

## Cold Tolerance of *Megacopta cribraria* (Hemiptera: Plataspidae): An Invasive Pest of Soybeans

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Subject Editor: Melody Keena

Received 17 April 2017; Editorial decision 26 July 2017

### Abstract

Kudzu bug, *Megacopta cribraria* Fabricius (Hemiptera: Plataspidae), first discovered in the United States in 2009, is an invasive pest of soybeans. From 2013 to 2016, Maryland has been the northern limit of its distribution in the United States. We sought to determine the physiological cold temperature limits, timing of movement to overwintering locations, and to characterize overwintering microhabitat temperature. We measured supercooling point (SCP) on three populations from distinct USDA plant hardiness zones in Maryland and Virginia between October and December of 2015. The average SCP across all sample months and populations was  $-12.6^{\circ}\text{C}$  and no consistent trend of month or population location were observed. Additionally, we assessed the lower lethal temperature to kill 50% of the population ( $\text{LLT}_{50}$ ) at the same population locations in October and November 2015. The average  $\text{LLT}_{50}$  over both months and all three population locations was  $-5.1^{\circ}\text{C}$ . Again, no consistent trend based on population location was observed but we did find a modest depression in the  $\text{LLT}_{50}$  values between October and November. We observed that kudzu bug overwinters in leaf litter and begins to move into the litter in late November to early December. Leaf litter moderates day to night temperature differences and was warmer than ambient temperature by an average of  $0.7^{\circ}\text{C}$ . Evidence suggests that the cold tolerance of the kudzu bug limits its distribution north of Maryland.

**Key words:** Kudzu bug, supercooling point, lower lethal temperature, microclimate, overwintering

Kudzu bug, *Megacopta cribraria* Fabricius (Hemiptera: Plataspidae), is a recent invader to the United States arriving in Georgia in 2009 (Suiter et al. 2010). Distribution had expanded to Maryland by 2013 but has since not moved any farther northward (Grant et al. 2014, Leslie et al. 2014). Kudzu bug can be extremely damaging at high densities to soybeans. Yield reductions ranged up to 59% in untreated plots with densities up to 182 nymphs and adults per plant (Seiter et al. 2013). In recent years, kudzu bug populations are declining in much of the Southeast United States. From 2013 to 2016, the kudzu bug populations in Georgia have declined due to factors including an egg parasitoid, *Parateleonomus saccharalis* Dodd (Hymenoptera: Platygasteridae), an entomopathogenic fungus, *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Hypocreales: Calvicipitaceae) and winter kill (Gardner and Olson 2016). In Maryland, there are no records to date of either *B. bassiana* or *P. saccharalis* in the state. Thus, a lack of observations of kudzu bug populations north of its 2013 range, even though host plants are available, suggests they have reached their northern limit. It appears based upon climate conditions that physiological temperature limits may be the determining factor of their northern limit.

Cold tolerance, the physiological ability to withstand winter temperatures, can be a powerful tool in assessing winter mortality rates as well as possible geographical range limits (Sinclair et al. 2015).

Many insects face a physiological barrier along the northern extent of their range. Mapping the physiological lower lethal limits can elucidate the potential range expansion of a new invasive or range shifts under climate change (Crozier 2003). Lower lethal limits aid in predictions of winter mortality, which serve in predicting fluctuations in population levels (Cannon 1998). Thus, knowledge of kudzu bug lethal temperature limits could inform the prediction of potential colonization areas as well as annual density within the current distribution. Kudzu bug overwinters as adults in leaf litter and under tree bark but little else is known about their overwintering behavior (Lahiri et al. 2015). To date, no studies have quantified their lower physiological temperature limits in the United States.

Cold tolerance is often divided into two strategies: freeze-tolerant and freeze-avoidant (Bale and Hayward 2010). Freeze-tolerant insects can withstand ice nucleation in the body cavity whereas freeze-avoidant cannot. For freeze-avoidant species, ice nucleation is fatal and often their lower lethal temperature (LLT) is well above their freezing point (Sinclair et al. 2003, Bale and Hayward 2010). A common method to assess cold tolerance is to quantify the supercooling point (SCP). SCP is the lowest temperature possible before the onset of ice crystallization in the body. Often SCP is used to determine the broad difference between freeze-tolerant and freeze-avoidant species

(Bale and Hayward 2010, Denlinger and Lee 2010). Freeze-tolerant species can withstand temperatures below their SCP; in contrast, freeze-avoidant species cannot and often die at temperatures above their SCP (Bale and Hayward 2010). For predictive purposes, the SCP of a freeze-avoidant species can be an absolute geographic range boundary where such minimum temperatures occur.

In addition to SCP, LLT limits can estimate a wide range of winter mortality. The LLT to kill 50% of the population ( $LLT_{50}$ ) is a common measure of lethal temperature mortality but is dependent on exposure time (Sinclair et al. 2015). LLT can predict winter survival under specific conditions, especially those in which the SCP would be uninformative such as temperatures above the SCP in a freeze-avoidant species. In biocontrol agents,  $LLT_{50}$  values correspond with range edges where establishment is patchy because of the winter mortality (Byrne et al. 2004).

