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ORIGINAL PAPER

Laboratory rearing of *Halyomorpha halys*: methods to optimize survival and fitness of adults during and after diapause

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Abstract Laboratory colonies are necessary to conduct year-round research on the invasive brown marmorated stink bug, *Halyomorpha halys* (Stål), a severe agricultural and nuisance pest in the USA. When adults are collected in the fall to either start or supplement colonies, they require a period of cold storage before they resume sexual development and egg production. There is a lack of understanding of how to collect and store diapause-triggered adults in the laboratory. A series of experiments in 2013–2015 assessed survival and fecundity of stink bugs collected from different locations and stored under different temperatures and durations. We found that a minimum of 7 weeks is necessary to break diapause and that a substantial proportion of adults can survive when stored at constant 9 °C, even for periods longer than needed to terminate diapause. Adults survived significantly better at 6 and 9 °C than at 3 °C in storage for 7 weeks. Longer durations up to 34 weeks in storage reduced adult survival and significantly affected survival rates, timing of first egg laying, and overall fecundity. Location where adults were collected at overwintering sites in the fall had a significant impact on survival in cold storage and colony performance. Adults collected from soybean fields in mid-September and fed in the laboratory for 2 weeks before storage had lower survival than adults collected in October at aggregation sites and stored immediately. The food sources available to *H. halys* adults at collection locations for nutrition and sequestration of sufficient energy reserves going into diapause are discussed.

Keywords Stink bug · BMSB · Diapause · Colony rearing · Cold storage

Key message

- When *Halyomorpha halys* adults already triggered for diapause are brought into the laboratory, they need a period of time in cold storage before they resume egg production.
- We showed that a minimum of 7 weeks is necessary to break diapause and that a substantial proportion of adults can survive when stored at constant 9 °C, even for periods longer (at least 34 weeks) than needed to terminate diapause.
- Survival in cold storage was impacted by storage temperature and the location where adults were collected in the fall.
- Adult survival rates and fecundity of surviving adults reared in laboratory colony decreased and egg laying begins more quickly with longer periods of storage.

Introduction

The brown marmorated stink bug, *Halyomorpha halys* (Stål), is an invasive stink bug originally from Southeast Asia that was introduced into the USA in the mid 1990s (Hoebeke and Carter 2003). It has been detected in 43 states and is considered a severe agricultural and nuisance pest in

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Special Issue: The brown marmorated stink bug *Halyomorpha halys* an emerging pest of global concern.

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nine eastern states (http://www.stopbmsb.org). As a highly polyphagous insect, *H. halys* feeds on many hosts (Leskey et al. 2012a, b; Bergmann et al. 2016) once it emerges from overwintering sites. Toward the end of summer, as day length shortens and temperature begins to drop, diapause-destined adults aggregate in large numbers on the sides of buildings, under bark, and/or on rocky outcroppings at high elevations (Watanabe et al. 1994; Hoebeke and Carter 2003). *H. halys* experiences facultative diapause (Saulich and Musolin 2012), which is seemingly triggered by shorter day length (Watanabe 1979; Niva and Takeda 2002, 2003) during the nymphal stages (Nielsen, unpublished), although which nymphal stage specifically triggered is unknown. Adult females entering diapause are sexually immature and require an overwintering period before they can reach sexual maturity (Nielsen, unpublished).

Laboratory colonies are necessary to conduct year-round research (Medal et al. 2012; Dingha and Jackai 2017; Rosen et al. 2016) and must be maintained continuously to provide sufficient numbers of eggs, nymphs, and adults. However, loss of the colony, declines in egg production, and lack of required numbers of insects for planned research have been a major problem when this pest cannot be collected in the field. One backup strategy is to collect adults from field populations when densities are high later in the growing season or when they begin to aggregate in large populations in overwintering areas. Although large numbers of adults can be collected this way later in the year, they are usually already triggered for diapause and therefore must experience an 'overwintering' period before being brought out of storage to supplement the laboratory colony. Leopold 2007 suggests that taking advantage of an insect's dormancy behaviors is one approach to consider when addressing the challenges of the continual rearing of insects, and methods of cold storage have been used for other insect colonies, such as the codling moth (Bloem 1997) and southwestern corn borer (Davis 1983). However, production of a consistent supply of high-quality *H. halys* adults has been hampered by a lack of understanding of how to collect and store diapause-triggered adults in the laboratory. To address this issue, we evaluated different field collection and rearing methods to optimize survival and fitness characteristics of diapause-triggered *H. halys* collected from the field.

