WTFRC Project Number:

Project Title: Cold Tolerance, Diapause, and Survival of Brown Marmorated Stink Bugs
    (*Halyomorpha halys*)

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Total Project Request: **Year 1:** $34,800   **Year 2:** $33,200
Other funding sources: None   **WTFRC Collaborative expenses:** None

Item	2016-2017 July1 - June 30	2017-2018 July 1 – June 30
Salaries		
Benefits		
Wages	28560	28800
Benefits	1940	1950
RCA Room Rental		
Shipping		
Supplies	2800	950
Travel	1500	1500
Plot Fees		
Miscellaneous		
Total	34800	33200

Footnotes: Benefit rate is 9% for CWU academic year, 3% for summer.

Budget Explanation:

The salary requested will support a graduate student during the summer and academic year as she performs the activities outlined in this proposal. Irwin's laboratory already has a functioning respirometry system so funds are requested only for scrubbing chemicals (i.e., Ascarite and Drierite) and occasional small parts such as fittings and tubing. The laboratory is fully equipped for cold tolerance measurements so no significant costs will be incurred. Other than some inexpensive chemicals, Dr. Neven is not requesting any funds. Other expenses include a lumite outdoor insect cage (#1412C, BioQuip Products) and "Bug dorms" (#1462C, BioQuip Products). Both are requested to raise BMSB in captivity and within an outdoor field enclosure. Mileage is requested to defray the costs of travel to locate sites for collection and monitoring of BMSB.

OBJECTIVES

1) Describe characteristics of diapause in this species, including the seasonal timing of metabolic suppression and arrested development, and the cues for diapause induction (e.g., critical photoperiod).
2) Measure seasonal changes in cold tolerance for overwintering adults (e.g., determination of supercooling points and chill-intolerance survivorship).
3) Identify any potential links between diapause timing and seasonal changes in cold tolerance.
4) Describe overwintering site preferences, including microclimate, and measure winter survival under field conditions (including selected natural sites and an enclosure study).

SIGNIFICANT FINDINGS

Objective 1: We were able to successfully characterize the seasonal timing of diapause for BMSB in Eastern Washington, and provide the first verified measurement of critical photoperiod (e.g. photoperiodic threshold) for this species.

- Metabolic rates of field-maintained BMSB fell as they went into diapause (the insect version of hibernation) in late-October through November.
- Both metabolic rate and reproductive status of laboratory-maintained BMSB indicate that the critical photoperiod (that is, day length) of 13.5h induces diapause in this species.
- The critical photoperiod of 13.5h is necessary but not sufficient (that is, a required prerequisite) for diapause induction to occur naturally in BMSB.

Objective 2: Seasonal changes in the cold tolerance of overwintering adult BMSB were successfully quantified within Eastern Washington populations (from Walla Walla and Yakima).

- The supercooling point (SCP, the temperature at which ice forms in the body) of adult BMSB fell as Fall moved into Winter.
- Males supercooled better than females, with a mean SCP of $5.79 \pm 0.2°F$ (mean $\pm$ standard error) in males and $9.26 \pm 0.2°F$ in females.
- Supercooling points for BMSB were not below the minimum freezing temperatures experienced in Eastern Washington during our study and, thus, full mortality of the outdoor population was observed during both sampling seasons.
- BMSB in Eastern Washington are chill-intolerant (that is, individuals may die even before ice forms in their bodies), as has been observed in other parts of the United States.
- Temperatures of 14 to 5°F cause 50% mortality of naturally cold-acclimated BMSB, which are much higher than normal winter temperatures in this area.

Objective 3: There were no strong links between diapause and cold tolerance indicated in adult BMSB.

- Diapause induction proved to be necessary but not a sufficient stand-alone factor to induce high levels of supercooling ability in BMSB. A period of cold-acclimation is also necessary to achieve maximum supercooling ability.

Objective 4: A naturally selected overwintering site in Walla Walla, WA and our outdoor enclosure in Yakima, WA were successfully monitored for temperature throughout the fall and winter.

- BMSB sought refuge beneath the cedar-shingle siding of a residential house in Walla Walla, WA, within a few blocks of Franklin Park, a known BMSB 'hot spot'.
- Temperature probes placed internal and external the shingle siding recorded minimum air temperatures of 3.9°F and 1.3°F respectively, below the observed minimum SCP values, and

within the range where probability of survival is significantly decreased due to chill intolerance.
- Temperature probes at the Yakima enclosure experienced lower minimum temperatures than the Walla Walla site, with a recorded minimum of -0.6°F.