Further considerations in evaluating cold tolerance include the variation across latitudinal gradients and physical factors such as body mass and gender. Gender and body mass are well known factors that affects cold temperature physiology, with gender causing bimodal distributions and larger masses increasing the SCP of an individual (David and Vannier 1996, Salin et al. 2000, Renault et al. 2002, Hahn et al. 2008). Geographically, SCP and LLT tend to increase moving equatorially (Addo-Bediako et al. 2000). For invasive insects, thermal plasticity is often limited due to founder effects (Colautti et al. 2010). However, given time, local adaptation may cause a shift in thermal tolerance or behavior. The brown marmorated stink bug, *Halyomorpha halys* Stål (Hemiptera: Pentatomidae), has a significantly different yearlong mean SCP between Minnesota and Virginia. When analyzed seasonally, the Minnesota population acclimated to cold weather sooner but the mean SCP during winter was not statistically different between the two locations (Cira et al. 2016). In Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), geographically distinct populations did not show a difference in SCP but did show an adapted behavior to avoid cold temperatures (Izzo et al. 2013). These cases highlight the importance of behavior and timing of induction cues along with physiological response as factors of cold tolerance.

Microhabitats often influence the survival of insects over the winter. Many insects take advantage of burrowing or moving closer to the soil for the geothermal buffering of the earth (Sinclair et al. 2003, Bale and Hayward 2010). For insects near urban developments, seeking shelter in homes or underground pipes can provide a refuge from cold temperatures (Labrie et al. 2008, Inkley 2012). The timing of the movement to these microhabitats can be critical for the survival of an insect. For instance, a cold front experienced before moving to protected areas could increase the mortality of a population (Ungerer et al. 1999). Characterization of both the timing of movement to and the temperature buffering of microhabitats are important in accurately predicting winter mortality or distributions through measuring ambient temperature.

Climate change will also affect the realized distributions of insects bound by cold tolerance. Winter temperatures are warming at a faster rate than summer temperatures and a conservative model, RCP 4.5, predicts a 3°C winter warming by the end of the century (IPCC 5th Report, 2013). The rapid warming of winters potentially allows for a relaxation of cold tolerance boundaries thus extending the possible range of an insect.

As the kudzu bug has great economic damage potential to soybean growers, we determined its cold tolerance to predict future population densities and range shifts. First, we determined the SCP and  $LLT_{50}$  physiological response across gender, population, and fall months. This information informs the geographic pattern and

variation of the populations along the northern range limit. Second, we observed the timing of movement to and location of overwintering to clarify behavioral actions of bugs during fall and into winter. Third, we measured microhabitat temperature of overwintering sites relative to ambient temperature to aid in predictive modeling of microclimatic temperatures experienced by the kudzu bug. Finally, we evaluated the physiological limits for synchrony with the current distribution and future distributional shifts under climate change scenarios.

## Materials and Methods

### Measurement of SCP

SCP methods are congruent with a review of measuring cold tolerance by Sinclair et al. (Sinclair et al. 2015) as well as in experimental papers (McDonald et al. 2000, Crozier 2003, Khani and Moharramipour 2010, Coleman et al. 2014). Adult kudzu bugs were collected once a month in the first week of each month from October to December of 2015 at three population locations: Prince George's County, 38.780685, -76.99818; Calvert County, 38.432125, -76.55215; and Suffolk County, 36.781478, -76.57712. Each population location corresponded to a different USDA Plant Hardiness Zone (7A, 7B, 8A, respectively) (Fig. 1). Adults were captured on kudzu, *Pueraria montana* (Lour.) Merr. (Fabales: Fabaceae), at each site with a sweep net and brought back to the University of Maryland, College Park in a 34 × 34 × 60 cm mesh popup cage (Bioquip, Rancho Dominguez, CA). Cages were kept at room temperature, each with an edamame, *Glycine max* var. 'Midori Giant' (L.) Merrill (Fabales: Fabaceae), plant. Plants were in the reproductive stage of development and were grown in chambers at the University of Maryland.

Twenty bugs, 10 male and 10 female, were used to determine SCP from each population each month. To measure the SCP, an adult was placed in a 10 ml glass vial with half of a standard cotton ball at the bottom. A coiled thermocouple wire (Model TT-K-24-SLE, Omega, Norwalk, CT) was depressed against the dorsal side of the insect. Thermocouple wires were attached to a thermocouple hub (Model USB-TC, Measurement Computing, Norton, MA) and temperature was recorded through a software program (Tracer Daq, Measurement Computing, Norwalk, CT). Vials were placed into a refrigerated recirculating water bath (Model AP07R-40-A11B, PolyScience, Niles, IL) filled with a cooling bath fluid (Model Polycool HC-50, PolyScience). The bath was set to run from room temperature to -25°C at a cooling rate of 0.277°C/min. SCP was determined as the temperature immediately preceding the exothermic temperature increase from the onset of ice nucleation (Sinclair et al. 2015). After assessing the SCP, each bug was dried for 24 h in an oven and weighed.

Population, month, and gender for SCP were analyzed with an ANOVA and Tukey's honest significant difference (HSD) post hoc test on significant terms. Suffolk County in December was excluded from analysis as the fungus *B. bassiana* killed the population. The effect of body mass on SCP was run for each sex and population by linear regression (JMP Pro version 11, SAS Institute, Cary, NC).

### Measurement of $LLT_{50}$

$LLT_{50}$  methods are consistent with those outlined in a review of measuring cold tolerance by Sinclair et al. (Sinclair et al. 2015) as well as in experimental papers (McDonald et al. 2000, Crozier 2003, Chen and Kang 2004, Khani and Moharramipour 2010, Morey et al. 2012). Adult kudzu bugs were collected at the same time and