Materials and methods

Effect of storage duration on BMSB survival and fitness during and after diapause

Separate experiments were conducted in 2013 and 2015 to evaluate adult survivorship when held diapausing in cold storage for different durations, as well as within the laboratory colony after diapause termination. In 2013, 8166 adults in total were collected by hand and placed in 3.8-L cardboard containers at two University of Maryland research and education centers at Keedysville (WMREC, 39°30′38.68″N, 77°44′13.66″W) and Clarksville (CMREC, 39°15′14.89″N, 76°56′34.45″W) on October 2. Pre-diapause adults were collected from the outside surfaces of buildings where they were seeking overwintering sites. Adults were held at room temperature until the following day, when 53 cohorts of on average 110 adults (~50:50 ♀:♂) were placed in 3.8-L cardboard containers with screened lids and filled with paper towels as a resting substrate. Subsets ranging from 6 to 18 cohorts of each collection location were removed after 5, 7, 9, 11, and 14 weeks of cold storage 9 °C, and the number of dead and live adults was recorded per cohort. In 2015, adults were collected by placing shelter traps attached 1 m above the ground on tree trunks near a rock outcropping area on Sugarloaf Mountain (SLM, 39°15′37.73″N, 77°23′22.54″W) at Dickerson, MD. Each trap was a standard Langstroth hive box (41.3 by 16.8 cm) with a fixed bottom, entrance holes on each side, and removable lid. Each box was filled with 30 plastic foundation sheets spaced roughly 1 cm apart to provide a resting substrate for adults. The SLM location, about 120 m higher than the surrounding farmland, is a well-known site where *H. halys* adults aggregate in protected areas. A total of 13,491 adults were obtained from weekly collections from October 8 to November 4. From these collections, 47 cohorts were established, each apportioned to approximately 250 unsexed adults by weight. Each cohort was placed in a 3.8-L cardboard container filled with cornstarch-based packing peanuts as a resting substrate. In this experiment, cohorts were stored much longer than those in 2013 to determine if adults survive to the following spring when laboratory colonies may need additional adults to increase egg production. Subsets of 6–14 cohorts were removed after 11, 19, 30, and 34 weeks of cold storage, and each adult was sexed and recorded as dead or alive (adults that showed no normal signs of activity after 30 min were considered dead). In both experiments, cohorts were cold stored in constant darkness in a cooler at 9 °C and 70% RH.

After diapause termination in both experiments, groups of surviving adults pooled over cohorts for each period of storage were randomly assigned (~50:50 ♀:♂) to colony populations reared in mesh cages (60 × 30 × 35 cm) at 25 °C and 70% RH. Adults were fed three times weekly with potted green bean plants, *Phaseolus vulgaris* L., green bean pods, and raw sunflower seeds. For each storage duration treatment, four colonies averaging 126 adults per cage were reared in 2013–2014, whereas 6–14 colonies of either 75 or 150 adults were reared per cage in 2015–2016. In both experiments, each caged population was examined three times weekly to record adult survival and egg laying until survival dropped below 20% and egg laying ceased.

Effects of collection location and storage temperature on BMSB survival and fitness during and after diapause

Adults were collected from September 22 to October 6, 2014 at four locations in Maryland. At WMREC and CMREC, stink bugs were collected from the outside surface of buildings during the afternoon. At SLM, adults were collected using shelter traps near the rock outcropping area. At Rupert Nurseries (RN), a collection was made on October 2 and consisted of adults attracted to a pheromone lure without a killing agent over a 7-day period. In total, 879, 824, 808, and 376 adults were collected at the WMREC, CMREC, SLM, and RN locations, respectively. Since collections were seasonally and temporally similar, we assume that each population of stink bugs had an equal amount of time to acquire food and stored energy for overwintering. Therefore, any differences in survival were likely due to the type and availability of host plants providing food around each location.

Each collection was brought back to the laboratory where adults were sexed and allocated in cohorts of 24 bugs (~50:50 ♀:♂). Each cohort was placed in 0.5-L cardboard containers containing cornstarch-based packing peanuts as a resting substrate. Cohorts from each collection location were randomly assigned to each of the three temperature treatments (3°, 6°, and 9 °C). Ten to 12 cohorts per collection location were stored at each temperature, except for the RN collection for which only five cohorts were stored per temperature due to fewer collected adults. Each cohort was treated as an experimental unit. Replicate cohorts from the four collection locations were stored in constant darkness in a Percival environmental chamber at each temperature. To equalize possible microenvironmental gradients within each chamber, cohort containers of each collection location were evenly distributed among shelves in the chamber. Moderate humidity of 40–50% was maintained by placing containers of water at the bottom of each chamber.

