observation Cages (#1452A, BioQuip Products) to allow for sufficient air ventilation and prevent injury from elevated temperatures, and transported to an outdoor shelter in Yakima, WA (46 °.60’3.5176” N latitude and -120 °.61’6.6340” W longitude). *H. halys* reared and maintained outdoors were kept within BugDorm 2 rearing cages (#1462C, Bioquip Products), with the outdoor cages housed within a large luminite outdoor cage measuring 6 x 12 x 6’ (#1412C, Bioquip Products), all secured within a wooden shelter to ensure the security of the colony and prevent the potential for escape. This outdoor enclosure allowed for the stink bugs to experience the naturally occurring environmental temperatures experienced within the field. Temperature dataloggers (HOBO Pro v2, Onset Computer Corporation) were placed within and outside the outdoor enclosure to monitor both ambient-air temperature (°C) and light intensity (units)/photoperiod throughout the seasonal transition of fall to winter (October – February). This population of *H. halys* were sampled at three-week intervals throughout Fall and Winter for experiments of both cold tolerance (*e.g.* cold-tolerance strategy and supercooling point), and diapause regulation (*e.g.* metabolic rate and reproductive status).

A portion of the collected *H. halys* adults were transported to the Central Washington University Vivarium, a facility designed to contain animals and pathogenic material, thus appropriate for the quarantine of *H. halys* within Kittitas county. The facility includes negative pressure air flow (filtered before venting to outside), multiple-door entry to prevent escapes, limited-access security, and individually temperature- and photoperiod-controlled chambers. These laboratory colonies were housed within BugDorm 1 rearing cages (#1452, Bioquip Products), and kept under constant environmental conditions: a reproducing Long-day light regime of 16L:8D, a temperature at 25 °C, and 60 ±5% relative humidity. A portion of adults were assigned to a breeding colony and housed separately. Offspring produced by the breeding colony supplemented the field-collected laboratory populations. Combined these remaining individuals were designated toward experiments. This laboratory population of *H. halys* was sampled for experiments of both cold tolerance (*e.g.* cold-tolerance strategy and supercooling point), and diapause regulation (*e.g.* critical photoperiod, metabolic rate and reproductive status).

All stink bug colonies were reared/maintained on raw organic vegetables (tomatoes, carrots, green beans, snap peas, watercress shoots, as were available), organic almonds, and organic figs and water, which was refreshed every other day. Nymphs hatched from the reproductive breeding colonies were housed separate of the adults within smaller clear plastic containers with mesh lids for proper air ventilation, with nymphs of like developmental stage grouped together to prevent predation.

Field measurements of microclimate at naturally-selected and artificial (field enclosure) overwintering sites were necessary to understand how our local climate affects the winter survival of *H. halys.* These monitored populations (from both Yakima, WA and Walla Walla, WA) also served as a source for season-long collection of *H. halys* for tests determining cold tolerance and diapause. Winter temperatures experienced by *H. halys* were also monitored from a naturally chosen overwintering site on the outside of a residential home near Pioneer Park in Walla Walla, WA. This site featured cedar shingle siding where *H. halys* were found to aggregate within the crevices underneath the exterior paneling of the structure. This house is within a few blocks radius of Pioneer Park (46.0662 °N, 118.3178 ° W), a well-established site of *H. halys*. A dual-probed temperature datalogger (add make/model) was also placed at this site to monitor the internal temperature beneath the exterior paneling, as well as the external ambient-air temperature from October 2016 to June 2017.

Diapause regulation

Metabolic Rate

To characterize diapause within this species the timing of diapause-induced metabolic suppression was determined in both the outdoor population (dictated by seasonal timing and natural cues), and the laboratory population (dictated by modified photoperiod while temperature remained a constant), following the methods of Irwin *et al.* (2001) and Lester and Irwin (2012). I monitored the seasonal changes in metabolic rates observed in the outdoor population by measuring the CO_2 production of stink bugs in three-week intervals throughout the fall and winter seasons beginning in mid-August to late-January (n = 7 per week). Similar changes in metabolic rate were monitored in the laboratory population, but sampled at intervals dictated by the Light-dark (LD) regime as described below, in our attempts to identify the critical photoperiod for diapause induction. The total number of individuals analyzed for the outdoor and laboratory populations was n = 56 and n = 107 respectively.

Individuals were pre-weighed and inserted into one of seven glass respirometry chambers, with an additional chamber that was run empty as a control, all enclosed within an incubator with no light present. All eight chambers included a section of pipe cleaner ~ 4cm in length to provide the insect with a textured surface to grasp and encourage a resting state. CO_2 production was measured using flow-through, positive-pressure respirometry (Sable Systems International) at 5, 10, and 15 °C with a one-hour holding time to achieve temperature equilibrium before data collection. Each chamber was flushed with fresh air for 5 min, before CO_2 enrichment was measured for 15 min using a Licor Li-6251 CO_2 analyzer. These production values were transformed via integration (Sable Systems Expedata) to determine the volume of CO_2 enrichment per unit time for each individual, then divided by body mass for a final calculated value of CO_2 per gram per hour. Three different temperatures were used to allow for the analysis of metabolic response to temperature, which is an indicator of metabolic suppression associated with diapause. 15 °C was used as the upper limit to approximate the resting metabolic rate that occurs during normal activity, which should be consistent throughout the study period, regardless of seasonal temperatures. Any depression observed at this temperature would indicate that environmental temperature was not the sole regulator of metabolism, and the insect was therefore experiencing endogenous metabolic suppression.

Reproductive Status

Female stink bugs from both the photoperiodic threshold and metabolic rate experiments were evaluated for reproductive arrest, a common indicator of diapause, via dissection of the gonads. Immediately following metabolic rate and cold tolerance measurements, each stink bug was dissected and evaluated for its reproductive status, with reproductively arrested females indicated by the presence of immature oocytes (Niva and Takeda, 2002). Insect legs, wings, and scutellum were removed prior to dissection, and each insect was pinned ventral side-down in a petri dish. General Ringer's solution was used for all dissections.

Critical Photoperiod

Adult *H. halys* were exposed to decreasing day-length to determine the critical photoperiodic cue for diapause induction as proposed by Šlachta *et al.* (2002). Late instar nymphs are known to be photo-sensitive (Niva & Takeda date), so to ensure that newly emerged adults were allowed adequate sensitive exposure to the experimental photoperiods, individuals from the breeding colony reared to the 5th instar were transferred from the CWU Vivarium (temperature 23 °C at 60% humidity) to an incubator (temperature 20 °C at 60% humidity). The relative age of the 5th instars were tracked via age groups, in which one age group is defined as all egg clusters laid within a two-week period. Upon transfer from the vivarium to the incubator, the available photoperiod of 16L:8D was decreased to the next lowest designated LD regime of 15.5L:8.5D. The photoperiod was incrementally reduced there-after in light-hour duration by 30-min intervals, to end at a day-length of 12L: 12D. This step-wise progression allowed the individuals to transition from photoperiods known to encourage reproduction, to photoperiods that could potentially serve as a token stimulus for diapause induction. The duration of each photoperiod was determined by observed natural seasonal changes in daylight duration experienced in Yakima, WA, and required a total of 105 days**.** At the transition of one LD regime to the next, individuals were sampled from each assigned age group for a total sample size of n = 10, in which both metabolic rate and reproductive status were measured as described above.