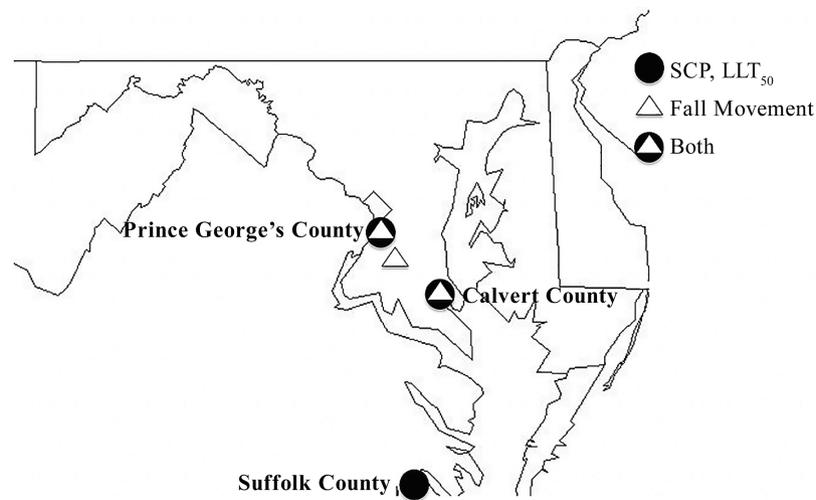


Fig. 1. Map of Maryland and Virginia with population locations of kudzu bugs collected for SCP,  $LLT_{50}$ , and fall movement studies.

kept in the same manner as the SCP experiment. December was excluded due to low population densities. To determine the  $LLT_{50}$ , each population and month was tested using 8 replicates of 5 adults for 5 minimum temperatures. Minimum temperatures used were  $-10$ ,  $-6$ ,  $-4$ ,  $-2$ ,  $+2^{\circ}\text{C}$  to achieve a range of 100% to 0% death. Each group of 5 adults was placed in a 10 ml glass vial and topped with half of a standard cotton ball before capping. For a minimum temperature treatment, all vials, 8 per population, were placed into a refrigerated bath (Model AP07R-40-A11B, PolyScience) filled with a low temperature bath fluid (Model Polycool HC-50, PolyScience). One additional vial was placed into the bath with a thermocouple wire (Model TT-K-24-SLE, Omega) to monitor temperature. The 9 vials represented one run in the bath. Each run was cooled from room temperature to one of the minimum temperatures ( $-10$ ,  $-6$ ,  $-4$ ,  $-2$ ,  $+2^{\circ}\text{C}$ ) at a rate of  $0.15^{\circ}\text{C}$  per min. The run was held at the minimum temperature for 12 h, which corresponds to the longest natural cold duration in Maryland (Grant unpublished). After the 12-h period, the bath was warmed to room temperature at a rate of  $0.15^{\circ}\text{C}$  per min. Upon reaching room temperature each vial of bugs was transferred to a petri dish with a ventilated lid containing an edamame leaflet (*G. max* var. 'Midori Giant') wrapped on the cut end with a moistened cotton ball. Twenty-four hours after the end of the run, insects were checked for mortality. Death was defined as a lack of movement when provoked with an artist brush. Counts of dead individuals in each petri dish were recorded.

A logit model of the death counts was run with variables for month, population, minimum temperature, and all interactions (month  $\times$  population, month  $\times$  minimum temperature, population  $\times$  minimum temperature, and month  $\times$  population  $\times$  minimum temperature). Parameter significance was checked with the effect likelihood ratio test. The  $LLT_{50}$  with upper and lower 95% CI was calculated for each month and population combination from the modeled 50% probability of death (JMP Pro version 11, SAS Institute).

### Fall Movement Observations

To determine timing of fall movement to overwintering locations and overwintering microhabitat sites, a field cage was stocked with kudzu bug and observed over the fall of 2015 and 2016. The mesh  $1.8 \times 3.6 \times 1.8$  m cage was erected outside the greenhouse complex at the University of Maryland. The bottom of the cage was covered with a fine weed cloth and edges sealed with a combination of tape and bricks.

In 2015, eight gray  $45 \times 35 \times 12$  cm bins with drainage holes in the bottom were arranged in the cage. Four bins were filled with approximately 8 cm of potting soil and the other 4 were filled with soil and topped with 8 cm of leaf litter collected from kudzu sites. A large holed ( $1 \times 1$  cm) mesh was used to cover and secure the leaf litter. Additionally, eight kudzu plants in large pots were placed in the cage, four supported on 1.5-m plant stakes and the other four left to naturally twine. Prior to placement in the cage, the kudzu plants were propagated at the University of Maryland greenhouse complex from vine cuttings taken from a nearby kudzu patch. A total of 2,661 kudzu bug adults collected from Calvert County, Maryland and Suffolk County, Virginia were released into the cage. The cage was checked twice a week from 22 October 2015 to 19 December 2015 for location of bugs. A score of  $3 = 30+$ ,  $2 = 10-30$ ,  $1 < 10$ ,  $0 =$  no bugs visible was recorded for each plant and the cage sides. On 14 January 2016, the cage was destructively sampled and total number of bugs in each bin, plant, and cage floor was counted. Plant counts included the number of bugs found in the vines, leaf litter and soil surface of the pots. Leaf litter counts included the number of bugs found in the leaf litter and soil surface of the bins and soil counts included the number of bugs found in the soil. For analysis, plants, leaf litter, soil, and cage were grouped.

The experiment was repeated in fall, 2016 with modifications. Seven grey  $45 \times 35 \times 12$  cm bins with drainage holes in the bottom were placed in the cage. The bins were filled with 12 cm leaf litter collected from a kudzu site in Maryland and covered as before. Six potted and staked kudzu plants, as well as two trays of smaller kudzu plants, were also placed in the cage. Approximately 1,300 kudzu bugs collected from Anne Arundel County, Maryland were released into the cage. The cage was checked twice a week from 2 October 2016 to 15 December 2016 for location of bugs. Plants and cage locations were scored as before. On 19 January 2017, the cage was destructively sampled and number of bugs was counted as before.