After 7 weeks in storage, which is sufficient time to terminate diapause based on preliminary studies, containers were removed and held for 3–4 h at room temperature to arouse stinkbug activity, after which the number of live and dead males and females in each replicate cohort was recorded. Additionally, the body length and width of each stink bug were recorded using a digital caliper to the nearest one tenth of a millimeter. Using the surviving adults from each location (except RN which had too few adults survive cold storage) and temperature group, seven colonies containing 50–80 adults (~50:50 ♀:♂) were established for each temperature group and reared for 65 days to record adult survival and egg laying, similar to the procedures used above.

Effects of field-collected adults reared in the laboratory on survival during diapause in cold storage

Another experiment was conducted in 2014 to compare survival of field-collected adults fed in the laboratory before entering diapause with survival of adults collected at overwintering sites and stored immediately. Adults were collected by hand from soybean plants at the CMREC and WMREC locations on September 16 and brought to the laboratory where they were reared in the mesh colony cages described above and fed green bean pods and raw sunflower seeds for two weeks. On October 2, adults seeking overwintering sites were collected from the outside surfaces of buildings at the same locations. The following day, ten replicate cohorts of 25 adults of approximate equal sexes were randomly placed in 0.5-L cardboard containers containing cornstarch-based packing peanuts as a resting substrate. All containers were stored in the same environmental chamber for 7 weeks at 9 °C and then removed to record survival of males and females.

Statistical analyses

Survival of cohorts removed from cold storage was expressed as the percentage survival of males, females, and sexes combined. Body size of dead and alive adults was computed as an index of [(length) x ($width^2$)]. All data sets were tested before analysis for normality and homogeneity of variances using Shapiro–Wilk and Spearman rank correlation tests. For percentage data not meeting the assumptions of ANOVA, the arcsine transformation was used prior to analysis, and variances were partitioned accordingly. Survival data from the storage experiments were analyzed by mixed model ANOVA to test each variable for main effects (collection location, storage duration, storage temperature, or sex) and all interaction effects, with each cohort as an experimental unit. The egg laying performance of the colony populations following storage was expressed as days to first egg mass and mean number of egg masses per female. The mixed model ANOVA tested each variable for main effects of storage duration and temperature, with location treated as a random blocking factor and each colony as an experimental unit. Means were separated following a significant F test by using the Tukey option ($p < 0.05$). All main and interaction means (±SE) reported were computed from the untransformed data (SAS 2013).

For the survival data, the LIFETEST procedure using the product-limit method estimated nonparametric estimates of the survivor functions for each colony. In each analysis, adults alive at termination of the colony were treated as censored events, and output functions were

expressed as survival time estimates of the 25th, 50th, and 75th percentiles and mean survival. Because LIFETEST has no options for treating replicate data sets or random effects, the output metrics from LIFETEST were then analyzed by mixed model ANOVA to test for main and interaction effects of the fixed factors, with collection location in the 2014 colony experiment treated as a random factor. An exception was the 2013 colony experiment, which was analyzed by LIFETEST using combined survival data across colonies after the number of dead adults over time was adjusted for the initial colony size, and the Log-Rank option tested whether survivor functions were different among storage week groups (SAS 2013).

Results

Effect of storage duration at 9 °C

The two-way interaction effect was not significant for the 2013 data; however, there were significant differences in the main effects due to weeks of storage and collection location. Collection location had a significant effect on adult survival ($F_{(1,38.6)} = 12.43$, $p = 0.001$), and percent survival was higher for adults collected at the WMREC location (mean 41.5% ± 4.82) compared to those collected at CMREC (25.5% ± 5.79) (see Fig. 1). Survival of adults from both locations declined significantly with an increase in storage duration ($F_{(4,22.8)} = 12.98$, $p < 0.001$). Although the interaction was not significant, overall trends in differences among interaction means suggest that survival of the CMREC adults declined at a higher rate.

Results of the 2015 data (see Fig. 2) show a similar decrease in percent survival with increasing storage duration. While both males and females displayed similar declines in survivorship over time, males consistently exhibited higher mortality than females. However, since SLM adults were stored much longer at the same temperature than those collected in 2013, it is particularly noteworthy that 40–60% of adults survived after 30–34 weeks in storage. Although variation in experimental design prohibits direct statistical comparison, differences in relative survival suggest that the SLM adults collected in 2015 better survived than adults collected at CMREC and WMREC in 2013.

