

Induction of Cold Hardiness in an Invasive Herbivore: The Case of Hemlock Woolly Adelgid (Hemiptera: Adelgidae)

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Abstract

As a measure of cold hardiness, we tested the supercooling points or freezing temperatures of individual hemlock woolly adelgids (*Adelges tsugae* Annand) collected from 15 locations across the north to south range of the adelgid in eastern North America at different times during two winters. Adelgids from the northern interior locations with USDA hardiness zones of 5B–6B had lower supercooling points than adelgids from more southern or more coastal locations (zones 7A and 6B), where minimum winter temperatures were higher. Supercooling points reached a minimum in February in northern but not in southern locations. Laboratory experiments demonstrated that adelgids exposed to colder temperatures (–12 °C) had lower supercooling points after 3 d and adelgids held at 10 °C had higher supercooling points than did adelgids held at 2 °C for the same period. Extending these periods to 7 d produced no further change in supercooling points. Adelgids at northern sites had much lower supercooling points in February 2015 following at least 10 d of much colder weather than adelgids from those same sites in February 2016 following much warmer weather. The induction of cold hardiness produced much year-to-year variation in cold hardiness, especially in northern sites, in addition to concurrently and previously demonstrated genetic differences in cold hardiness between northern and southern adelgid populations. Consequently, the cold temperatures required to kill hemlock woolly adelgids will vary year to year and week to week based on exposure to prior temperatures.

Key words: supercooling, overwintering mortality, acclimation, invasive species, *Tsuga canadensis*

Many insects avoid death by freezing in winter by producing cryoprotectants such as glycerol and other polyols in their hemolymph, which reduce the temperatures at which they freeze to values well below 0 °C (Zachariassen 1985). The temperature at which such insects freeze is known as the supercooling point (Bale 1987, Lee et al. 1987). The production of cryoprotectants is a physiological response to cold temperatures (Lee et al. 1987). Induction of cold hardiness due to prior exposure to cold temperatures or acclimation has been previously demonstrated in a number of insect species (Bale 1987, Lee et al. 1987). Here we demonstrate it for the first time in the hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae).

The hemlock woolly adelgid is an invasive forest insect in eastern North America that was introduced to eastern Virginia prior to 1951 from Japan (Havill et al. 2006). Insects invading a new region often face climatic conditions that are different from their countries of origin. That is certainly true of hemlock woolly adelgid, which originated from near Osaka, Japan, a location that experiences very

mild winter temperatures (Havill et al. 2006). Since its accidental initial introduction to Virginia, hemlock woolly adelgid populations have spread south to Georgia and north to upstate New York and southern parts of Vermont, New Hampshire, and Maine (Orwig and Foster 1998). Hemlock woolly adelgids are transported by wind, mammals, birds, and humans (McClure 1990). The adelgid has caused high mortality of eastern hemlock (*Tsuga canadensis* Carrière) and Carolina hemlock (*T. caroliniana* Engelm.) in many stands throughout the eastern United States (Orwig 2002). Damage from the adelgid has been most severe in the southern and mid-Atlantic states, where substantial hemlock decline has occurred in as little as 4 yr (Eschtruth et al. 2006, Ford et al. 2012). This has caused major changes in tree species composition in hemlock-dominated forests (Jenkins et al. 1999, Kizlinski et al. 2002, Orwig et al. 2002, Stadler et al. 2005, Eschtruth et al. 2006).