In addition to the caged study, observations of kudzu bugs were made at field habitats during the fall, 2015. Once a month from October to December, three sites (Fig. 1) were searched for the location of bugs within the kudzu patch. At each site, five random points were chosen and a  $0.5 \times 0.5$  m section of vine, leaf litter, and topsoil were collected and bagged separately from each point. Counts of nymphs and adults were assessed in the lab from collected samples.

### Microhabitat Temperature Measurements

In a kudzu patch in Calvert County Maryland, five stakes were deployed with two temperature monitors (Model DS1921G iButton, Maxim Integrated, San Jose, CA) on each. One monitor was within the leaf canopy of the kudzu vine and the other in the leaf litter. An additional monitor measuring ambient temperature was suspended protected from direct sunlight on the north side of a tree within the kudzu patch. Monitors recorded temperature once an hour from 17 December 2014 to 19 December 2016. Here, we provide results for the winter only, December through March.

### Application of Temperature to Future Range Expansion

Average minimum winter temperature was obtained from the USDA plant hardiness zone map. The temperature range of each hardiness zone was adjusted for the average winter difference in microhabitat temperature. Isolines were mapped at the top edge of the corresponding zone in which the LLT<sub>50</sub> and SCP values fall. This was then compared to the current kudzu bug distribution from EDDMaps (EDDMaps 2016). Predicted climate RCP 4.5 from the IPCC (IPCC 5th Report 2013) was applied to determine the change in plant hardiness zones. A new temperature isoline was added to represent the possible change in geographic range for the kudzu bug.

## Results

### Measurement of SCP

The overall SCP across populations, month, and gender was  $-12.6 \pm 3.5^\circ\text{C}$  ( $\bar{x} \pm \text{SE}$ ). SCP was not significantly affected by gender but was affected by population and month ( $F = 2.77$ ;  $df = 1,154$ ;  $P = 0.098$ ;  $F = 6.54$ ;  $df = 2,154$ ;  $P = 0.0019$ ;  $F = 19.19$ ;  $df = 2,154$ ;  $P \leq 0.0001$ ; respectively). SCP values for the Prince George's, Calvert, and Suffolk populations were  $-12.2 \pm 0.4^\circ\text{C}$ ,  $-11.5 \pm 0.4^\circ\text{C}$ , and  $-13.7 \pm 0.5^\circ\text{C}$ , respectively. Only the Suffolk population was significantly different among the sites by a Tukey's HSD ( $P \leq 0.05$ ). SCP values for October, November, and December were  $-13.0 \pm 0.4^\circ\text{C}$ ,  $-14.0 \pm 0.4^\circ\text{C}$ , and  $-10.2 \pm 0.5^\circ\text{C}$ , respectively. December was significantly different among the months by a Tukey HSD ( $P < 0.05$ ) (Table 1).

Dry mass of the bugs differed by gender and population but not month ( $F = 10.46$ ;  $df = 1,154$ ;  $P = 0.0015$ ;  $F = 6.88$ ;  $df = 2,154$ ;  $P = 0.0014$ ;  $F = 1.44$ ;  $df = 2,154$ ;  $P = 0.24$ ; respectively). Mass ( $\bar{x} \pm \text{SE}$ ) of females was significantly heavier than males ( $10.3 \pm 0.4$  mg and  $8.7 \pm 0.4$  mg, respectively;  $t = -3.23$ ;  $df = 154$ ;  $P < 0.05$ ). The mass per bug of the Suffolk and Prince George's populations were significantly greater than the mass per bug of the Calvert population ( $10.1 \pm 0.5$  mg,  $9.8 \pm 0.4$  mg, and  $8.3 \pm 0.4$  mg, respectively; Tukey HSD,  $P < 0.05$ ). Individual mass did not correlate with SCP for Prince George's ( $F = 0.54$ ;  $df = 1,59$ ;  $P = 0.47$ ) and Suffolk ( $F = 1.17$ ,  $df = 1,39$ ;  $P = 0.29$ ) populations, however there was a significant negative correlation in the Calvert population ( $F = 5.51$ ;  $df = 1,59$ ;  $P = 0.02$ ). Across all populations, male and female masses were significantly negatively correlated with SCP ( $F = 7.56$ ;  $df = 1,81$ ;  $P = 0.0074$ ;  $F = 4.20$ ;  $df = 1,77$ ;  $P = 0.044$ ; respectively).

### Measurement of LLT<sub>50</sub>

The Suffolk population in November was dropped from analysis due to a high incidence of death due to a fungal infection of *B. bassiana* in the kudzu bug population. Model parameters for month, temperature, population, and population by temperature significantly affect kudzu bug survival (Table 2). Probability of surviving

**Table 1.** Temperatures are shown for the SCP and LLT<sub>50</sub> for three kudzu bug populations in Maryland and Virginia over 3 months of fall, 2015

Month	Population	SCP ( $\bar{x} \pm \text{SE}^\circ\text{C}$ )	LLT <sub>50</sub> ( $^\circ\text{C}$ )	LLT <sub>50</sub> 95% CI ( $^\circ\text{C}$ )	
				Lower	Upper
October	Prince George's	$-13.1 \pm 0.7$	-5.1	-5.8	-4.5
	Calvert	$-11.4 \pm 0.6$	-4.1	-5.0	-3.2
	Suffolk	$-14.6 \pm 0.5$	-4.8	-5.3	-4.4
November	Prince George's	$-13.3 \pm 0.8$	-6.7	-7.7	-5.9
	Calvert	$-13.8 \pm 0.8$	-5.0	-6.0	-4.0
	Suffolk	$-15.0 \pm 0.4$	—	—	—
December	Prince George's	$-10.1 \pm 0.8$	—	—	—
	Calvert	$-9.1 \pm 0.4$	—	—	—
Overall		$-12.6 \pm 3.5$	-5.1	-6.0	-4.4

December was not measured for the LLT<sub>50</sub> due to insufficient numbers for testing; furthermore, Suffolk was dropped from the SCP analysis in December and LLT<sub>50</sub> in November due to a fungal infection of *B. bassiana*. LLT<sub>50</sub> and 95% CI for month and population combinations are predicted temperatures at 50% probability of survival modeled through a logit regression.