RESULTS & DISCUSSION

In our temperate climate, winter conditions affect the population dynamics of BMSB. In other stink bugs, photoperiod plays a key role in regulating the seasonal timing of diapause and plays a major role in limiting northward range expansion (Musolin & Numata 2003). Similarly, the northward expansion of BMSB may be limited in regions where cold weather arrives before diapause is induced via shorter photoperiods. Metabolic rates of BMSB from a naturally-acclimated outdoor population underwent significant metabolic suppression (an indicator of diapause) throughout the sampling period. When measured at three measurement temperatures, all treatments significantly differed across months in 59°F ($F_{(7)}$ = 6.4, P <0.001), 50°F ($F_{(6)}$ = 3.9, P <0.05), and 49°F ($F_{(7)}$ = 2.8, P <0.05). Metabolic rates showed no significant difference between sexes over time for 59°F ($F_{(1)}$ = 0.29, P = 0.59), 50°F ($F_{(1)}$ = 1.75, P = 0.19), and 49°F ($F_{(1)}$ = 3.87, P = 0.056) (Fig. 1). The interaction term between 'date' and 'sex' was evaluated, and did not significantly improve the model. These results demonstrate that adult BMSB in eastern WA are able to fully transition into a diapause state that is prepared to overwinter.

A population of BMSB that enter diapause later than induced by day-length alone, 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 being killed by cold weather early in the fall. In some cases (for example, the Pitcher Plant Mosquito (*Wyeomyia smithii*)) the timing of seasonal development presents the most immediate impediment to range expansion in the temperate zone (Bradshaw et al. 2000, 2001). In the Southern Green Stink Bug (*Nezara viridula*) in Japan, the critical photoperiod for diapause induction proved to be maladaptive, preventing the species from moving northward. In colder regions, individuals were unable to enter diapause before seasonal cold temperatures inflicted high rates of mortality (Musolin 2007). Furthermore, diapause has been found as a prerequisite to maximum cold tolerance in the southern green stink bug (*Nezara* viridula) (Slachta et al. 2002). Determining the relationship between diapause and cold tolerance in BMSB has remained a knowledge gap in understanding how this pest will biologically function and survive within a region.

Prior research has assumed that a critical photoperiod (day length) of 13.5h is adequate to induce diapause in BMSB, but our study is the first to experimentally verify the accuracy of this value (Watanabe 1979; Nielsen et al 2016). Throughout the determination of the critical photoperiod, temperature was held constant at 68°F as the available light hours gradually decreased from 16L:8D to 12L:12D at a rate matching the natural seasonal changes of day-length in Yakima, WA. Metabolic rates of stink bugs from the artificially-acclimated laboratory population, measured at the incubation temperature of 59°F differed significantly across light hour availability ($F_{(7)}$ = 4.35, p < 0.001). Metabolic rates did not differ significantly between sex ($F_{(1)}$ = 3.78, P > 0.05) (Fig. 2), and the interaction between 'light hour' and 'sex' was also evaluated and did not significantly improve the model. BMSB are known to go into reproductive diapause prior to overwintering (Niva & Takeda 2003; Nielsen & Hamilton 2009), and the probability of female reproductivity observed from the same regime of decreasing photoperiod, also differed significantly across light hour availability ($X^2_{(8)}$= 15.53, P-value = 0.049) (Fig. 3). As the days grew shorter, BMSB showed a state of both metabolic and reproductive suppression (absence of mature oocytes during dissections) (Fig. 4), within the same light hour range of 14h to 13h (Fig. 2 & 3). Under controlled conditions (i.e., constant temperature with a step-wise decrease in photoperiod), our results strongly support that a critical photoperiod of 13.5h can induce suppression of metabolism and reproduction in BMSB.

Prior research has used the 13.5h day-length as the critical photoperiod to model population phenology and dynamics across various geographic regions (Nielsen et al. 2016), but our data suggest that this is too simplistic. These models assume that diapause is driven by photoperiod alone, independent of temperature in natural settings (Watanabe 1979; Yanagi & Hagihara 1980; Nielsen et al. 2016). Our data suggest that a critical photoperiod may be necessary, but is not sufficient to induce diapause in BMSB. Other seasonal factors such as food availability and fluctuating temperatures interacting with declining day-length, could potentially extend the point of diapause induction for BMSB, as observed in our outdoor population. This interaction of multiple environmental factors potentially influences the point at which BMSB begin to induce diapause. Metabolic suppression occurred naturally later in the season (Oct – Nov) (Fig. 1), where seasonal photoperiods were shorter than the laboratory determined critical photoperiod of 13.5h.