Cold Tolerance

Cold Tolerance Strategy

Measures of cold tolerance strategy in *H. halys*, were modelled after the methods of Šlachta *et al.* (2002) and Cira *et al.* (2016). Three groups of insects were used: (1) field-maintained adults from the outdoor colony representing the natural course of seasonal cold-acclimation, (2) non-diapausing adults produced in the laboratory (reared at a long-day 16L:8D regime at 23 °C), and (3) diapausing adults produced in the laboratory (reared at a short-day 12L:12D regime at 23 °C; Niva & Takeda 2002). Groups 2 and 3 were established from field-collected 5th instar *H. halys* (F2 generation) from late-August through September of 2017, that were brought into the lab and maintained at the desired LD regimes as they emerged into adults. These two groups underwent a cold-acclimation process consisting of a gradual decrease in temperature over the course of 15 days: 20 °C (2days) → 15 °C (2 days) →10 °C (2 days) → 5 °C (2 days) → 0 °C (7 days). Photoperiod regimes were maintained throughout the cold-acclimation process from 20 °C to 5 °C, according to the particular LD regime group (either LD or SD), then transitioned into continuous darkness at the final holding phase of 0 °C. Once the cold-acclimation period was finished, insects were tested for levels of chill intolerance.

H. halys were randomly assigned to one of three temperature treatments (-5, -10, -15 °C). In group 1 (field-maintained, naturally acclimated adults), all three treatments were sampled at three-week intervals beginning early-September into late-February 2017. The laboratory-sourced groups 2 and 3, were sampled at the end of the artificial cold-acclimation period. For all three groups, insect cooling and thermocouple temperature monitoring were done using the same cooling methods. Stink bugs were weighted and placed in a 50mL glass test tube, with a copper-constantan thermocouple held in place against the bug's body using a foam plug, and then sealed with an addition foam plug. Body temperature of each bug was monitored using a (USB-TEMP Measurement Computing get model version) data logger, and the output continuously tracked during cooling using data acquisition software (TracerDAQ). The test tube was placed into a refrigerated alcohol cooling bath (RTE-740, Neslab) to reach the desired minimum temperature treatment. For each trial, the temperature was reduced from room temperature to the desired temperature treatment at a rate of 0.5 °C/minute, held at this minimum for 10 minutes, then allowed to warm to room temperature at the same rate. At room temperature, each insect was provisioned with food and water, and placed within individual plastic containers to be monitored daily for mortality over the course of four days. Mortality was defined as any insect that lacked any physical response to being gently prodded with a small brush. Mortality for this analysis also included moribund insects, as defined as having the inability to right oneself after ~10s, or the action of coordinated crawling. The percent mortality was collected for each temperature treatment throughout the sampling period from fall into winter. Additional individuals from each laboratory population were tested for supercooling ability. For this experiment, group 1 sampled n = 48 adults, while groups 2 and 3 consisted of n = 30 – 50 adults per group.

Supercooling Point

We monitored supercooling ability through the seasonal changes in the naturally-acclimated outdoor population, and within the laboratory population undergoing artificial cold-acclimation. The outdoor population was sampled at three-week intervals throughout the fall and winter seasons from late-September to late-January, with these sampling occurrences then grouped by season (Fall = Sept – Nov, Winter = Dec – Feb) for statistical analysis. The laboratory population was sampled after a gradual cold-acclimation process (described above) and separated by light-dark (LD) regime (long-day or short-day). Insect cooling and thermocouple temperature monitoring were done using the same methods as described above for cold tolerance strategy. Cooling bath temperature was reduced from 0 to -40 °C, at a rate of 2 °C per minute. The supercooling point was indicated by the exothermic latent heat of fusion.

Data Analyses

All statistics were run utilizing R version (get version) in Rstudio version (get version) with an α value of 0.05. Shapiro-Wilk tests for normality of residuals and a Levene test for homogeneity of variance across groups indicated no violations of ANOVA assumptions. Two-factor ANOVA was used to analyze metabolic rate (log-transformed), and supercooling point data. Multiple pairwise comparisons between groups were analyzed using least-squares means, and graphically represented as least-squares means including 95% upper and lower confidence intervals, with the exception of field temperature data. Paired t-tests were used to compare field-temperature data.

The relationships between survival and temperature (via chill intolerance), and reproductive status and light-hour regime were determined using a binary logistic regression model. Multiple pairwise comparisons were analyzed using least-squares means in R with in inverse logit link argument to produce appropriate predicted probability yields (rather than predicted log yields). I evaluated all potential interaction terms within all models, and finding none to be significant, therefore they were excluded from the models.

RESULTS

Diapause Regulation

Metabolic Rate and Reproductive Status

Metabolic rates of stink bugs, from the naturally-acclimated outdoor population, measured at incubation temperatures of 15, 10, and 5 °C all significantly differed across months in 15 °C ($F_{(7)} = 6.4$, p-value < 0.001), 10 °C ($F_{(6)} = 3.9$, p-value < 0.05), and 5 °C ($F_{(7)} = 2.8$, p-value < 0.05) (Fig. 1). Metabolic rates showed no significant difference between sexes over time for 15 °C ($F_{(1)} = 0.29$, p-value = 0.5), 10 °C ($F_{(1)} = 1.7$, p-value = 0.19), and 5 °C ($F_{(1)} = 3.8$, p-value = 0.056). Metabolic suppression was observed to occur beginning late-October through the month of November.