**Table 2.** Logit regression model results for LLT<sub>50</sub> experiment on kudzu bug survivorship from three populations located in Maryland and Virginia during October and November 2015 at five minimum temperatures ( $-10$ ,  $-6$ ,  $-4$ ,  $-2$ ,  $+2^\circ\text{C}$ )

Model term	df	Test statistic	<i>P</i>
Month (M)	1	5.31	<b>0.0212</b>
Temperature (T)	1	127.06	<b>&lt;0.0001</b>
M*T	1	0.21	0.6448
Population (P)	2	21.06	<b>&lt;0.0001</b>
M*P	1	1.08	0.2983
T*P	2	13.67	<b>0.0011</b>
M*T*P	1	0.50	0.4753

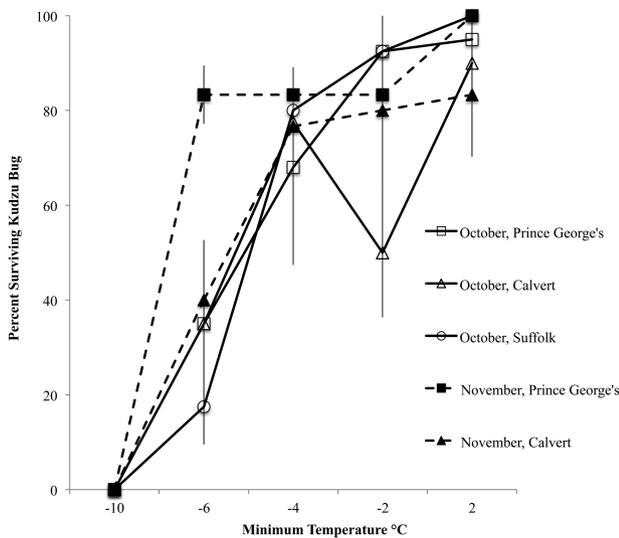
All bolded *P* values are considered significant ( $\alpha = 0.05$ ).

increased in November suggestive of seasonal cold acclimation (Fig. 2). Populations showed differences but did not consistently follow a latitudinal trend (Fig. 2). Predicted LLT<sub>50</sub> values ranged from  $-4.1^\circ\text{C}$  from the Calvert population in October to  $-6.7^\circ\text{C}$  from the Prince George's population in November. The 95% CI of the Prince George's population in November did not overlap with any population in October but did overlap with the Calvert population in November (Table 1). Overall, LLT<sub>50</sub> across populations and months averaged  $-5.1^\circ\text{C}$  with a lower and upper CI of  $-6.0^\circ\text{C}$  and  $-4.4^\circ\text{C}$  respectively.

### Fall Movement Observations

In the 2015 cage study, a general decline in median score of bugs was observed from late October through the middle of December (Fig. 3). By 12 December 2015, bugs were no longer observed on the plant surfaces or cage walls. Most of the movement of bugs to protected overwintering sites occurred in November (Fig. 3). At the time of the destruction of the cage, 58% of the insects were recovered. Of the recovered, 35% were found alive (Fig. 4). Survival rates in each area were 36% in leaf litter, 35% in plants, 21% in cage, and 14% in the soil.

In the 2016 cage study, median score of bugs was higher throughout October and November than in December on the plants and



**Fig. 2.** Three kudzu bug populations in Maryland and Virginia were assessed over October and November during the fall of 2015. Eight replicates of kudzu bug groups consisting of five individuals were lowered to and held at a minimum temperature (-10, -6, -4, -2, +2) for 12 h. After warming, groups were allowed to recover for 24 h before mortality was assessed. Average percentage of surviving kudzu bugs with SE is shown per minimum temperature tested. Suffolk was dropped from the analysis in November due to a fungal infection of *B. bassiana*.

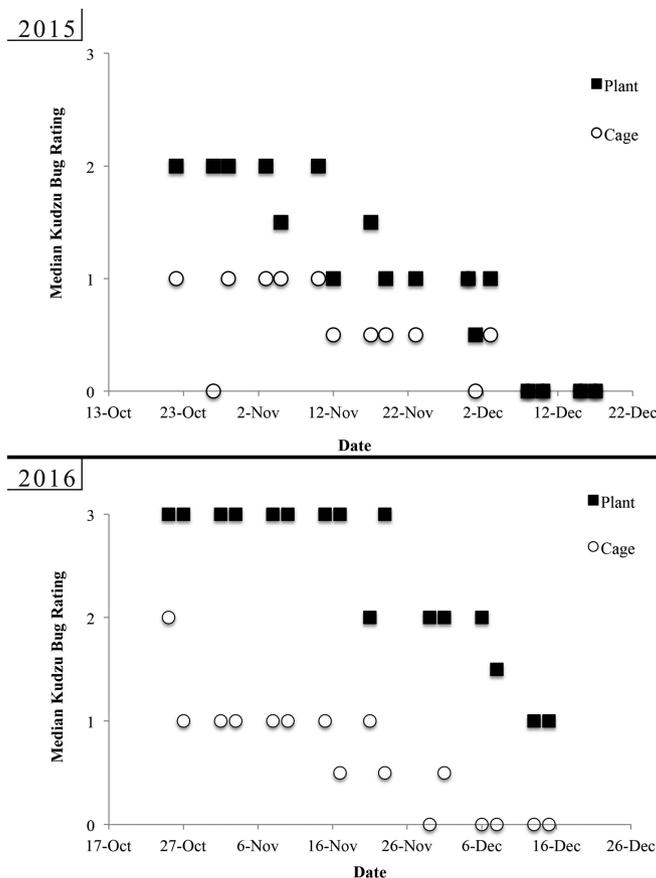
cage. Movement to overwintering locations occurred primarily from mid-November to early December (Fig. 3). At the time of the cage destruction, 77% of the insects were recovered of which 9% were alive (Fig. 4). Survival rates in each area were 10% in plants, 10% in leaf litter, and 6% in the cage. In both years kudzu bug adults were observed to often overwinter in small groups, less than 10, within the recesses of dead leaves.