Mathematical models, based on climatic variables, predicting potential range expansion of BMSB demonstrated that minimum monthly temperature plays a significant role in determining range (Zhu et al. 2012). Supercooling ability is found in many insects, a process where no ice forms in the body even though it may be at temperatures well below freezing. The supercooling point (temperature at which ice forms in the body) serves as the theoretical temperature minimum at which insects can survive because once ice forms, the insect will die. Recent research by Cira et al. (2016), observed supercooling points (SCP) of BMSB in the eastern United States (Virginia & Minnesota), and found that the region of cold-acclimation, rather than geographical origin, strongly determined SCP ability. Our research performed in Washington State, showed similar supercooling points during the fall and winter to those found in Virginia and West Virginia. Our research also compared the SCP values of individuals from a naturally-acclimated outdoor population and an artificially-acclimated laboratory population. These comparisons allowed for a better understanding of how photoperiod and temperature influence cold tolerance in BMSB.

Supercooling points from the naturally-acclimated outdoor population showed significant difference between seasons (Fall = Sept – Nov, Winter = Dec – Feb) ($F_{(2)}$ = 3.32, P = 0.041), while SCP from the artificially-acclimated laboratory population differed significantly between Light/Dark Hour (LD) regimes (LD = 16L:8D, SD = 12L:12D) ($F_{(1)}$ = 5.74, P = 0.023). An analysis of both populations combined showed SCP values with significant differences across LD regime ($F_{(3)}$ = 4.55, P = 0.0048), sex ($F_{(1)}$ = 9.49, P = 0.0026), and mass ($F_{(2)}$ = 4.62, P = 0.033) (Fig. 5). The addition of body mass as a covariate did not reduce the significance between sexes in the model. Supercooling points measured within the 'fall' group, had a mean SCP of 9.3 ± 0.7°F in females and 5.8 ± 0.7°F in males, while the 'short-day' group had a mean SCP of 4.9 ± 1.0°F) in females and 1.4 ± 1.2°F in males (Fig. 5). Our results for supercooling ability show that neither males or females supercool well enough to survive natural minimum temperatures recorded in eastern Washington during the sampling period (Fig. 7 & 8). The 'short-day' group were observed to have supercooling points significantly lower than field individuals measured during the coldest recorded instances in winter. This data suggests a relationship between cold-tolerance and diapause like those found in the Italian striped bug (*Graphosoma lineatum)* (Šlachta et al. 2002), in which diapause is necessary for developing cold tolerance, but is not the only driving factor. A subsequent process of cold acclimation is also necessary to achieve the maximum levels of cold tolerance in this species. The 'short-day' group illustrates this relationship, in which an acute and rapid cold-acclimation regime resulted in SCP values significantly lower than even those observed in the field population. The long-term and fluctuating rate of cold acclimation experienced by the field populations only produced SCP values similar to the non-diapausing 'long-day' lab population. No prior research has successfully provided quantifiable support to the relationship between diapause and cold tolerance in this species.

Supercooling is a known mechanism to survive adverse winter conditions, and no present research has found any true bug (Heteroptera) to survive freezing (Saulich & Musolin 2012). Though BMSB can supercool to very low temperatures, recent research by Cira et al. (2016) determined that the cold-tolerance strategy of BMSB is chill intolerance, in which adults die at significantly higher temperatures than they freeze. Our research produced comparable results. Survival rates began to

decline when temperature was reduced to 23°F, and showed a significantly reduced probability of survival between 14 and 5 °F. These temperatures at which survival declines are much higher than reported for supercooling ability. Naturally cold-acclimated adult BMSB in eastern Washington, exposed to minimum cooling temperatures of 32, 23, 14 and 5°F, showed significant differences in the probability of survival across temperature ($X^2_{(3)}$ = 9.02, P-value = 0.028), sex ($X^2_{(1)}$ = 6.22, P-value = 0.012), and mass ($X^2_{(1)}$ = 5.09, P-value = 0.024). BMSB survival dropped significantly below a 50% threshold between the minimum temperatures of 14 and 5°F (Fig. 5).