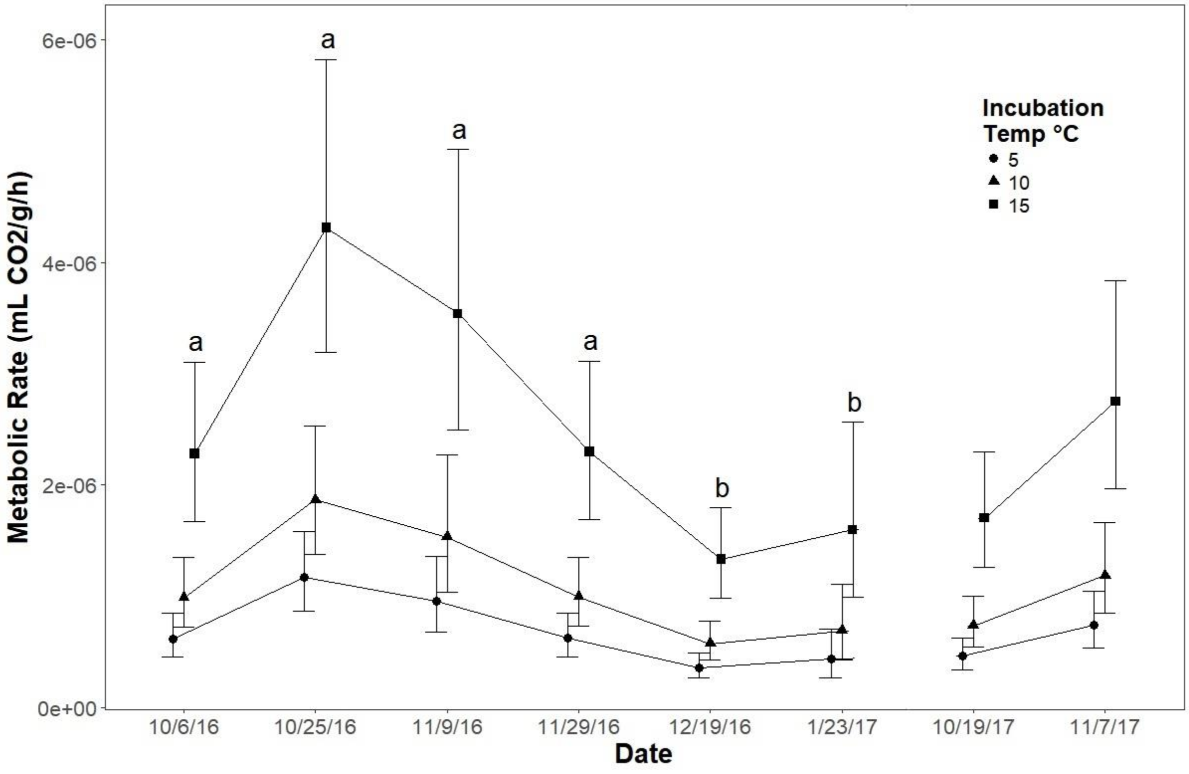

Figure 1. Mean metabolic rates including upper and lower confidence intervals, measured at 5, 10, and 15 °C of overwintering adult *H. halys*.

Metabolic rates of stink bugs, from the artificially-acclimated laboratory population, measured at 15 °C differed significantly across photoperiod ($F_{(7)} = 4.35$, p-value < 0.001) (Fig. 2). Metabolic rates did not differ significantly between sex ($F_{(1)} = 3.7$, p-value > 0.05). The photoperiod range of 13.5L:10.5D – 12L:12D produced both a significant metabolic (Fig. 2) and reproductive ($X^2_{(8)} = 46.65$, p-value < 0.05) (Fig. 3) suppression from those observed from the photoperiod range known to support reproduction (*e.g.* 16L:8D – 14L:10D). Dissections of female *H. halys* indicated both non-reproductive and reproductive individuals (Fig. 4).

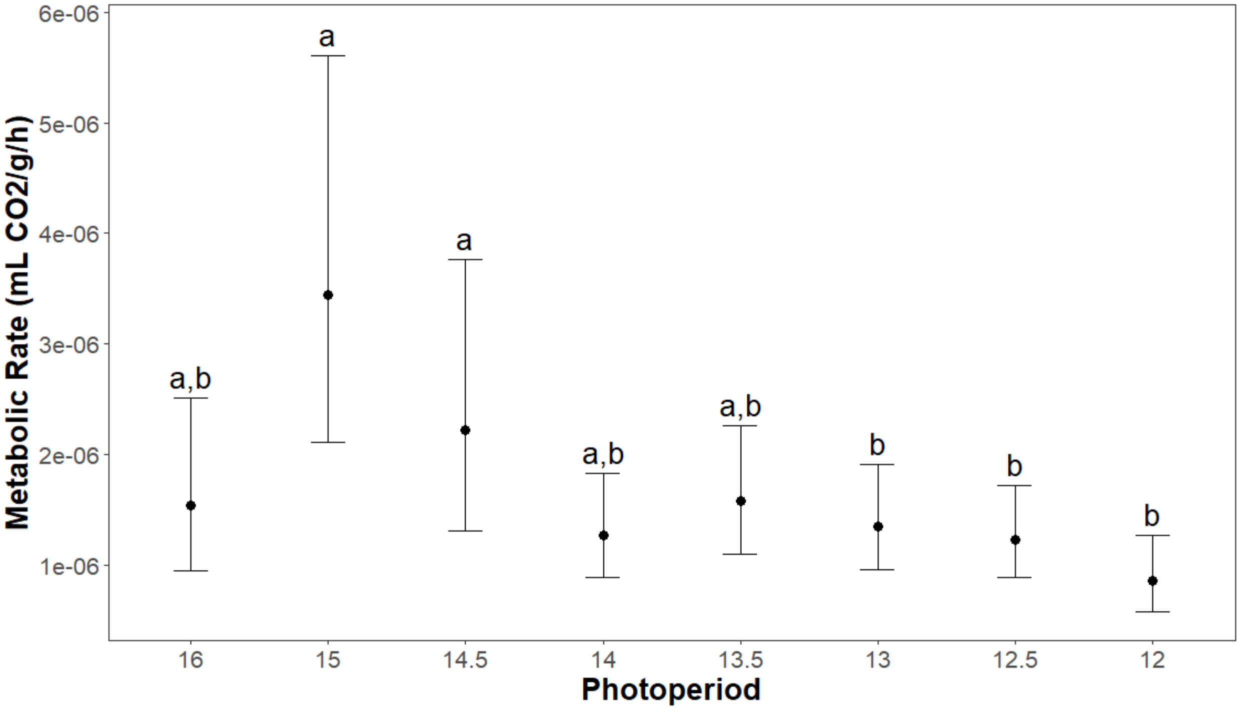

Figure 2. Mean metabolic rate including upper and lower confidence intervals, measured at 15 °C of artificially-acclimated adult *H. halys*. Metabolic rates differed significantly across light hour availability ($F_{(7)} = 4.3$, p <0.001). Metabolic rate response to incubation temperature showed an initial increase in metabolic rate during the photoperiod of 15L:9D, followed by a decreasing trend throughout the photoperiod transition to 12L:12D.