In the field study during fall, 2015, a decline in bugs was observed from October to December in the vine samples and a corresponding increase in bugs found in the leaf litter samples. October showed nearly all bugs in the kudzu vines (Fig. 5). By November, bugs were still mostly found in the vines; however, bugs began to appear more in the leaf litter as well. December saw a reversal in which leaf litter was the dominant location of bugs. Although vines were observed to harbor a few bugs in December, the number was greatly reduced from the previous month. December was the first observation of a kudzu bug in the soil.

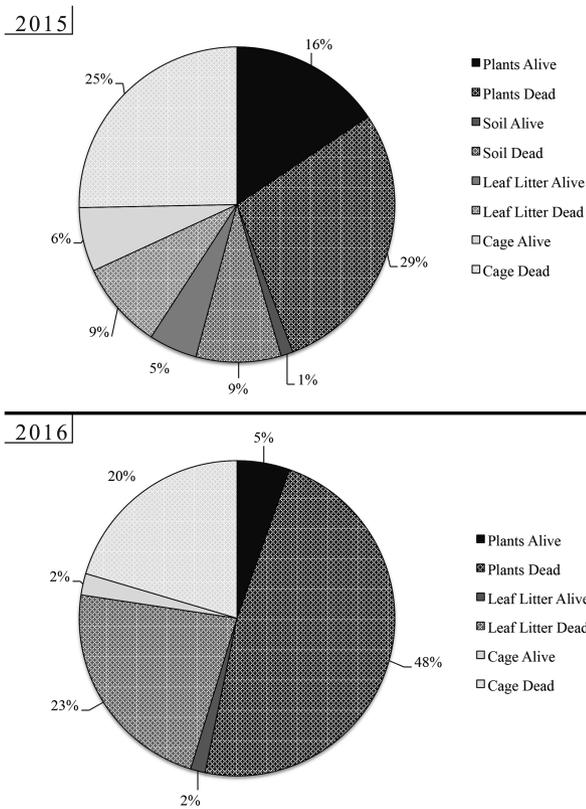
**Microhabitat Temperature Measurements**

Temperature changes between day and night were most variable in the vine and ambient monitors. Leaf litter showed some thermal buffering to changes in temperature (Table 3). On average vines were colder than the ambient temperature whereas on average leaf litter was warmer. The greatest buffering differences in the leaf litter occurred in January and February.

Recorded hourly ambient temperature at the kudzu site exceeded the LLT<sub>50</sub> value of -5.1°C a total of 475 times between December



**Fig. 3.** (2015) A cage stocked with >2,000 kudzu bugs was sampled between 22 October and 19 December 2015. Kudzu bugs in the cage were provisioned with kudzu plants and overwintering refugia. Scores of 3 = 30+, 2 = 10–30, 1 <10, and 0 = no kudzu bugs visible were given to each plant and cage wall. Median score of kudzu bug observed on the plants and cage walls is depicted over observation date. (2016) The cage stocked with +1,300 kudzu bugs was monitored between 25 October and 15 December 2016. Kudzu bugs were provisioned and scored similar to 2015. Median score of kudzu bug observed on the plants and the cage walls is depicted over date.

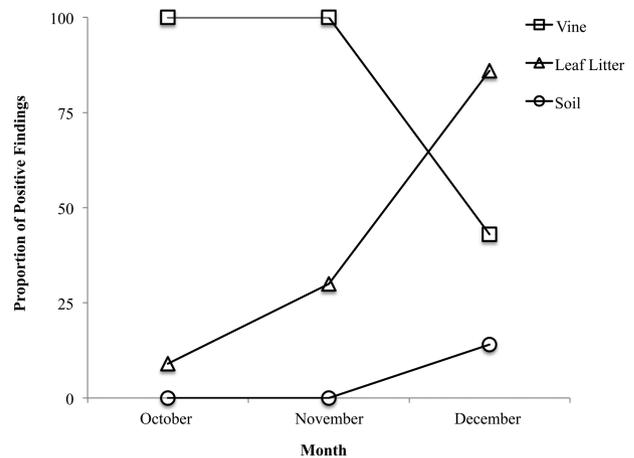


**Fig. 4.** (2015) Kudzu bugs were released in a cage containing kudzu plants, bins with leaf litter and bins with soil. Individuals were allowed to move to an overwintering location over the fall of 2015. On 14 January 2016 the cage was sampled to count individuals in each overwintering area. Percentages of kudzu bugs found per location are subdivided into dead (hashed) versus alive (solid). The category plants include the vines, leaf litter, and soil surface of each pot. The leaf litter category represents the leaf litter in the bins and soil category the soil in the bins. (2016) The cage was similarly set up in 2016 except for the exclusion of bins with soil. Kudzu bugs were allowed to move to overwintering locations over the fall of 2016 and the cage was sampled on 19 January 2017.