To relate our experimental findings to BMSB populations overwintering in nature, we monitored ambient air temperatures at our field-maintained outdoor enclosure in Yakima, WA (Fig. 7), and a naturally-selected overwintering site in Walla Walla, WA (Fig. 8). At the latter site, we monitored air temperatures on the interior and exterior of cedar-shingle siding on a residential home. The observed minimum interior and exterior temperatures were 3.9°F and 1.3°F with an average temperature difference between the two of 2.5°F throughout the sampling period. While we were not able to directly monitor the mortality of overwintering BMSB at this site, observations of both the homeowners and a licensed pest manger (Dr. Albert Grable), detected negligible populations of BMSB emerging from the structure in the spring, which supports out assumptions of high mortality due to chill intolerance and minimum SCP (Fig. 7). BMSB are known to select for cool, dry, enclosed spaces as overwintering sites as a way to avoid contact with wet conditions, and man-made structures provide these exact specifications (Lee et al. 2014).

Our research emphasizes the importance of human structures in successful overwintering of BMSB in eastern and central Washington. BMSB in our area can complete their life cycle, enter diapause, and become cold tolerant. However, normal winter temperatures will kill BMSB – only with an artificially heated site could they survive. Growers may be able to minimize BMSB populations by carefully inspecting buildings and other thermally-buffered structures where BMSB might successfully overwinter.
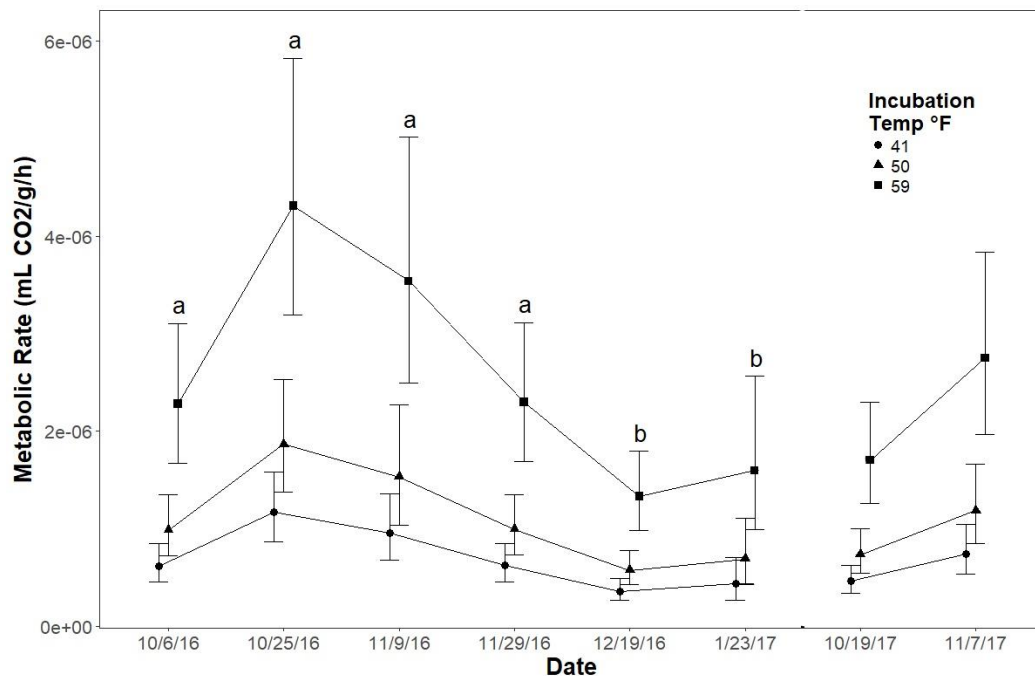

Figure 1. Mean metabolic rates including upper and lower confidence intervals, measured at 41, 50, and 59°F of overwintering adult brown-marmorated stink bugs (*Halyomorpha halys*). Metabolic rates of stink bugs, from the naturally-acclimated outdoor population, significantly differed across months in 59°F ($F_{(7)}$ = 6.4, P <0.001), 50°F ($F_{(6)}$ = 3.9, P <0.05), and 41°F ($F_{(7)}$ = 2.8, P <0.05). When