Damage from the adelgid has slowed substantially, however, in its northern range. After 20 yr of adelgid infestation in Massachusetts, significant hemlock mortality has accumulated

much more slowly than in the south (Paradis 2011, Orwig et al. 2012). Colder winter temperatures in the north, which cause greater overwintering mortality of the adelgid, have been accepted as the likely explanation for this slow pace of hemlock mortality in northern stands infested with the adelgid (Skinner et al. 2003, Shields and Cheah 2005, Trotter and Shields 2009). Studies by Skinner et al. (2003) showed that adelgids collected during winter from southern states suffered higher mortality when exposed to cold temperatures than adelgids collected from northern states. A common garden study by Butin et al. (2005) with adelgids collected from Maryland (hardiness zone 6B) and from Massachusetts (hardiness zones 5B and 6A) showed that the adelgids from Maryland had higher cold-induced mortality than those from Massachusetts after they were reared together for two generations (one year) at a site in Massachusetts. These results suggested that the adelgid in northern locations had evolved some degree of cold hardiness. In other words, the difference in cold hardiness between northern and southern populations is at least partially genetic.

Hemlock woolly adelgids feed on the ray parenchyma cells of hemlock twigs by inserting their stylets at the base of needles (Young et al. 1995). As the overwintering sistens generation mature in late fall, they secrete a white, protective wool-like wax, beneath which they oviposit in late winter (McClure 1989, 1991). The eggs hatch in March, April, or May (depending on latitude and elevation), producing the springtime progrediens generation (McClure 1989, Joseph et al. 2011). Some progredientes (plural of progrediens) become winged sexuparae. In their native Japan, the sexuparae disperse to spruce (*Picea* spp.) and initiate a sexually reproducing generation. In North America, however, none of the offspring of these sexuparae survives on the native spruce species. Thus, only parthenogenic reproduction occurs in North America (McClure 1989). The progrediens crawlers settle on hemlock shoots produced in the previous year. They mature and oviposit in May or June, and eggs hatch in May, June, or July. The resulting sistens crawlers enter a period of summer aestivation, where feeding and development cease. Feeding and development resume in October, and continue throughout winter. The adelgids reach maturity in late winter or early spring.

Here we further explore the causes of the differences in cold hardiness between northern and southern populations of the adelgid by analyzing the freezing temperatures or supercooling points of adelgids collected from 15 locations, spanning the range of adelgid from north to south in the eastern United States at different times during the winter. We also report the results of laboratory experiments, in which adelgids were exposed to different cold temperatures for 3 and 7 d to assess the degree to which cold hardiness could be induced by exposure to cold.

Materials and Methods

Adelgid Collection

To determine the variation in cold hardiness across their introduced range, we tested the supercooling point of hemlock woolly adelgid from 15 sites spanning northern Georgia to New York and Massachusetts (Fig. 1; Table 1). Hemlock twigs infested with adelgids were removed from branches within a height of 2 m from haphazardly selected trees at each site. The samples consisted of small branches (~25–35 cm in length) with a moderate–high density of live, apparently healthy adelgids on branch tips of the most recent growth. The samples were placed in insulated boxes and transported by car or via overnight mail to Amherst, MA.

Measuring Supercooling Points

Supercooling tests were conducted within 2 d of sample collection. Prior to the test, samples were held in a Percival growth chamber (Perry, IA) at 2°C. The samples consisted of small branches (~25–35 cm in length) with a moderate–high density of live, apparently healthy adelgids on branch tips of the most recent growth. To measure its supercooling point, an individual adelgid was selected from a sample branch, the waxy outer covering (“wool”) was removed with fine-tipped forceps, and the individual was attached to the end of a K-type thermocouple sensor using clear tape. The thermocouples were placed into a container containing small brass beads to facilitate heat conduction. The container was submerged in a supercooling bath (Neslab RTE-140) and the temperature of the bath was slowly reduced from a starting point of 5°C down to –35°C over the course of 3 h (~1°C change every 5 min). The temperature of each adelgid was recorded in 1-s intervals using a multichannel thermocouple recorder (Physitemp Inc., NJ). The point at which the adelgid froze produced an obvious spike in the temperature as a result of the heat of fusion. We used the temperature in the second before the thermal spike as the supercooling point. The supercooling points of 20–50 adelgids per site per sampling event were measured. Twenty adelgids per site were sufficient to estimate the trends we were seeking to demonstrate, but we did more of them whenever time and the number of adelgids available on our sample twigs permitted. These sites were sampled in February, March, and December 2015, and in February 2016, but we were not able to collect from every site in each of the 4 mo. A list of sites used in the four sampling events can be found in Table 1.