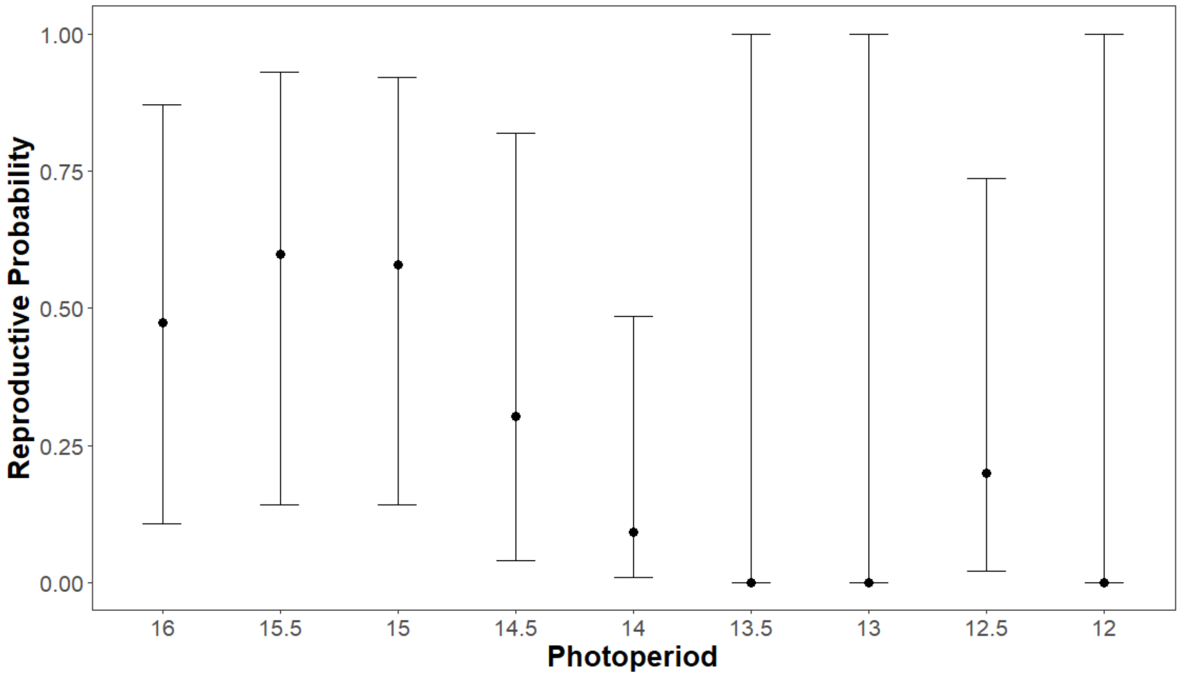

Figure 3. Proportion of reproductively active overwintering adult female *H. halys*. Female reproductive status was significantly reduced beginning within the 14L:10D to 13L:11D range.

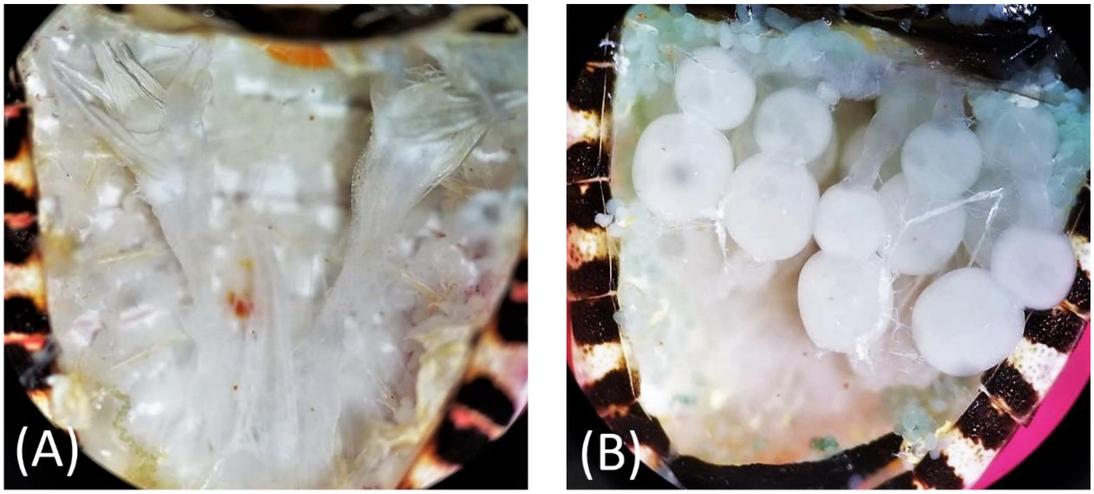

Figure. 4 Dissections of female *H. halys* indicating the presence of (A) previtellogenic oocytes and (B) vitellogenic oocytes.

Cold Tolerance

Cold Tolerance Strategy

Throughout the supercooling experiments, all individuals that froze died. Naturally and artificially cold-acclimated adult Brown-marmorated stink bugs exposed to a series of minimum cooling temperatures above the point of freezing, show significant differences in the probability of survival across temperature treatments ($X^2_{(3)} = 51.6$, $p < 0.01$) (Fig. 5). Survival rates began to decline when temperature was reduced to -5 °C and showed a significantly reduced probability of survival between the two lowest temperature treatments (10 °C & 15 °C). Predicted survival rates significantly dropped between -10 °C and -15 °C in the field (73% to 6%) and laboratory (86% to 14%) populations.

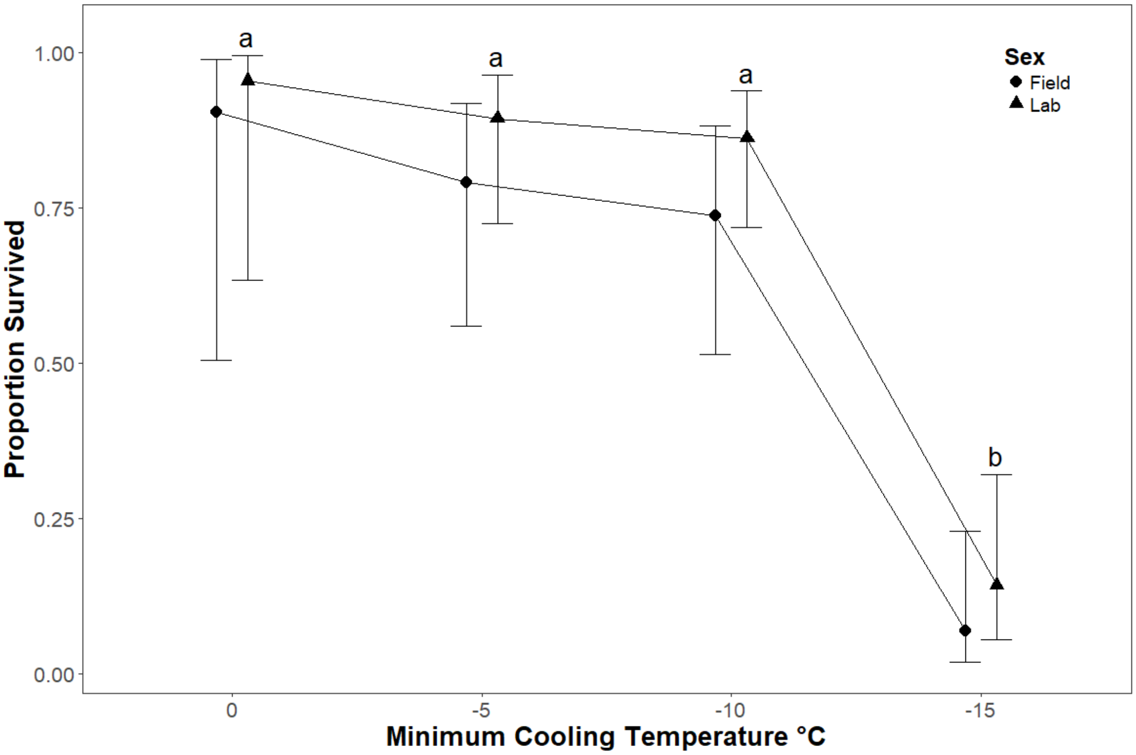

Figure 5. Naturally and artificially cold-acclimated adult *H. halys* exposed to a series of minimum cooling temperatures, show significant differences in the proportion survived across temperature treatments ($X^2_{(3)} = 51.598$, $p < 0.01$).