Table 1 presents the effects of storage duration on mean survival and egg laying in colonies of stored adults from both experiments. Five of the six replicate colonies reared from adults stored for five weeks in 2013 did not produce eggs after 40 days and were returned to cold storage, on the assumption that diapause was not completed. Only one cage with adults stored for 5 weeks was reared for 85 days until survival dropped below 20%. In this cage, mean survival was 44.6% reaching 50% mortality on day 46, and egg laying was

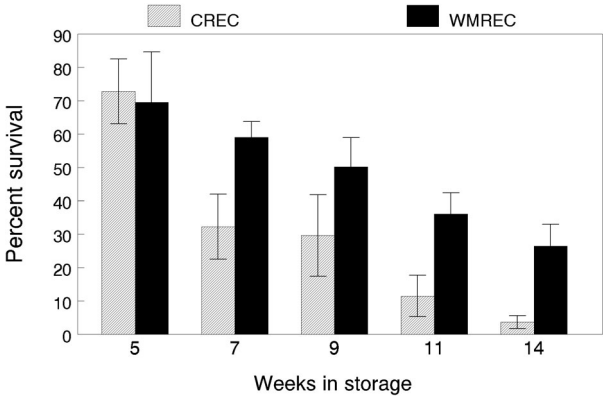

Fig. 1 Percent survival of *H. halys* adults collected from CMREC and WMREC farms in 2013 during 5–14 weeks of storage at 9 °C. The interaction effect was not significant, but collection location had a significant effect on adult survival ($F_{(1,38.6)} = 12.43$, $p = 0.001$) and survival declined significantly with an increase in storage duration ($F_{(4,22.8)} = 12.98$, $p < 0.001$)

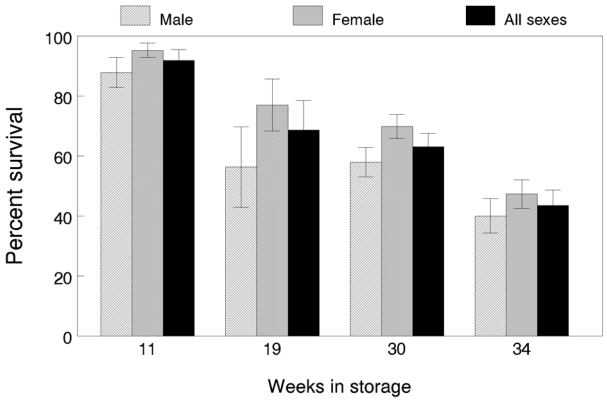

Fig. 2 Percent survival of *H. halys* males, females, and all sexes pooled collected from Sugarloaf Mountain (SLM) in 2015 during 11–34 weeks for storage at 9 °C. Male and female survival declined significantly with an increase in storage duration (male $F_{(3,43)} = 8.6$, $p < 0.001$; female $F_{(3,43)} = 14.9$, $p < 0.001$)

very irregular, with the first egg mass recorded at day 63 and an overall average of 0.32 egg masses per female. All other colonies with adults surviving each storage period were maintained for more than 70 days. In both experiments, mean survival was significantly lower for adults that were held longer in cold storage (2013: $\chi^2 = 126.3$, $df = 4$, $p < 0.001$; 2016: $F_{(1,13.7)} = 29.27$, $p < 0.001$). Significant storage duration treatment effects for time in days to 25, 50, and 75% median mortality for both sexes combined were similarly observed in 2015–2016, with greater mortality of adults held longer in cold storage; however, no significant difference in survival distributions between sexes was observed. In both experiments, females oviposited the first egg mass in significantly less time with increasing durations of diapause storage (2013: $F_{(4,11)} = 11.05, p < 0.001$; 2016:

Table 1 Survival functions and egg laying endpoints of caged colonies of *H. halys* adults that survived diapause after different durations of storage at 9 °C

Year treatments	Mean % survival[b]	Days to first egg mass[a]	Egg masses per female[a]
2013 Storage durations			
5 weeks	44.6 ± 2.9[a]	63.0[a]	0.32[a]
7 weeks	48.2 ± 1.5[ab]	38.8 ± 1.0[b]	1.09 ± 0.36[a]
9 weeks	39.8 ± 1.2[abc]	33.0 ± 1.2[bc]	0.71 ± 0.10[a]
11 weeks	35.2 ± 1.4[bc]	33.0 ± 2.1[bc]	0.68 ± 0.34[a]
14 weeks	31.0 ± 1.1[c]	30.0 ± 2.5[c]	0.51 ± 0.13[a]
2015 Storage durations			
11 weeks	59.4 ± 1.9[a]	37.0 ± 6.8[a]	0.51 ± 0.13[a]
19 weeks	49.7 ± 1.1[b]	28.2 ± 0.7[a]	0.51 ± 0.04[a]
30 weeks	29.9 ± 1.1[c]	20.5 ± 0.8[b]	0.33 ± 0.05[b]
34 weeks	30.0 ± 1.0[c]	21.8 ± 1.4[b]	0.30 ± 0.08[b]

[a] Means (±SE) within each column in experiment year followed by the same letter are not significantly different at the $p < 0.05$ level

[b] Survival means for the 2013 storage durations are estimated based on the survival distribution pooled over all colonies in each treatment and compared between treatments (Log-Rank test: $\chi^2 = 126.3$, $df = 4$, $p < 0.001$). All other means are averages across replicate colonies within each treatment group

$F_{(1,13.8)} = 18.03$, $p < 0.001$), but each female produced fewer egg masses. Both experiments showed declining fecundity with longer storage durations, except that differences were statistically significant only for the 2015–2016 experiment ($F_{(1,14.5)} = 4.16$, $p = 0.026$).