Differences in the supercooling point of adelgid among the various source populations were analyzed using regression of supercooling points against the mean minimum winter temperature (coldest day of the year) experienced at each collection site over the past 10 yr (2006–2015). Daily temperature data were obtained from the nearest NOAA weather station to each site. Regression analyses were conducted in R (R Core Team 2015). Differences in supercooling points between months at each site were analyzed by ANOVA coupled with Tukey’s HSD test SAS (Proc GLM, SAS 9.3, SAS Institute 2012).

Induction of Cold Hardiness in Laboratory Experiments

In order to understand how quickly the adelgid can respond to the onset of cold temperatures, we preconditioned the adelgids by holding them in Percival growth chambers for 3 or 7 d at one of three different temperatures (2°C (control), –12°C, and 10°C) before we measured their supercooling points. To do this, branches containing adelgid sistens were collected from eastern hemlock trees in Amherst, MA, on 20 January 2016. Supercooling points of these adelgids were obtained using the methods described above. Data from this experiment were analyzed by ANOVA coupled with one-sided *T*-tests to determine the statistical difference between the supercooling points of the –12°C and 10°C preconditioning treatments from the controls. We also regressed the supercooling points versus exposure temperature for each of the two durations. The analyses were conducted in R (R Core 2012).

Results

There was a seasonal trend evident in the supercooling points of adelgids from northern locations (Fig. 2A), wherein they were much lower in February 2015 than in March 2015 or December 2015 (Table 2). The coldest temperatures of the year typically occur in January or February (Table 2). At northern sites, supercooling points were much lower in February 2015, than they were in