Supercooling Point

The outdoor population consisted of individuals sourced from Yakima, WA and Walla Walla, WA, and were housed within the outdoor enclosure in Yakima, WA. Supercooling points of adult *H. halys* did not significantly differ between populations ($F_{(1)}$ = 0.16, p = 0.68). Supercooling points from this naturally-acclimated outdoor population showed significant difference between seasons ($F_{(2)}$ = 3.32, p = 0.041), while supercooling points from the artificially-acclimated laboratory population differed significantly between LD regimes ($F_{(1)}$ = 5.74, p = 0.023) (Fig. 6). Both populations tested also showed significant differences between sex ($F_{(1)}$ = 5.54, p = 0.021; $F_{(1)}$ = 9.49, p = 0.0026).

An analysis of both populations combined showed supercooling points that differed significantly across light-hour regime ($F_{(3)}$ = 4.55, p-value = 0.0048), sex ($F_{(1)}$ = 9.49, p-value = 0.0026), and mass ($F_{(2)}$ = 4.62, p-value = 0.033) (Fig. 6). Neither sex nor mass were significant covariates in this analysis. Supercooling points measured within the 'fall' group had a mean SCP of -12.6 °C ±0.381 in females and -14.5 ±0.402 in males, while the 'short-day' group had a mean SCP of -15.1 ±0.57 in females and -17.0 ± SE 0.66 in males. (Fig. 6), indicating that the 'short-day' group having significantly lower SCP values than field individuals measured during the coldest recorded instances of winter.

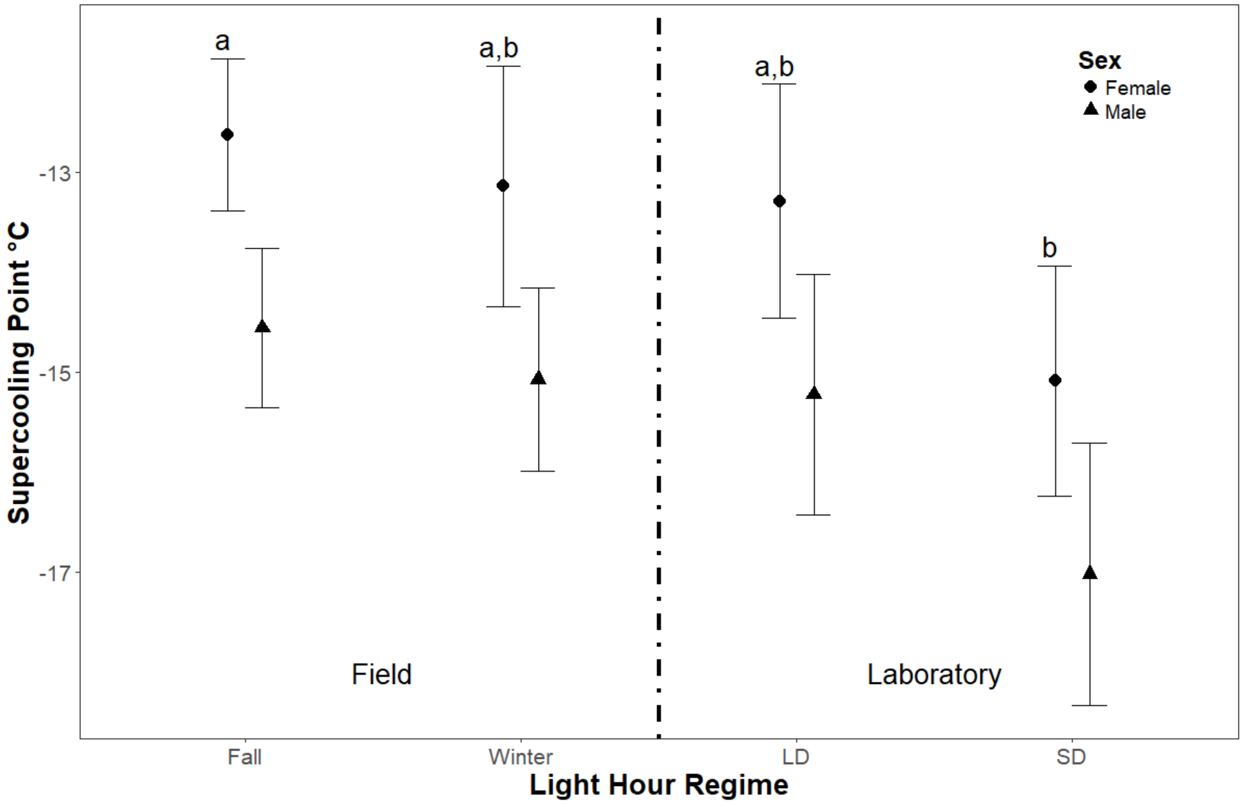

Figure 6. Supercooling points differed significantly across light-hour regime, sex and mass ($F_{(3)} = 4.55$, $p = 0.0048$; $F_{(1)} = 9.49$, $p = 0.0026$; $F_{(2)} = 4.62$, $p = 0.033$) in adult *H. halys.* A pairwise comparison of light-hour regime grouped by sex showed significant differences between the 'fall' and 'short-day' groups for both female and male stink bugs ($t = 3.845_{(103)}$, $p = 0.0012$; $t = 3.845_{(103)}$, $p = 0.0012$).

Overwintering Site Characteristics

Temperature samples were collected at two field sites from October 2016 to April 2017 in Yakima and Walla Walla, WA. Both sites were outfitted with two temperature data loggers to measure both internal and external ambient air temperatures. Temperature data differed significantly in Yakima ($t_{(168)} = -29.6$, $p < 0.05$), and in Walla Walla ($t_{(170)} = -27.9$, $p < 0.05$). Daily minimum ambient air temperature at the outdoor shelter in Yakima, WA experienced a minimum of -18.14 °C outside the shelter, and -14.96 °C within. Daily minimum ambient air temperature at the overwintering site in Walla Walla, WA experienced a minimum of -16.31 °C.