Effects of storage temperature

Survival was much lower for adults collected in pheromone traps at the RN site, with mean survival averaging 0, 1.5 ± 0.92, and 9.6 ± 2.4% for cohorts stored at 3, 6, and 9 °C, respectively. Due to the low survival and different conditions under which these adults were collected, the RN data were not included in the analysis of the other three collection locations. The two-way interaction was not significant for the 2014 data; however, there were significant differences due to storage temperature and collection location. Averaged across interaction means shown in Fig. 3, percent survival of adults stored for 7 weeks was significantly influenced by the collection location ($F_{(2,150)} = 65.6$, $p < 0.001$) and storage temperature ($F_{(2,150)} = 9.4$, $p < 0.001$). Pooled across temperatures percent survival of adults collected at WMREC (52.3% ± 4.16) was significantly lower compared to adults collected at CMREC (94.3% ± 0.94) and SLM (96.0% ± 0.80). Pooled across the three locations, overall survival of adults stored at 6° and 9 °C for 7 weeks was not statistically different (averaged 90.1% ± 2.98 and 98.2% ± 0.53, respectively) but significantly higher than adults stored at 3 °C (78.7% ± 3.85).

Colony performance following diapause showed significant differences in adult survival due to storage temperature and collection location (Table 2) but no significant interaction effects among these factors. Mean survival was significantly lower in colonies with adults stored at the lower temperatures ($F_{(2,36)} = 10.1$, $p < 0.001$). Moreover, the estimated days to the 25th percentile of mortality based on the survival distribution of replicate colonies were also significant for storage temperature ($F_{(2,35.2)} = 14.5$, $p < 0.001$). Colony adults stored at 3°, 6°, and 9 °C reached the 25% median mortality level at 25, 37, and 43 days, respectively. This indicated that adults stored at the lower temperatures died at a higher rate earlier in the colony than adults stored at 9 °C (Fig. 4). Similar to the storage survival results, location of collection significantly impacted colony survival after storage for seven weeks

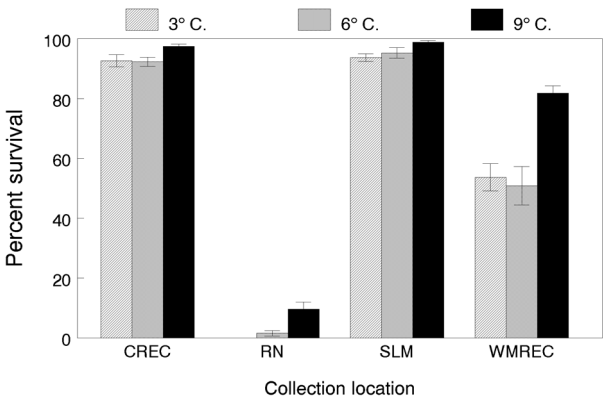

Fig. 3 Percent survival of *H. halys* adults collected from different locations and stored for 7 weeks at 3, 6, or 9 °C. ANOVA did not include the RN data due to low survival. The interaction was not significant, but survival of adults stored was significantly influenced by the collection location ($F_{(2,150)} = 65.6$, $p < 0.001$) and storage temperature ($F_{(2,150)} = 9.4$, $p < 0.001$)

($F_{(2,13)} = 5.3$, $p = 0.021$). Adults from WMREC died in colony at a significant higher rate leading up to the 25% median mortality level than adults from the other two locations. Colony adults from CMREC, SLM, and WMREC reached the 25% mortality level at 39, 44, and 22 days, respectively (Fig. 5). Survival probability curves of male and female adults in colonies were similar among the three temperature treatments, but the overall mean survival pooled across temperatures was significantly higher for females compared to males ($F_{(2,35.9)} = 6.7$, $p = 0.014$).

Despite differences in adult survival, storage temperature had no overall significant effect on fecundity, with the first appearance of egg laying around 31–33 days, and similar egg masses per female (Table 2). However, the main effect for collection location pooled over temperatures showed significantly lower number of eggs per female in colonies that originated from adults collected from the WMREC location ($F_{(2,9)} = 4.8$, $p = 0.038$).