comparing two sample points, those assigned different letters (i.e. a – b) are significantly different from each other.
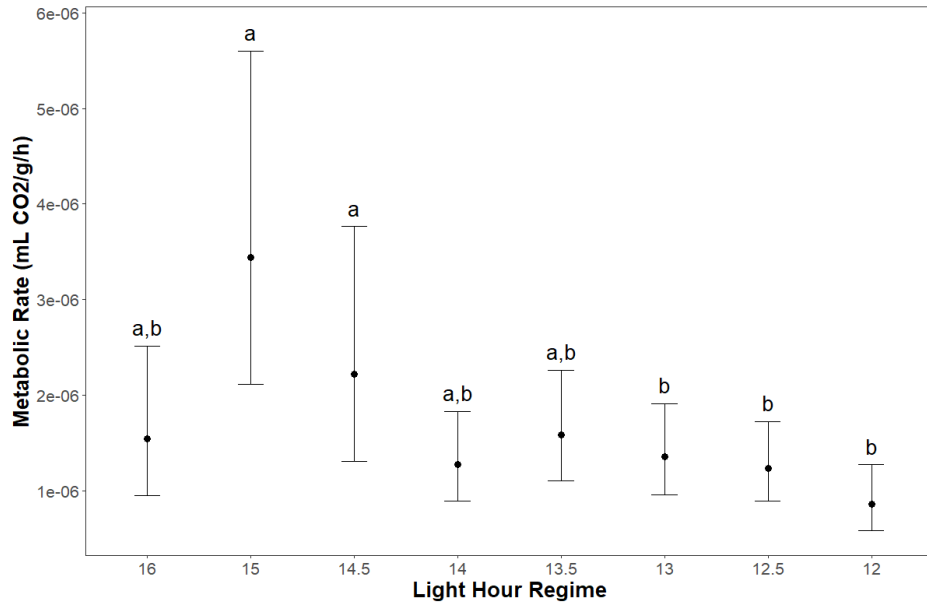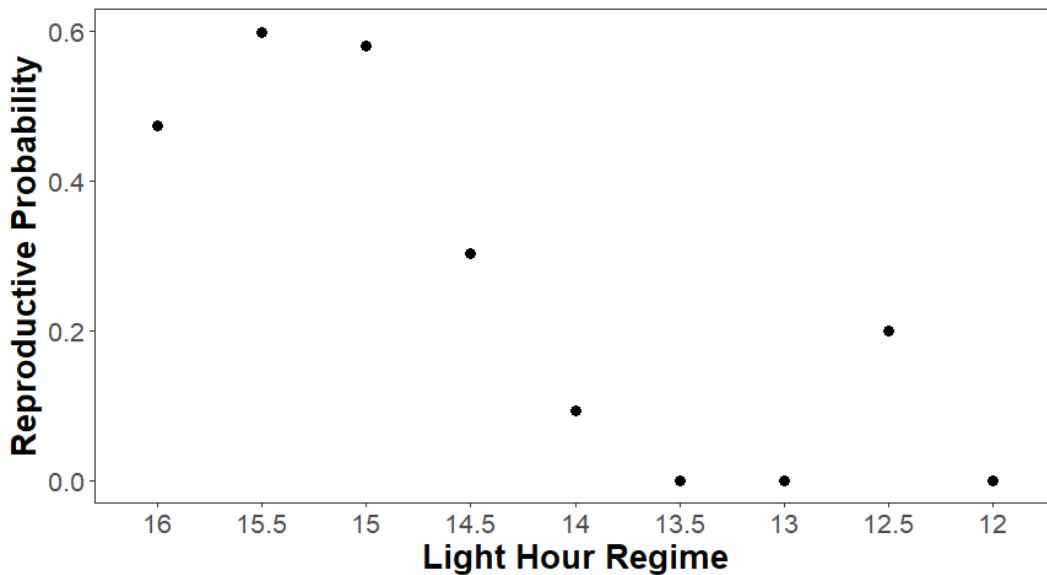

Figure 2. Mean metabolic rate including upper and lower confidence intervals, measured at 59°F of adult brown-marmorated stink bugs (*Halyomorpha halys*). Metabolic rates differed significantly across light hour availability ($F_{(7)} = 4.35$, P <0.001). Metabolic rate response to incubation temperature showed an initial increase in metabolic rate during the photoperiod of 15 h, followed by a decreasing trend throughout the photoperiod transition to 12 h. When comparing two sample points, those assigned different letters (i.e. a – b) are significantly different from each other.