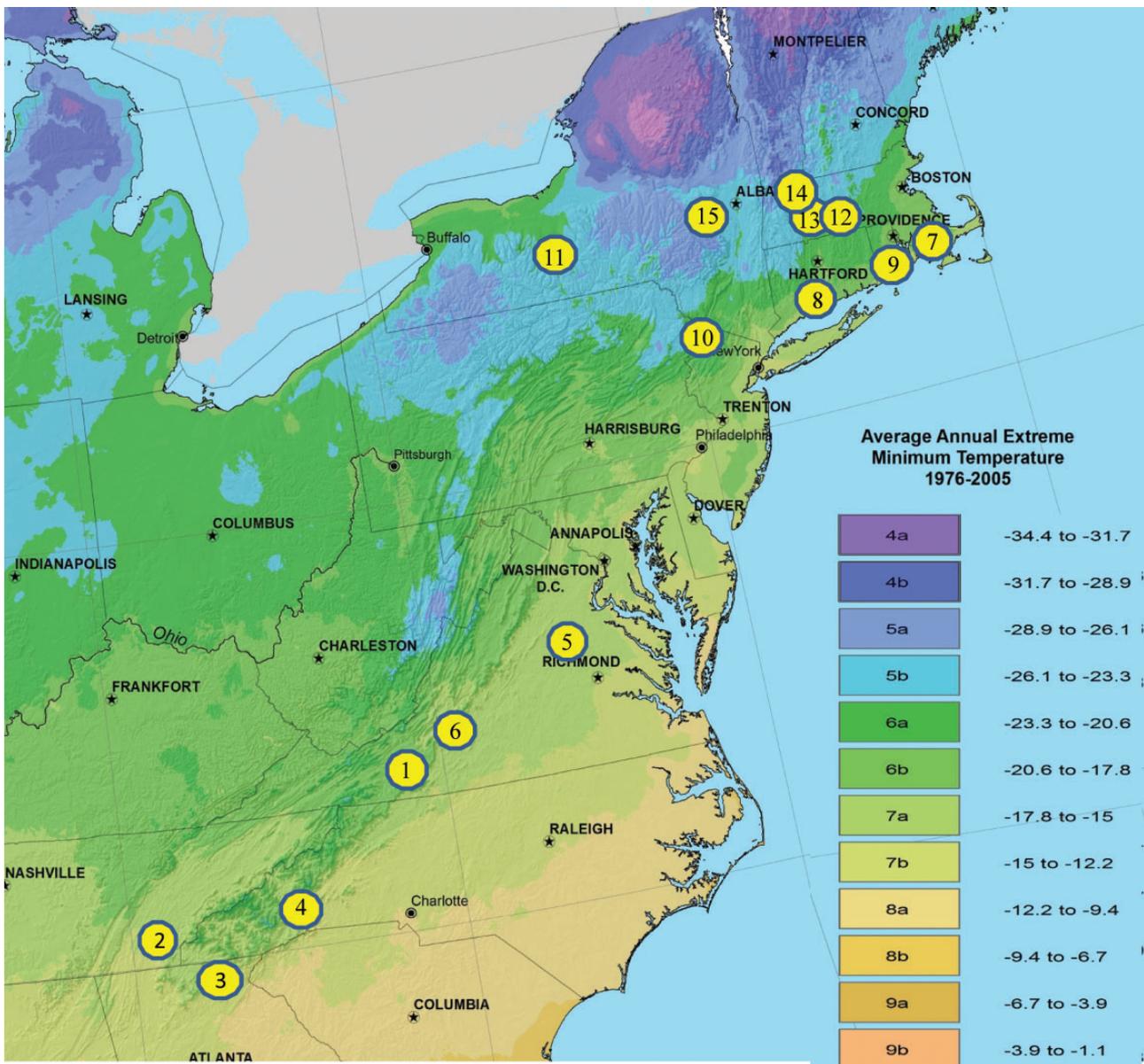


Fig. 1. Hemlock woolly adelgids collection locations plotted on a USDA plant cold-hardiness zone-maps based on average minimum winter temperatures (1976–2006). Site numbers from Table 1 are given inside yellow circles.

February 2016 (Fig. 2A). No such trends were evident in the southern locations (Fig. 2B). There was a corresponding difference in mean temperatures between the 2 yr, particularly in the 10 d preceding the February samples (Table 2). At the three northern interior sites (Fig. 1; Table 2), the difference was 9.8–10.5°C warmer during this period in 2016 compared with 2015. In contrast, the temperatures at the southern sites were 2.9–0.2°C colder in 2016 than in 2015 during this same period (Table 2). At the northern coastal site in Wareham, MA, which is in the same hardiness zone (6B) as the two Virginia sites, the early February temperature was 7.28°C warmer in 2016 than 2015 (Table 2), and the difference in supercooling points was correspondingly intermediate (Fig. 2A). The sites presented in Fig. 2 and Table 2 were those for which we had obtained samples from at least three, if not four, of the four different sample months.