On multiple occasions ambient air temperature dropped below the average supercooling point (-13.88 °C) observed from the adult *H. halys* that were housed at both sites throughout the sampling season. By the end of January 2017, high mortality in the outdoor enclosure in Yakima was observed from both the Yakima (100%) and Walla Walla-sourced (87%) populations. While the overwintering mortality in Walla Walla was not directly monitored, only very few *H. halys* emerged from the structure in the spring.

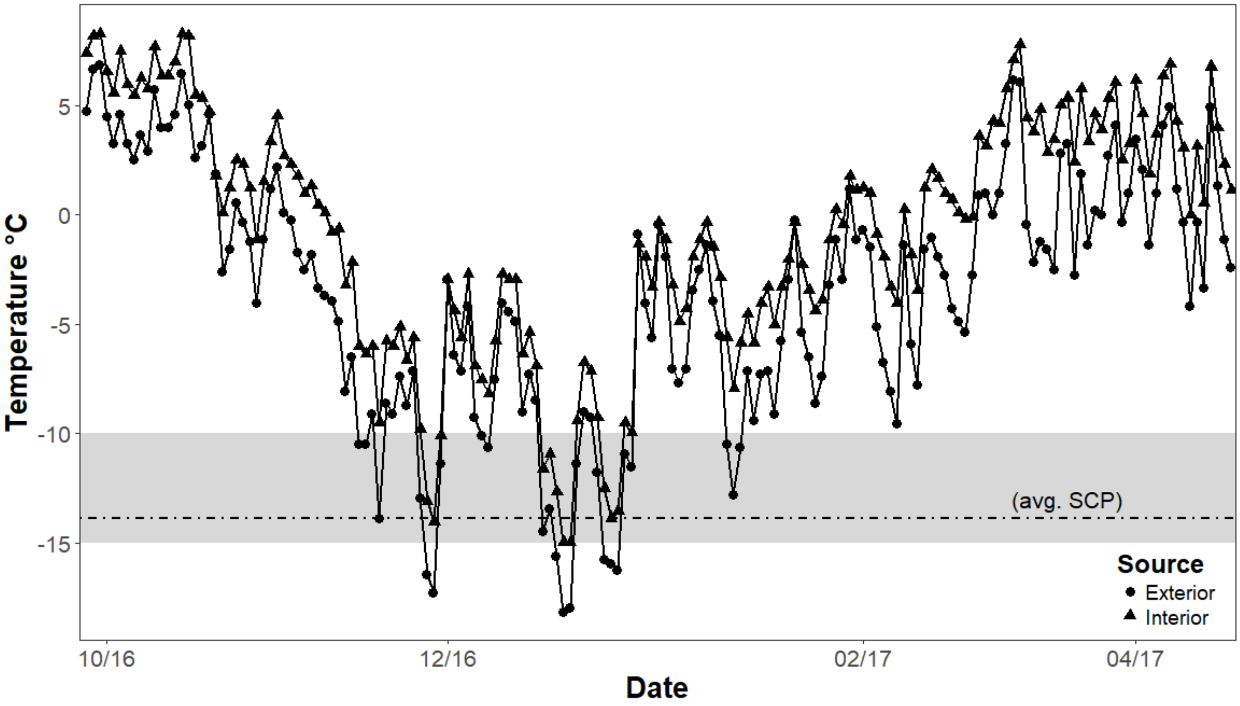

Figure 7. Daily minimum temperature of the outdoor enclosure field site in Yakima, WA from 10/28/16 to 4/15/17. The minimum ambient air temperature observed was -18.14 °C externally, and -14.96 °C within the enclosure. The dashed line indicates observed mean supercooling point of -13.88 °C for adult *H. halys*, and the shaded area indicates temperature range at which the least-squares means probability of survival via chill intolerance decreases from 76% to 6%.

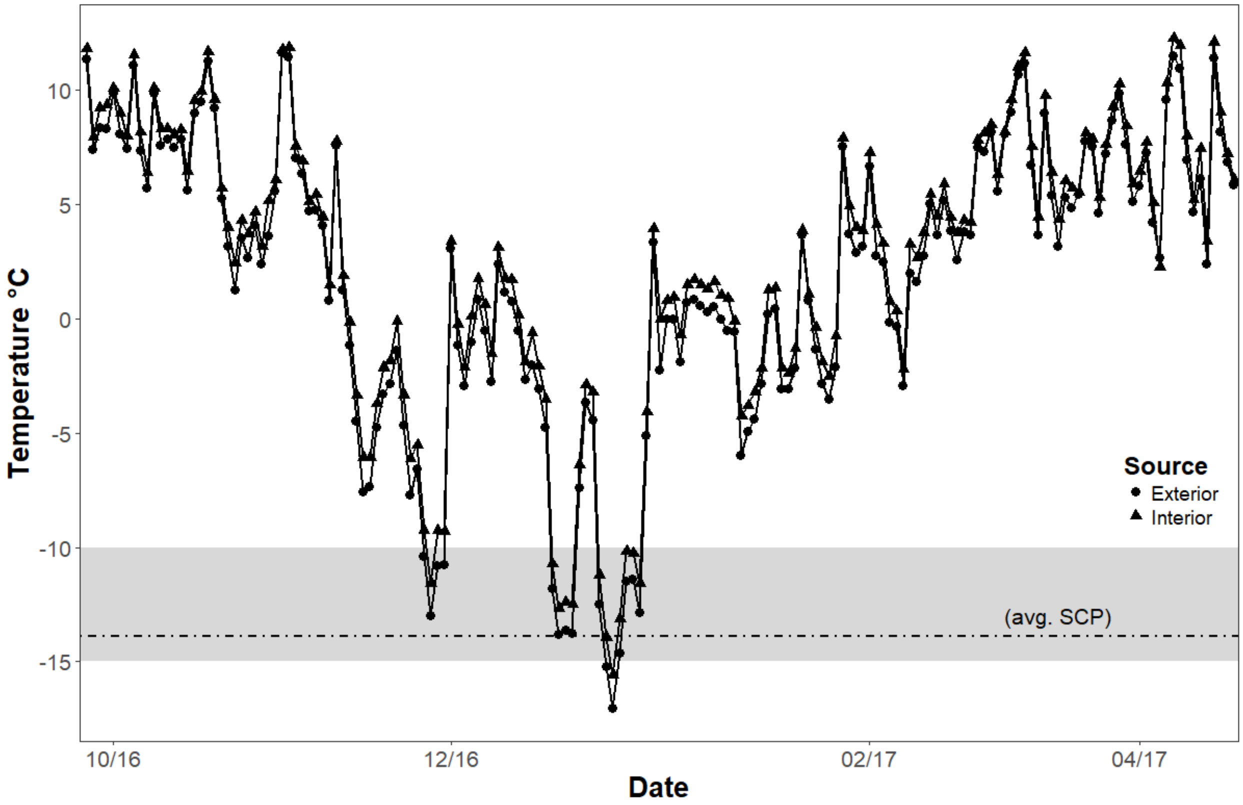

Figure 8. Daily minimum temperature at a naturally-selected overwintering site of *H. halys* in Walla Walla, WA from 10/27/16 to 4/15/17. The minimum ambient air temperature observed was -18.14 °C externally, and -14.96 °C underneath the cedar siding. The dashed line indicates observed mean supercooling point of -13.88 °C for adult H. halys, and the shaded area indicates temperature range at which the least-squares means probability of survival via chill intolerance decreases from 76% to 6%.