Figure 3. Probability of female Brown-marmorated stink bugs (*Halyommorpha halys*) being in a reproductive state at different day-lengths. The probability of reproductive BMSB differed across LD regime ($X^2_{(8)} = 15.53$, P-value = 0.049).
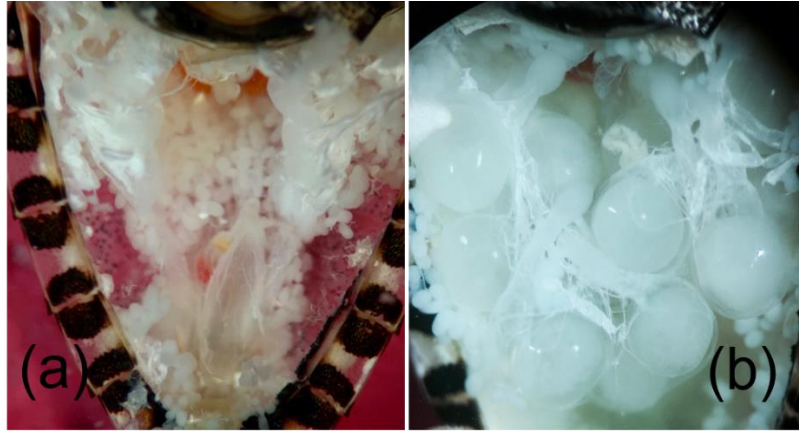
Figure 4. Female BMSB dissections demonstrating (a) lack of mature oocytes indicating a non-reproductive female (reproductive suppression during diapause) and (b) presence of mature oocytes indicating a reproductive female.
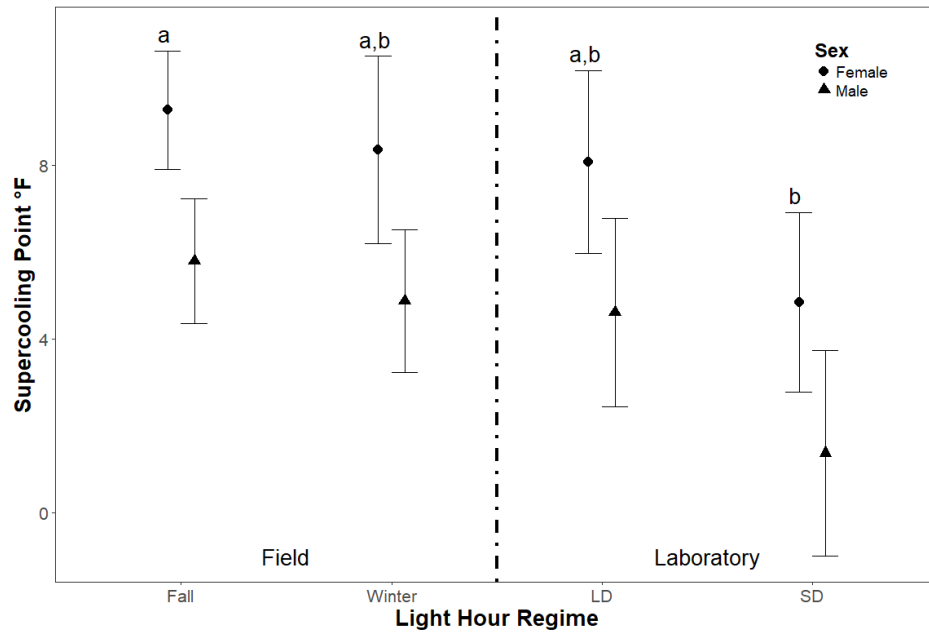