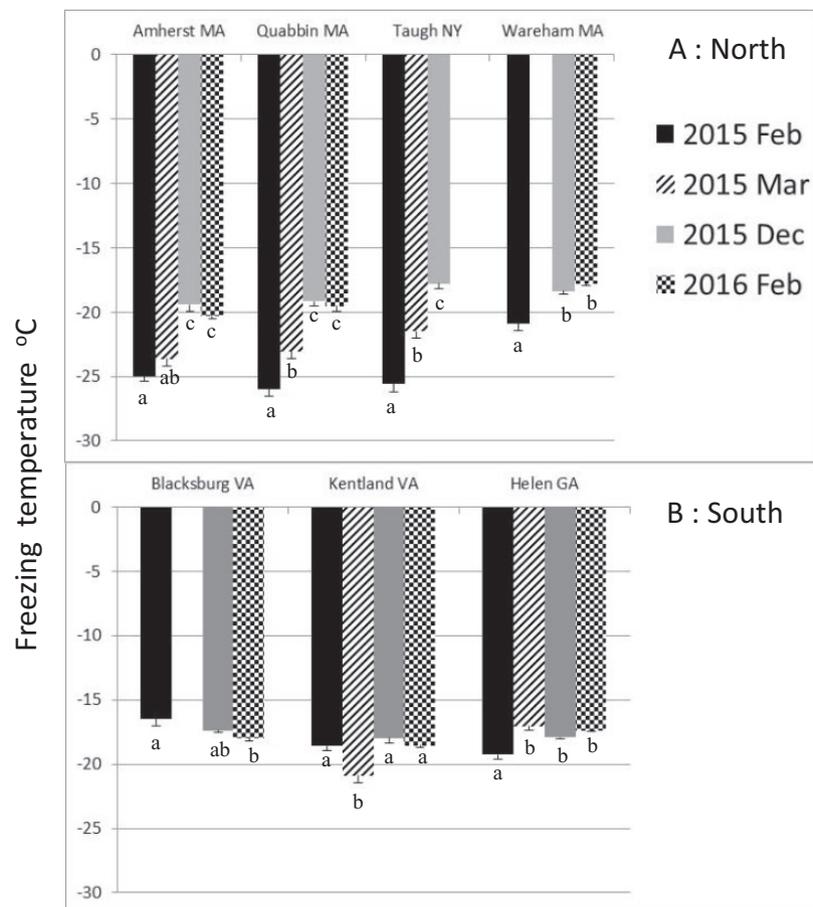
There was a significant positive correlation (Table 3) between the supercooling points of adelgids and the average coldest yearly

temperature over the past 10 yr at the site of collection in February 2015 (Fig. 3A), March 2015 (Fig. 3B), December 2015 (Fig. 3C), and February 2016 (Fig. 3D). The slope of the regression line for February 2015 (Fig. 3A; Table 3) was significantly steeper than the slope for February 2016 (Fig. 3D; Table 3), because of the much lower supercooling points at the northern sites in February 2015. In each of these samples, we tested 20–50 adelgids and there was always a large variation of supercooling points spanning at least 10°C at most locations, as is evident throughout (Fig. 3). This was true even though all the adelgids in each sample typically came from the same hemlock twig or twigs.

In the laboratory induction experiment using hemlock woolly adelgids collected from around Amherst, MA, in January 2016, we found that the supercooling point of adelgids increased with preconditioning temperatures of -12°C , 2°C , and 10°C (Fig. 4; $df = 2,331$; $F = 26.35$; $P < 0.001$; Table 3) after an exposure of only 3 d. A one-sided t-test showed that the mean supercooling points of

Table 1. Location, latitude, longitude, plant hardiness zone, average daily minimum winter (December–February) temperature over the past 30 yr, and average absolute minimum each year over the past 10 yr at different hemlock woolly adelgid collection sites

Site no.	Site location	State	Latitude	Longitude	Hardiness zone	Mean min. daily temp (°C)	Mean absolute winter min. (°C)
1	Kentland	Virginia	37.20747	−80.5894	6B	−1.2	−15.7
2	Hiawasee	Tennessee	35.16148	−84.4817	6B	−1.9	−13.0
3	Helen	Georgia	34.78576	−83.75800	7B	−2.1	−12.5
4	Bent Creek,	North Carolina	35.46545	−82.65554	7A	−3.8	−13.8
5	Powhatan	Virginia	37.60162	−77.76826	7A	−4.7	−14.4
6	Blacksburg	Virginia	37.39543	−80.41462	6B	−5.1	−16.2
7	Wareham	Massachusetts	41.76437	−70.71539	6B	−5.2	−18.7
8	Hamden	Connecticut	41.38869	−72.90330	6B	−5.4	−19.0
9	Kingston	Rhode Island	41.48076	−71.52256	6B	−5.6	−21.9
10	Del. Water Gap	Pennsylvania	40.92925	−75.14279	6B	−7.8	−21.9
11	Taughannock Falls	New York	42.53611	−76.61154	5B	−7.9	−23.9
12	Quabbin	Massachusetts	42.27940	−72.34807	5B	−8.3	−21.8
13	Amherst	Massachusetts	42.39208	−72.53099	5B	−8.7	−22.7
14	Shelburne Falls	Massachusetts	42.60424	−72.73312	5B	−9.5	−22.3
15	Mine Kill	New York	42.43328	−74.46283	5B	−9.7	−25.5