DISCUSSION

Within this species, the metabolic changes observed throughout the fall and winter seasons follow patterns of metabolic suppression commonly found in other temperate insect species. The metabolic rates of naturally acclimated adult brown marmorated stink bugs was endogenously suppressed, giving strong evidence of diapause. Induction was observed to occur in early-October, followed by a preparation phase, as indicated by metabolic rate changes prior to maximal metabolic suppression. This “preparation” phase of pre-diapause (Koštál 2006) is characterized by a rapid period of elevated respiration, followed by a gradual decrease to residual metabolic levels (*i.e.* diapause initiation phase) (Fig. 1). These results suggest that in this region, *H. halys* undergoes diapause induction in early-October, and preparation throughout the remainder of October into early-November, to fulfill physiological processes presumably necessary for effective winter survival (*e.g.* accumulation of energy stores, biosynthesis of cryoprotectant and the limiting of energetic metabolism) (Hand & Podrabsky 2000; Koštál *et al.* 2004; Koštál *et al.* 2007). Given the mortality rates observed during early-winter and the limited sample populations available at the time of this study, we were unable to continue metabolic measurements through to the end of winter and at spring emergence. Additional sampling would allow a more comprehensive picture of the entire diapause process *H. halys* undergoes, and better inform the population dynamics of the subsequent growing season.

In the fall, *H.* halys goes through common changes associated with diapause, with some difference in the timing of events than has been previously assumed. Photoperiod is the primary abiotic cue for diapause induction in many species of insects, including pentatomids. Prior research for this species has based phenology and population dynamics research on the assumption that a single critical photoperiod serves for both the spring termination and fall induction of diapause. Phenological studies regarding diapause termination, have assumed a critical photoperiod of 13.5L:11.5D, while population modelling combined with field observations offered 12.7L:11.3D as a better estimate of the critical photoperiod at latitudes found in the United States (Watanabe 1979, Nielsen *et al.* 2016, Nielsen *et al.* 2017). A daylength of 12.7h occurs within this region in mid-September, but my study observed a photoperiodic response to occur in early-October when the daylength ranged from 11.3h – 11h (Fig. 1) While a two-week difference in diapause induction might not seem severe (particularly in more southern latitudes), northern regions can experience rapid seasonal changes (*e.g.* temperature drops) within this relatively short time frame, and can challenge the physiological preparedness of a species. To assume that diapause is driven by photoperiod alone, independent of other environmental factors is too simplistic. Other seasonal factors such as food availability and fluctuating temperatures interacting with declining photoperiod are known to alter a photoperiodic response in other species (Takeda & Chippendale 1982; Let *et al.* 1993; Saulich & Musolin 2012).

In other insects, including pentatomids, temperature modifies the photoperiodic effect of diapause induction: elevated temperatures suppress the tendency to diapause while cold temperatures encourage diapause, even in the presence of an assumed critical photoperiod (Danilevski 1965; Saulich & Musolin 2012). In these scenarios, an effective critical photoperiod that successfully induces at least 50% of individuals into diapause would need to be re-evaluated. In addition, host-plant factors such as availability, nutritional value, and even the phenophase of the food source (*e.g.* seeds vs. leaves) can influence whether insects experience photoperiod sensitivity as the winter season approaches. A study into the pitcher plant mosquito (*W. smithii*) found that less food availability resulted in higher probabilities of diapause (Istock *et al.* 1995;

Saulich & Musolin 2002). This interaction of multiple environmental factors potentially influences the point at which *H. halys* begins to induce diapause within this region. Warmer temperatures and the presence of food sources (*e.g.* seed pods) were observed nearing the end of fall collection efforts. However, more experiments are required to identify specific cues, or combination of cues, that may be responsible for a more precise timing of diapause induction in *H. halys.*

Laboratory manipulations of relevant environmental cues for diapause induction further supports that notion that a complex interaction of factors influences the timing of diapause induction in *H. halys.* Under controlled laboratory conditions where temperature and food availability were consistent, photoperiod sufficiently induced diapause at a critical photoperiod longer than what I observed to occur in nature. Within artificially-acclimated laboratory populations, photoperiod alone effectively allowed for both metabolic (Fig. 3) and reproductive suppression (Fig. 4) in *H. halys* in photoperiods ranging from 13.5L:10.5D to 12L:12D. This is much longer than the observed 11.3L:12.7D to 11L:13D range in the field population. The difference between laboratory and field results, indicates that a critical photoperiod is necessary but not sufficient to induce diapause within this species under natural conditions.

Additional support for the timing of diapause induction was achieved through the systematic measurement and observed synchrony of reproductive arrest and metabolic suppression. Reproductive arrest is known to occur in *H. halys* prior to overwintering (Niva & Takeda 2003; Nielsen & Hamilton 2009) as determined through female dissections and the observed previtellogenic state of female *H. halys* found in or near overwintering sites (Nielsen *et al.* 2017). My study observed a similar timing of reproductive arrest, with the addition of the coinciding metabolic rates of individuals experiencing each experimental photoperiod. These simultaneous measurements offer more definitive support for diapause than prior studies have provided. However, due to the availability of individuals during testing, sample size was relatively low (n = 4-8) for female dissections that assessed reproductive status, thus additional testing would offer greater clarification to the significance of each LD regime on reproductive suppression.

While diapause effectively allows *H. halys* to reduce its seasonal winter energy expenditures, the ability to withstand potentially fatal cold temperatures of this region ultimately depends on the process of cold tolerance which occurs with diapause. Some individuals die before freezing. Survival rates of adult *H. halys* decreased as the minimum cooling temperatures were decreased. This suggests that this species is chill intolerant, which has been verified in east-coast populations. A LT50 between the temperatures -10 °C and -15 °C indicates that even prior to death by freezing, *H. halys* are not well adapted to survive at low temperatures common in central Washington. Such low survival rates would.