2014 and December 2016 (Table 3). Temperatures in leaf litter exceeded the  $LLT_{50}$  threshold 40 times and only occurred in January and February of 2016 (Table 3). Duration of consecutive hours below  $LLT_{50}$  threshold varied and equaled or surpassed 12 consecutive hours in 35% of occurrences in the leaf litter. The mean SCP of  $-12.6^{\circ}\text{C}$  was exceeded 25 times in the ambient temperature with all occurrences in February 2015 (Table 3).

**Application of Temperature to Range Expansion**

A temperature difference of  $1^{\circ}\text{C}$  between ambient and leaf litter microhabitats was applied to the minimum temperature ranges in the USDA plant hardiness zone map. For the future SCP, a  $3^{\circ}\text{C}$  increase was applied to the minimum temperature after microhabitat adjustment. The mean SCP of  $-12.6^{\circ}\text{C}$  and mean  $LLT_{50}$  of  $-5.1^{\circ}\text{C}$  was



**Fig. 5.** Five replicate field samples of vine, leaf litter, and soil from three kudzu locations known to harbor the kudzu bug in central and southern Maryland over the fall of 2015. Positive samples are those in which at least one layer (vine, leaf litter, or soil) of a replicate had a kudzu bug. The percent of positive samples that contained bugs in each layer is depicted per month. Number of positive replicates in October was 11, in November was 10, and in December was 7.

**Table 3.** Hourly assessment of temperature in ambient, vine, and leaf litter habitats of a kudzu patch in Calvert County, Maryland from 17 December 2014 to 19 December 2016

Year	Month	Difference of habitats from ambient ( $\bar{x} \pm \text{SE}^{\circ}\text{C}$ )		Difference of day from night ( $\bar{x}^{\circ}\text{C}$ )		Hours below the SCP		Hours below the $LLT_{50}$	
		Litter	Vine	Litter	Vine	Ambient	Litter	Ambient	Litter
2014	Dec.	$1.3 \pm 0.2$	$-0.3 \pm 0.1$	0.8	3.4	0	0	7	0
2015	Jan.	$1.8 \pm 0.1$	$-0.2 \pm 0.1$	0.6	3.6	0	0	73	0
	Feb.	$3.1 \pm 0.2$	$1.2 \pm 0.1$	0.4	4.3	25	0	203	0
	Mar.	$-0.2 \pm 0.2$	$-0.4 \pm 0.1$	1.4	4.8	0	0	32	0
2016	Dec.	$-0.5 \pm 0.1$	$-1.0 \pm 0.0$	2.0	3.6	0	0	0	0
	Jan.	$0.8 \pm 0.1$	$0.3 \pm 0.1$	2.7	4.6	0	0	96	39
	Feb.	$0.4 \pm 0.1$	$-0.2 \pm 0.1$	2.4	3.4	0	0	46	1
	Mar.	$-1.0 \pm 0.1$	$-0.5 \pm 0.1$	3.9	6.0	0	0	1	0
	Dec.	$0.9 \pm 0.1$	$-0.6 \pm 0.1$	1.7	3.4	0	0	17	0
Overall		$0.7 \pm 0.0$	$-0.2 \pm 0.0$	1.8	4.1	2.7	0	52.8	4.4

A positive number indicates the litter or vine temperature was warmer than the ambient. The mean SCP used for comparison was  $-12.6^{\circ}\text{C}$  and the mean  $LLT_{50}$  used was  $-5.1^{\circ}\text{C}$ .



**Fig. 6.** Mean  $LLT_{50}$  of  $-5.1^{\circ}\text{C}$ , mean SCP of  $-12.6^{\circ}\text{C}$ , and future SCP predicted from the IPCC RPC 4.5 lines are based on the USDA plant hardiness zone boundaries adjusted for the leaf litter microhabitat temperature. The gray area shows 2016 distribution of the kudzu bug from EDDMaps.

used for creating the temperature threshold isolines. Based on these assumptions, the kudzu bug would be able to advance one plant hardiness zone by the end of the century (Fig. 6).

## Discussion

We sought to determine the cold tolerance of the kudzu bug and its relation to current and future distributions. We found that the kudzu bug is a freeze-avoidant species with a SCP value of  $-12.6 \pm 3.5^{\circ}\text{C}$  ( $\bar{x} \pm \text{SE}$ ) and  $LLT_{50}$  value of  $-5.1^{\circ}\text{C}$  with a lower and upper confidence limits of  $-6.0^{\circ}\text{C}$  and  $-4.4^{\circ}\text{C}$ , respectively. Furthermore, the kudzu bug has limited thermal plasticity and no apparent local adaptation between tested populations in northern Virginia and Maryland. The leaf litter, which the kudzu bug moves into between late November and early December to overwinter in Maryland, provides thermal buffering. The microclimate created by the leaf litter is not only on average warmer than the ambient temperature but is also more consistent in temperature between day and night. Given the cold temperature thresholds, which match the current northern distribution limit, the kudzu bug could be capable of expanding into southeast Pennsylvania and coastal regions as far north as Massachusetts by the end of the century based on projected temperature increases.

SCP values showed no effect of gender and inconsistent trends in the effects of collection month and location of population. Though differences in the response to cooling between genders can cause a bimodal SCP (Salin et al. 2000, Renault et al. 2002), there are other examples of gender not affecting the cold tolerance of an insect (Khani and Moharramipour 2010, Morey et al. 2012).

The effect of month on the SCP of populations does not show a consistent trend over the fall. In some insects, the SCP reflects increasing cold tolerance during the fall, e.g., the corn borer larvae, *Ostrinia furnacalis* Guenée (Lepidoptera: Crambidae), where the SCP decreases between September and December (Goto et al. 2001).