**Fig. 2.** Mean (+ SE) supercooling points of hemlock woolly adelgids collected from selected northern and southern locations in December 2015, February 2015, February 2016, and March 2015. Within each site, mean supercooling points that share the same lower case letter are not statistically different from other sample dates (one-way ANOVA, Tukey's HSD test, $\alpha = 0.05$).

adelgids in the -12°C treatment were lower than adelgids in the 2°C treatment ($t = 4.561$; $df = 203$; $P < 0.0001$), which were lower than that for adelgids in the 10°C treatment ($t = 2.468$; $df = 221$; $P = 0.007$). There was no change in the supercooling points among adelgids held at these temperatures for 7 d instead of 3 d (Fig. 4; $df = 1,331$; $F = 0.206$; $P = 0.65$; Table 3).

Discussion

Our laboratory demonstration of induction of cold hardiness among adelgids exposed to -12°C in as little as 3 d showed that adelgids can acclimate to cold temperatures by lowering their supercooling points over this time interval. It is important to note that this change

in cold hardiness was not due to killing off the least cold hardy amongst the test adelgids. The lowest preconditioning temperature (−12°C) used in this experiment (Fig. 4) was above the highest supercooling point of the adelgids from this region (Fig. 4 and site 13 in Fig. 3D). Despite these changes in supercooling points, there appeared to be no reduction in the variance of supercooling points

Table 2. Mean winter temperatures by month and mean temperatures during the first 10 d in February prior to February measurements at select northern and southern sites in 2014–2015 and 2015–2016

Site	Zone	Month	2014–2015	2015–2016	Difference ^a
Northern sites					
Amherst, MA	5B	Dec.	4.7	9.27	4.57
		Jan.	−1.09	2.38	3.48
		Feb.	−3.93	5.06	8.99
		Feb. 1–10	−3.67	6.83	10.5
Quabbin, MA	5B	Mar.	4.05	11.08	7.03
		Dec.	4.71	8.49	3.78
		Jan.	−1.51	1.61	3.12
		Feb.	−4.48	4.23	8.72
Tuag, NY	5B	Feb. 1–10	−4.56	6.28	10.84
		Mar.	3.19	10.02	6.83
		Dec.	2.58	8.76	6.18
		Jan.	−3.17	−0.22	2.95
Wareham, MA	6B	Feb.	−6.53	2.39	8.92
		Feb. 1–10	−3.56	6.28	9.84
		Mar.	1.4	9.75	8.35
		Dec.	8.28	11.92	3.64
Southern sites	Blacksburg, VA	Jan.	2.4	4.26	1.86
		Feb.	−1.13	5.88	7.01
		Feb. 1–10	−0.39	6.89	7.28
		Mar.	4.34	9.82	5.48
Blacksburg, VA	6B	Dec.	7.99	13.85	5.86
		Jan.	4.78	3.59	−1.19
		Feb.	2.9	5.75	2.85
		Feb. 1–10	9.00	7.39	−1.61
Kentland, VA	6B	Mar.	11.92	15.93	4.01
		Dec.	7.53	13.64	6.11
		Jan.	4.1	3.94	−0.16
		Feb.	2.42	6.07	3.65
Helen, GA	7B	Feb. 1–10	7.37	7.17	−0.20
		Mar.	11.83	15.63	3.8
		Dec.	10.38	13.69	3.32
		Jan.	6.77	5.45	−1.33
Helen, GA	7B	Feb.	5.06	7.06	2.00
		Feb. 1–10	9.22	6.28	−2.94
		Mar.	14.41	15.57	1.16

^aDifference = (2015–2016 mean) – (2014–2015 mean).