Figure 5. Mean supercooling points of adult brown-marmorated stink bugs (*Halyomorpha halys*) including upper and lower confidence intervals, that differed significantly across day length (LD = long day, SD = short day), sex and mass ($F_{(3)} = 4.55$, $P = 0.0048$; $F_{(1)} = 9.49$, $P = 0.0026$; $F_{(2)} = 4.62$, $P = 0.033$). When comparing two sample points, those assigned different letters are significantly different from each other for both males & females.
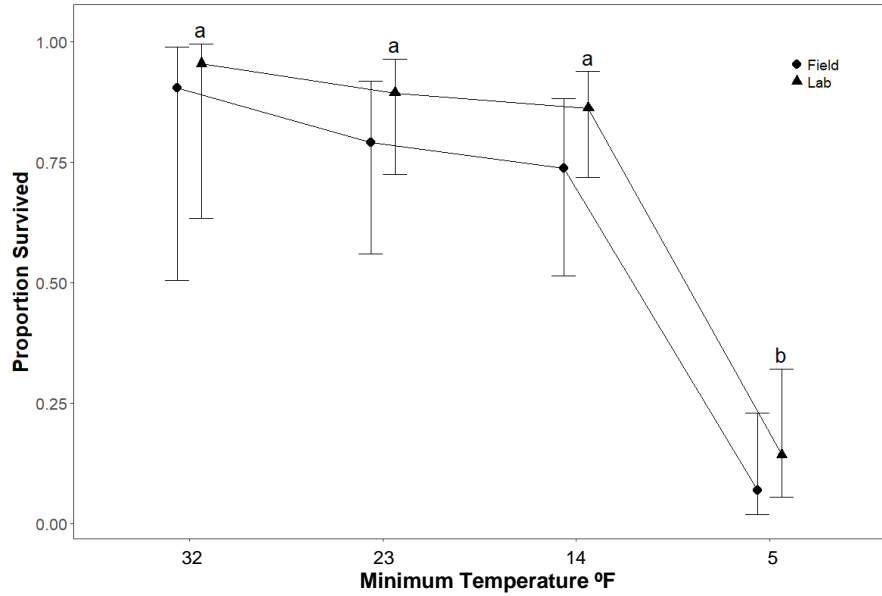
Figure 6. Survival of adult Brown-marmorated stink bugs (*Halyomorpha halys*) exposed to a series of minimum temperatures, show significant differences in the proportion survived across temperature treatments ($X^2_{(3)}$ = 51.598, p < 0.01). Survival dropped significantly with exposure to 5°F.
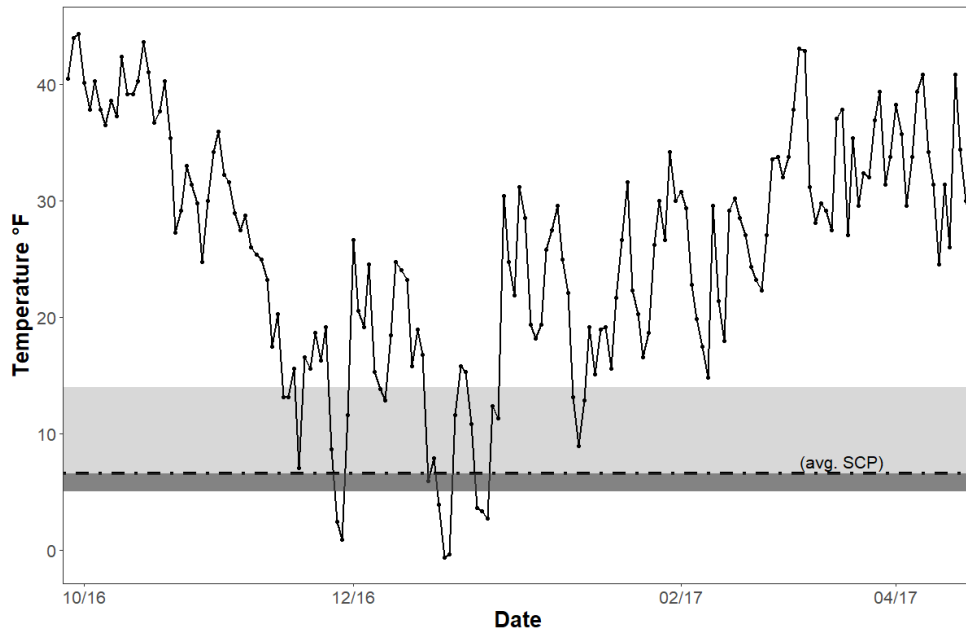

Figure 7. Daily minimum temperature (°F) recorded at an outdoor field enclosure in Yakima, WA from 10/28/16 to 4/15/17, with an observed minimum temperature of -0.65 °F on 1/6/17. The dashed line indicates the observed mean supercooling point of 6.6°F for BMSB, and the shaded area indicates the temperature range at which the probability of survival via chill intolerance is significantly reduced, with the shaded region below avg. SCP resulting in near full mortality.
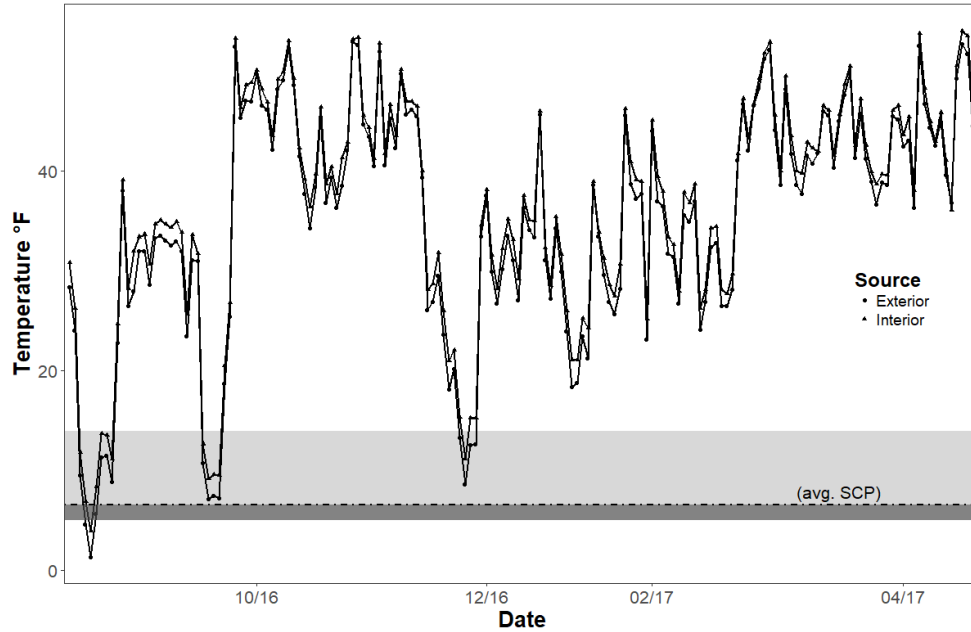
Figure 8. Daily minimum temperature observed in Walla Walla, WA, at a naturally-selected overwintering site of Brown-marmorated stink bug (*Halyomorpha halys*), from 10/27/16 to 4/15/17. BMSB selected the tight spaces beneath cedar-shingle siding as refuge, with observed minimum temperatures from the interior and exterior of 3.9°F and 1.3°F. The dashed line indicates the observed mean supercooling point of 6.6°F for BMSB, and the shaded area indicates the temperature range at which the probability of survival via chill intolerance is significantly reduced, with the shaded region below avg. SCP resulting in near full mortality.