In contrast, we observed warmer SCP values in December, which might be due to unusually warm temperatures experienced at the population locations in 2015. Additionally, the difference could be due to a change in the habitat of collected adults within the kudzu patch. In October and November, kudzu bugs had not yet begun to move to overwintering locations and all sampled bugs came from the kudzu vines directly. By December, many bugs had begun to move to the leaf litter for overwintering and sampled bugs included those from the leaf litter and vines. Insects in the leaf litter could have been at a different stage of cold accumulation, the seasonal change in cold tolerance, than those in the vines.

For population location, we expected a latitudinal gradient trend in which SCP is high in southern populations and reduced with increasing latitude. Examples of such gradients include the eastern five-spined engraver, *Ips grandicollis* Eichhoff (Coleoptera: Curculionidae) and the pea leafminer, *Liriomyza huidobrensis* Blanchard (Diptera: Agromyzidae) (Lombardero et al. 2000, Chen and Kang 2004). In fact, our results showed no latitudinal gradient between our population locations. SCP does not always follow a latitudinal gradient even in established pests within an invaded range. Colorado potato beetle was found to have no latitudinal pattern in SCP from Jalisco, Mexico to Vermont, United States (Izzo et al. 2013). Additionally, the SCP of the native harlequin bug, *Murgantia histrionica* Hahn (Hemiptera: Pentatomidae), showed no difference between populations in Maryland and Virginia (DiMeglio et al. 2016).

$LLT_{50}$  values showed an effect of month but no consistent latitudinal trend between population locations. As expected,  $LLT_{50}$  values decreased between October and November indicating a cold acclimation. Triggers for cold acclimation may include photoperiod or temperature responses (Clark and Worland 2008); however, the precise cues for the kudzu bug remain unknown. Similar to the SCP, there was no consistent latitudinal trend in the  $LLT_{50}$  values

between the three population locations. Location variation in  $LLT_{50}$  values is likely due to phenotypic plasticity within the populations. Plasticity can allow for survival and establishment during the time needed for an invasive species to achieve local adaptation (Moran and Alexander 2014).

Given the recent arrival of the kudzu bug to North America and the constricted single mitochondrial haplotype lineage (Jenkins and Eaton 2011), a founder effect may be limiting local adaptation. Kudzu bug populations in the United States were determined to derive from a population located on the subtropical island of Kyushu, Japan based on a phylogeographical analysis of mitochondrial DNA sequences (Hosokawa et al. 2014). As such, United States populations are likely experiencing high selective pressure to adapt to the temperate climate of Maryland. However, a genetic bottleneck might cause a barrier to rapid adaptation even under such a potentially high selective pressure. Additionally, it may take many more generations before a latitudinal cline develops in the United States. According to Moran and Alexander (2014), geographic clines for a trait often take between 50–150 generations in a newly invaded species.

Kudzu bugs in the cage experiment and at field sites were observed to overwinter in the leaf litter at the base of trees and dormant kudzu vines. This finding is consistent with a study published by Lahiri et al. (2015) in which they determined the kudzu bug was overwintering in the leaf litter. In Maryland, kudzu bugs were observed to move to overwintering locations in late November to early December in 2015 and 2016. Photoperiod is a likely cue for overwintering movement, although research is needed to confirm this hypothesis. In the brown marmorated stink bug short photoperiods are related to the overwintering induction cue (Niva and Takeda 2003, Cira et al. 2016).

Leaf litter provides thermal buffering for the kudzu bug against ambient temperatures. Leaf litter also moderates the diurnal temperature fluctuations which benefits the bugs by decreasing the metabolic rate thus reducing the use of stored energy reserves (Williams et al. 2012). We found that of the hours below the  $LLT_{50}$  in the ambient temperature during winter months of 2014–2015 and 2015–2016, only 8% were also below the threshold in the leaf litter, thus demonstrating thermal buffering of leaf litter. Additionally, although the ambient temperature reached the SCP temperature, there were no occurrences of the SCP temperature in the leaf litter. Thus, adjustments between microhabitat and ambient temperatures are crucial in modeling winter survival.

In the IPCC RPC 4.5 model, winter temperatures are predicted to increase by 3°C based on greenhouse gas concentration trajectories (IPCC 5th Report 2013) and the distribution of the kudzu bug will likely be able to expand northward through southeast Pennsylvania and up to coastal Massachusetts. Kudzu vines as well as soybean extend throughout the predicted range, thus host plant availability would not limit the range shift. The northern most reaches of the possible range would likely allow for establishment but would not allow populations to build to economically damaging levels. Maryland, the current northern limit, could see increased pest pressure of the kudzu bug in soybeans.

In summary, the kudzu bug is a freeze-avoidant species, as it cannot withstand ice nucleation and dies before the SCP temperature. Measured cold tolerance supports limited plasticity over local adaptation at this point in time. Cold tolerance can be slow to evolve due to a tight co-adaptation or the gene being pleiotropic in addition to bottlenecks (Kimura 2004). Plasticity has thus allowed the kudzu bug to become established but not expand or build to high densities at their northern distribution limit. Future climate shifts, particularly

with warmer winters, will likely allow the kudzu bug to expand northward. Producers can utilize knowledge of cold tolerance and specific winter temperatures within an integrated pest management plan to assess risk of future kudzu bug populations.

## Acknowledgments

We would like to acknowledge support from the Northeast Sustainable Agriculture Research and Education Program (#2014-38640-22161), the Maryland Soybean Board, and Hatch Project #MD-ENTM-1016. We appreciate helpful comments on the manuscript provided by Rebecca Wilson and Rebecca Eckert. Additionally, we thank Raina Kaji and Morgan Thompson for their technical help with the cage experiment.

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