In understanding cold tolerance, the supercooling point (temperature at which ice forms in the body) serves as the theoretical temperature minimum at which insects can survive. Recent research by Cira *et al.* (2016), observed supercooling points (SCP) of *H. halys* in the eastern United States (Virginia, West Virginia & Minnesota), and found that the region of cold-acclimation, rather than geographical origin strongly determined supercooling ability. My research in Washington state, showed similar average SCP values (-13.88 °C) for the fall and winter seasons combined, to those found in Virginia and West Virginia (-13.48 °C). In addition, my research compared SCP values of individuals from either a naturally-acclimatized outdoor population and an artificially-acclimated laboratory population (Fig. 6). These comparisons allowed for a better understanding of how photoperiod and temperature influence cold tolerance of this species. No prior research has successfully provided quantifiable support to the relationship between diapause and cold tolerance in *H. halys*.

Diapause has been found as a prerequisite to maximum cold tolerance in other pentatomids (Šlachta *et al.* 2002), and my data supports a similar relationship in *H.* halys: while diapause is a prerequisite for developing cold tolerance is not the only driving factor. A subsequent period of cold-acclimation is also necessary to achieve the maximum levels of cold-tolerance in this species. My data provides support for this interaction by (1) tracking the supercooling ability and metabolic rates of individuals in a naturally-acclimated outdoor population, and (2) comparing the supercooling abilities of outdoor individuals to those within a laboratory population. This approach helps to clarify how specific factors (*e.g.* photoperiod and temperature) contribute to winter survival preparation. While there were no significant differences in supercooling values across season (Fig. 5), the changes observed in the metabolic rates of individuals undergoing the same seasonal transition were both significant (Fig. 1) and followed patterns associated with each stage of the diapause process (*i.e.* induction, preparation, initiation, *etc.*). Other species have strong links between diapause and cold tolerance (*e.g.* mountain pine beetle (*Dendroctonus ponderosae*); Lester & Irwin 2012), but the lack of synchrony between metabolic rate and supercooling, may indicate that there is no strong and direct physiological link between cold tolerance and diapause in *H. halys.* A comparison of the SCP values of the field and laboratory populations also illustrates this weak linkage. Our ‘short-day’ laboratory group experienced a photoperiod (12L:12D) that is known to induce diapause in a laboratory setting (Watanabe 1979; Niva & Takeda 2003). This photoperiod, interacting with an acute and rapid cold-acclimation process, resulted in the lowest recorded SCP values, even lower than any SCP value observed in the outdoor population. Minimum mean SCP values in field and lab populations was -15.1 ±0.46 and -17.1 ±0.66 (males) and -13.14 ±0.60 and -15.1 ±0.57 (females), respectively (Fig. 6). We suspect, that fluctuating temperatures experienced by the outdoor population, had a negative influence on the ability for *H.* halys to become cold tolerant. As a result, the SCP values of the outdoor population were much closer to those observed in the non-diapausing ‘long-day’ laboratory population (Fig. 6).

My data suggest that it is not only the environmental factors themselves, but also the actual seasonal timing of these factors, that may greatly affect the ability of *H. halys* to successfully survive cold adverse winter conditions. The low temperatures experienced in nature during this study ultimately proved fatal to the field population, which experienced 100% mortality prior to the end of winter. While this population did experience naturally short photoperiods that were effective in suppressing metabolism (Fig. 1), the subsequent seasonal period there-after potentially failed to provide a sufficient cold-acclimation process that would encourage maximal levels of cold-hardiness, and ultimately survival. That is, *H. halys* experiencing natural outdoor conditions successfully fulfilled the prerequisites to enter diapause, but did not become fully cold tolerant, potentially due to an inadequate cold acclimation process. These findings offer up a rather complex question: was the reason for the low survival of *H. halys* under natural conditions the result of: (1) the postponed induction of diapause, (2) the lack of a subsequent cold-acclimation process, or (3) a combine of the two? A population of *H. halys* that experience postponed seasonal diapause induction, could lack adequate cold tolerance to survive the onset of winter temperatures. Diapause has been found to enhance cold tolerance in other insects (Denlinger 1991), and the timing of diapause plays a key role. Entrance into diapause too early cuts short the growing season, whereas entrance too late can leave the insects susceptible to higher mortality levels by cold weather early in the fall. In some cases, the timing of seasonal development presents the most immediate impediment to range expansion in the temperate zone (Bradshaw *et al.* 2000, 2001). This later than expected induction of diapause observed in *H. halys* may turn out to be maladaptive for an invasive population moving into more northern and cold regions. While this proved fatal for *H. halys* over the course of this study period, it may also have further reaching implications, contributing negatively to the ability for this pest to successfully establish within cold regions in the long-term.

The daily minimum air temperatures at both the artificial and naturally-selected overwintering sites (Fig. 7 & Fig. 8) provided greater clarity to the ineffective levels of cold tolerance acquired by *H. halys* in central Washington, and their inability to survive natural conditions. During this study, the natural cold-acclimation process experienced by *H. halys* was ineffective in producing a sufficient level of cold hardiness necessary to survive these regional conditions. On multiple days during the study period daily minimum temperatures were low enough to cause high rates of mortality in overwintering populations.

Our data indicate that under natural conditions *H. halys* does not effectively survive cold winters, however it does persist in cold regions by overwintering within thermally-insulated human-made structures, of which *H. halys* are known to utilize (Watanabe *et al.* 1994; Lee *et al.* 2014). There are however, certain disadvantages to overwintering for insects that includes factors such as immobility (*e.g.* potential predation), and other biological risks (*e.g.* starvation, desiccation and cold injury) that emphasizes the importance of good site selection (Leather *et al.* 1993). The data collected from the overwintering site in Walla Walla, WA provided an example of a potential outcome of overwintering site success and emphasized how the selection of an 'weak' thermal refuge can be detrimental to overall survival (Fig. 8). At the Walla Walla overwintering site, survival was low due to the cold, indicating a poorly selected overwintering site. However, high temperatures within overwintering sites may also cause high mortality. Low temperatures to reduce energy consumption is particularly important to many species at are non-feeding throughout the winter (Tauber *et al.* 1986). There is an energetic trade off to adapting to cold, freezing winter temperatures. Lower metabolic rates mean less seasonal energy loss, and thus helps in maintaining efficient energy conservation, leading to high survival and potential adult fecundity (Irwin & Lee 2000; Irwin *et al.* 2002). The selection of an overwintering site is of particularly great importance in species of insects that are not freeze tolerant and serves as an external physical defense against winter mortality. *H. halys* utilizes chemical and tactile cues to locate overwintering sites (Toyama *et al.* 2006) and this species has been detected to overwinter within the natural landscape (Lee *et al.* 2014). However, more research needs to be done to further investigate the microclimate variables (*e.g.* humidity, responses to thigmotaxis, light availability) aggregations of *H. halys* potentially select for within both natural and human-made overwintering sites that encourages higher rates of winter survival. Better assessment of microclimate will help to tease apart the exact environmental variables *H. halys* experiences, and how they tolerate adverse winter conditions, and aid in improved IPM tactics.

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