Effects of body size and sex

For all cohorts removed after storage in 2014, numbers of dead and alive adults and their body sizes were recorded by collection location and storage temperature. For survival, results showed no significant two-way interactions between sexes by location or temperature, indicating that these fixed factors had no differential influence on survival of males and females. However, the main effect for sex was significant ($F_{(1,142)} = 14.58$, $p < 0.001$), and females had a higher overall survival rate (90.7% ± 1.92) than males (84.4% ± 2.39). These results agreed with the colony performance of surviving adults from the stored cohorts in 2014. Survival distributions of male and female adults in colonies were similar among the three temperature treatments, as evidenced by a nonsignificant interaction effect. However, the overall mean survival pooled across temperatures was significantly higher for females compared to males ($F_{(2,35.9)} = 6.7$, $p = 0.014$).

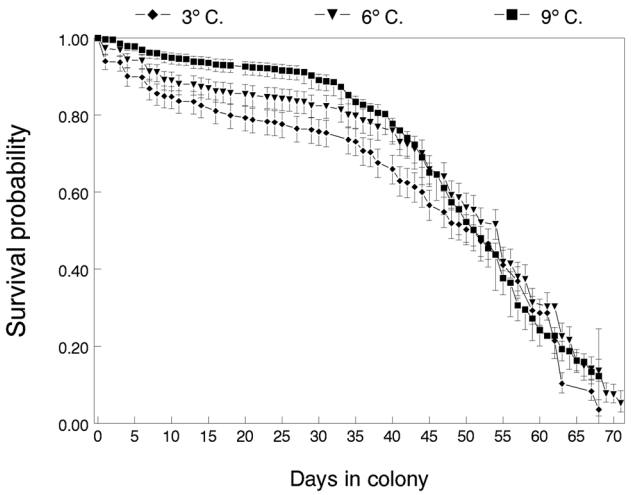

Fig. 4 Survival probability function curves of *H. halys* colonies reared from stored adults that survived storage for 7 weeks at 3, 6, or 9 °C, based on data pooled over collection locations (exclusive of RN data). Survival was significantly lower in colonies with adults stored at the lower temperatures ($F_{(2,36)} = 10.1$, $p < 0.001$). Specifically, significant differences in days to the 25th percentile of mortality were observed for storage temperature ($F_{(2,35.2)} = 14.5$, $p < 0.001$), demonstrating that adults stored at 3 °C died at a higher rate earlier in the colony than adults stored at 9 °C

Irrespective of sex, collection location, and storage temperature, the size of diapausing adults had a significant influence on their ability to survive cold storage. Live adults were significantly larger (length—13.77 mm ± 0.07; width—8.08 mm ± 0.04, and body size—914.7 mm^2 ± 12.7),

Table 2 Survival estimates and egg laying endpoints of caged colonies of BMSB adults from three collection locations that survived diapause after storage at three temperatures for 7 weeks

Treatments	Mean % survival[b]	Days to first egg mass[a]	Egg masses per female[a]
Temperature			
3 °C	41.5 ± 1.8[b]	31.8 ± 1.4[a]	1.07 ± 0.21[a]
6 °C	46.6 ± 1.5[a]	33.0 ± 1.2[a]	1.05 ± 0.11[a]
9 °C	48.9 ±1.5[a]	31.3 ± 1.4[a]	1.16 ± 0.13[a]
Collection location			
CMREC	48.5 ± 0.6[a]	30.2 ± 1.3[a]	1.28 ± 0.14[a]
SLM	50.0 ± 1.4[a]	32.0 ± 1.1[a]	1.17 ± 0.16[ab]
WMREC	42.6 ± 1.9[b]	34.0 ± 1.6[a]	0.82 ± 0.08[b]
Sex			
Female	47.7 ± 1.2[a]	–	–
Male	44.6 ± 1.4[b]	–	–

[a] Means (±SE) within each column of each treatment group followed by the same letter are not significantly different at the $p < 0.05$ level

[b] Survival means for each treatment group are averaged over nonparametric estimates of the survival distribution of replicate colonies