EXECUTIVE SUMMARY

Our investigations into BMSB achieved all our objectives. We sought to describe both diapause regulation and cold tolerance in BMSB in eastern and central Washington State, both factors which have major impacts on the success of BMSB as it continues to expand northward in the Pacific Northwest. Metabolic suppression and cessation of egg development (indicators of diapause) occurred during the fall and winter, with deep suppression occurring late-October into November. Both metabolic rate and reproductive status of laboratory-maintained BMSB demonstrated that diapause is initiated by a day-length of 13.5h. This is similar to the critical day-length for diapause estimated by other studies, but this is the first systematic measurement of this value.

A comparison of laboratory and enclosure populations suggests that the 13.5h photoperiod is indeed necessary for diapause induction, but it is not sufficient on its own to induce diapause. Other seasonal factors (for example, food availability and fluctuating temperatures) interacting with declining day-length can delay diapause induction for BMSB, as we observed in our enclosure population. This is important, because a population of BMSB that experience postponed seasonal diapause induction, could risk obtaining insufficient levels of cold tolerance to survive the onset of cold temperatures, leaving them at higher risk of mortality.

The cold tolerance of BMSB in eastern WA is comparable to those in other parts of the USA. Supercooling ability changed little from fall into winter, and although males supercooled better than females (5.7°F vs. 9.2°F), an average minimum SCP value of 6.6°F for adult BMSB is not enough to survive the minimum freezing temperatures experienced in the region. BMSB also succumbed to chilling injury (death caused at temperatures before ice forms in the body) at temperatures between 14°F and the average minimum supercooling point of 6.6°F. Given their sensitivity to chill injury and their high supercooling points, BMSB are unlikely to survive the winter in central and eastern Washington State. We measured minimum air temperatures of -0.6°F and 1.3°F at our field-maintained outdoor enclosure in Yakima, WA and at our natural overwintering site in Walla Walla, WA, respectively. These were lower than the average SCP of 6.6°F measured from field-maintained individuals throughout the sampling period. For the population we maintained outdoors in Yakima, the mortality rate was 100%. While we were not able to directly monitor the mortality of overwintering BMSB at the Walla Walla site, observations of both the home owners and a licensed pest manger (Dr. Al Grable), detected no BMSB emerging from the structure in the spring, which supports our assumptions of high mortality due to chill intolerance and minimum SCP. BMSB often select cool, dry, enclosed spaces as overwintering sites, and these are often human-made structures.

Implications for control of BMSB

Our research further emphasizes the importance of human structures, especially buildings, to protect BMSB from the coldest winter temperatures. The cold winters of central and eastern Washington will significantly reduce or even eliminate BMSB overwintering in unheated sites. However, populations will persist where they can find thermally buffered or heated overwintering sites. Unfortunately, we expect that the mild winters of western Washington will not cause significant winter mortality so BMSB will likely become established unless other factors limit the species distribution.

Given that BMSB cluster during the winter in large aggregations, management of overwintering sites could provide an important area of management for the species. Eliminating populations overwintering in nearby buildings (especially heated buildings), would reduce the populations size in the subsequent growing season throughout the state.