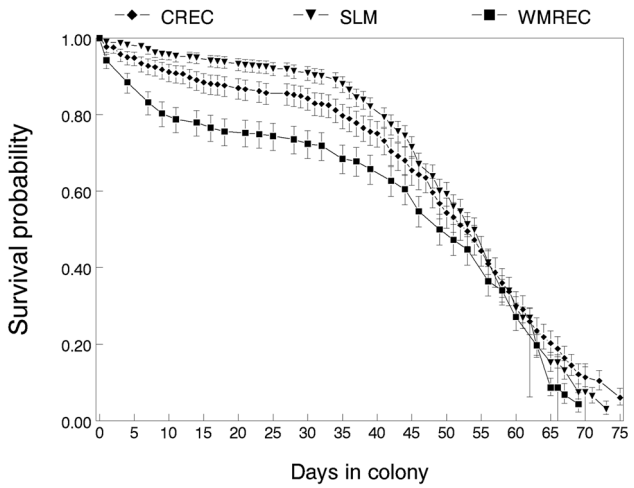

Fig. 5 Survival probability function curves of *H. halys* colonies reared from stored adults collected from different locations (*CMREC* Clarksville, MD; *SLM* Sugarloaf Mountain, *WMREC* Keedysville, MD), based on data pooled over storage temperatures. Adult collection location significantly influenced colony survival after storage for seven weeks ($F_{(2,13)} = 5.3$, $p = 0.021$). Adults from WMREC died in colony at a significantly higher rate leading up to the 25% median mortality level than adults from the other two locations

compared to dead adults (length—13.26 mm ± 0.09, width—7.75 mm ± 0.05, body size—813.13 mm^2 ± 16.7).

Effects of rearing field-collected adults before entering diapause

When *H. hays* populations are high in the fall, particularly in soybean fields, large numbers of adults can be collected, fed for several weeks to sequester the nutrient reserves to survive the diapause period, and then held in cold storage. We evaluated this approach and field-collected adults fed in the laboratory exhibited significantly lower survival during cold storage than adults collected at overwintering sites and stored immediately ($F_{(1,34)} = 50.47$, $p < 0.001$). Percent survival of laboratory-fed males and females averaged 41.7% ± 4.75 and 55.0% ± 4.23, whereas 86.7% ± 2.74 and 93.1% ± 1.81 of the males and females from overwintering sites survived, respectively. Relative differences in survival between the two treatment groups were similar for males and females.

Discussion

This is the first study to examine different approaches to collecting and storing *H. halys* for the purpose of producing adults to supplement continued rearing of a laboratory colony. We evaluated the effects of storage time, storage temperature, and collection site on survival of stored adults and their performance in a laboratory colony. Our results demonstrate that a substantial proportion of diapausing adults can survive when held in cold storage, even for periods longer than needed to terminate diapause. For example, 40% of the adults collected from Sugarloaf Mountain in 2015 survived for 34 week in storage at 9 °C. This period of quiescence or inactivity is 4–6 weeks longer than the overwintering period of *H. halys* in natural settings. Li et al. (2007) reported that most adults in overwintering sites become inactive below 9 °C, so it is unlikely that the mortality of adults stored at this temperature was due to over-chilling; instead, adults probably died from a combination of dehydration and metabolic depletion of their energy reserves (Hahn and Denlinger 2011).

Duration of cold storage at constant 9 °C significantly affected both survival and time to egg production of post-diapause *H. halys* in laboratory colonies. In 2013, the shortest storage period (5 weeks) resulted in egg laying in only one replicate cohort, and that cohort laid very few eggs after 8 weeks of colony rearing. Five weeks in storage is probably not an adequate amount of time to break diapause completely and trigger development of sexually mature *H. halys* females. After 7 or more weeks of storage, egg laying occurred earlier and more consistently across all colony cages, and the trend indicated that females laid eggs more quickly after longer periods of cold storage. However, survival and fecundity per female decreased significantly as the storage periods increased. For adults stored for 14 weeks, egg laying started 10 days earlier, but fecundity was reduced by one half compared to the first oviposition and total number of eggs laid by adults stored for 7 weeks. In 2016, SLM adults stored at 9 °C for 11–34 weeks showed even higher overall survival than adults collected at the two research farms. When brought out of storage and reared in laboratory colonies, these adults also laid eggs much quicker (as early as 22 days), but were significantly less fecund per female as the storage time increased. The metabolic strategies and physiological mechanisms that *H. halys* uses to survive lengthy cold periods of diapause are unknown. However, it is conceivable that females surviving longer storage may eventually start to develop sexual maturity at 9 °C, but exhibit less overall fitness due to depletion of stored energy reserves, thus enabling them to quickly mate and develop at least some eggs. In other insects, the energy reserves expended during diapause can have adverse effects on post-diapause fitness (Hahn and Delinger 2007).

Temperature significantly impacted *H. halys* survival while in cold storage though differences in survivorship were lower in magnitude than duration. Survival of adults stored at 6° and 9 °C for 7 weeks was not statistically different and was significantly higher than adults stored at 3 °C. Pooled over locations, adults stored at lower

temperatures showed significant lower survival when reared in colony, but temperatures had no effect on when the first eggs were laid or the average fecundity per female. Only a few studies have reported on the effects of temperature on overwintering behavior and survival of *H. halys*. Our observations are in agreement with Li et al. (2007), in that we observed only minor movements of adults in disturbed storage containers held at 9 °C but virtually no activity at the lower temperatures. In Japan, Kiritani (2007) observed a 13.5% increase in overwintering survival of *H. halys* for every 1 °C increase above 4 °C in areas where winter temperatures range from 2 to 6 °C, so it is not surprising that exposure to constant 9 °C during storage resulted in the highest survival rate. However, relative differences in survival among the three temperatures were lower than the changes in survival reported by Kiritani (2007), at least for adults stored for 7 weeks. Cira et al. (2016) determined that *H. halys* is chill intolerant and its ability to tolerate cold conditions during overwintering differs by season, sex, and location. In agreement, our results demonstrate that adult survival was significantly higher for females compared to males, and that those that did survive were larger in body size, suggesting that these individuals were capable of storing larger quantities of energy reserves. Cira et al. (2016) also reported that some effects of cold temperatures are location dependent for *H. halys*, specifically that stinkbugs in Minnesota were more cold tolerant earlier in the year when compared to Virginia adults. Although we consider 9 °C as the optimum storage temperature, lower temperatures may be more appropriate in locations where *H. halys* is acclimated and adapted to colder climates.

Interestingly, diapause-destined adults collected from different locations responded differently to both storage duration and temperature. In 2013, adults collected at WMREC survived significantly better than adults from CMREC across storage durations, yet this response reversed in the 2014 study. Furthermore, adults collected at the overwintering site on Sugarloaf Mountain showed overall higher survival during longer storage times, suggesting that these adults were more cold tolerant than those collected from buildings at the two research farms. *H. halys* aggregates at overwintering sites in response to shorter day length and lower temperatures in the fall, but adults must sequester energy reserves before moving to these sites. In most diapausing insects, the most important energy reserve is triacylglyceride fat which comprises 80–95% of the total lipid content (Hahn and Denlinger 2011). Since depletion of energy stores was almost certainly the main cause of mortality during long period of cold storage, the amount and quality of food sources available to *H. halys* adults leading up to their fall aggregation behavior likely had a major influence on their survival during diapause. The field-collected adults that were fed in the laboratory and those captured in pheromone traps probably did not have sufficient food reserves to survive the diapause period. For those adults collected during October at overwintering sites, we assume that they had ample time to acquire sufficient reserves to survive a lengthy diapause, and that local hosts near the aggregation were the major source of overwintering nutrition. Differences in survival of adults collected at the CMREC and WMREC may be attributed to the availability of high-quality host plants during September at these farms. During 2013 and 2014, large and small fruit crops, corn, and soybeans were the major crop hosts on the WMREC farm, whereas corn, soybean, and legume crops dominated the landscape at CMREC. Late maturing soybean fields are known to be attractive hosts of *H. halys* (Nielsen and Hamilton 2009; Venugopal et al. 2014) during September and probably provide the necessary lipid resources required for diapause. However, the availability of this host crop in the surrounding landscape varied by farm location and did not seem related to the cold tolerance differences observed in our studies. The feeding habits of adults prior to entering diapause on Sugarloaf Mountain are less understood because there was no evidence of high populations in soybean fields which dominated the landscape around the base of the mountain during September. We know that *H. halys* feeds on the fruiting bodies of numerous trees and shrubs (Nielsen and Hamilton 2009; Lee et al. 2013; Bergmann et al. 2016); therefore, it is possible that the tree and shrub fauna on Sugarloaf Mountain provides *H. halys* with excellent nutrition and allows them to prepare for diapause more successfully than the stinkbugs feeding on food resources on the research farms. This study draws attention to the importance of adequate nutrition and sequestration of sufficient energy reserves going into diapause as a key factor in diapause success for *H. halys*. This was clearly revealed by the very low survival of stink bugs that were collected from the field and fed in the laboratory for several weeks before entering diapause. Acebes-Doria et al. (2016) reported that *H. halys* nymphs had higher survival rates and generated larger, heavier adults when reared on mixed host diets, and also that certain hosts were better than others for nymphal survival. Further work is needed to determine what host plants are the best food sources available in the late season to prepare *H. halys* adults for successful diapause and overwintering. This information would be helpful for deciding where to collect diapause-destined adults to achieve the highest survival and overall fitness during both storage and later in colony. Taken together, findings of this study provide insight into the expected survival rates of *H. halys* adults under different storage conditions in the laboratory, so that the required number of adults needed for planned experiments or colony supplementation can be more accurately determined.

Author contributions

CT, PC, and GD conceived, designed, and conducted the research. GD analyzed the data. CT, PC, KH, and GD wrote the manuscript. All authors read and approved the manuscript.

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Compliance of ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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