Table 3. Regression coefficients of supercooling points estimated in different months plotted against average minimum winter temperature (Fig. 3) or versus exposure temperatures in the laboratory (Fig. 4) and one-tailed *T*-tests for slopes < 0

Date	Figure	Intercept (SE)	Slope (SE) ^a	<i>T</i> , slopes < 0	<i>P</i> -value
Feb. 2015	3A	−9.02 (0.78)	0.675 (0.041)a	16.48	<i>P</i> < 0.001
Mar. 2015	3B	−12.82 (0.85)	0.415 (0.045)b	9.18	<i>P</i> < 0.001
Dec. 2015	3C	−16.25 (0.393)	0.103 (0.021)c	4.99	<i>P</i> < 0.001
Feb. 2016	3D	−13.78 (0.476)	0.272 (0.026)d	10.28	<i>P</i> < 0.001
Treatment					
3-d exposure	4	−19.591 (0.147)	0.080 (0.017)	4.823	<i>P</i> < 0.001
7-d exposure	4	−19.501 (0.135)	0.082 (0.015)	5.449	<i>P</i> < 0.001

^aDifferent lower-case letters associated with slopes in Fig. 3 indicate they are significantly different from one another (Bonferroni-corrected *Z*-test, $\alpha = 0.05$).

either in the laboratory (Fig. 4) or in the field (compare February 2016 with February 2015 or December 2015; Fig. 3). If the cold hardiness evident in February 2015 or at −12°C in the laboratory (Fig. 4) were due to prior death of the least cold-hardy individuals, then we would expect to see a decline in the variance of supercooling points of those samples compared to the earlier dates or higher temperature samples. That did not happen, as is evident in Figs. 3 and 4.

Compared to the big differences in supercooling points between northern and southern adelgids evident in February 2015, the north to south differences in February 2016 were much smaller than those in February 2015, after a much milder January and early February. Indeed, there had been almost no change in supercooling between December 2015 (Fig. 3A) and February 2016 (Fig. 3D), and these were about the same magnitude shown in the common garden experiment in December 2015 (Lombardo and Elkinton, unpublished data). The implication is that the induction of supercooling evident in our data accounted for much of the north to south differences evident in February 2015 compared with February 2016.

The mean values of the coldest day of the year over the past 10 yr at each site (Table 1) were comparable with the supercooling points recorded in February 2015 (Fig. 3A). The large variation in supercooling points evident at all sites suggests that some adelgids would die everywhere nearly every winter due to cold temperatures. That conclusion is consistent with previous reports of north–south variation in overwintering mortality of the adelgid (Trotter and Shields 2009).

The supercooling points of adelgids from different sites in December 2015 were essentially indistinguishable from those we measured at the same time in adelgids from these same sites and reared for two generations in a common garden experiment in western Massachusetts (Lombardo and Elkinton, unpublished data). Our results from the common garden experiment show that adelgids have evolved cold tolerance, as they have moved north from the site of introduction near Richmond, VA, in the 1950s (Havill et al. 2006) and invaded New England in the late 1970s (McClure 1989, 1991). These results confirm those from the common garden experiment conducted by Butin et al. (2005), who showed that adelgids collected from three sites in eastern Maryland (hardiness zone 7A) had lower cold hardiness based on survival from laboratory cold shock after they were reared for two generations on adjacent trees near Amherst, MA, compared with those collected and reared from sites in Massachusetts (hardiness zones 6A and 5B). The implication is that cold hardiness of the adelgid has a genetic basis. Butin et al. (2005) argued that evolution of cold hardiness in this insect was possible despite its parthenogenetic reproduction in North America, because of the vast numbers of adelgids present. These genetic differences thus form the baseline of north–south differences in cold hardiness evident in December 2015 (Fig. 3A).

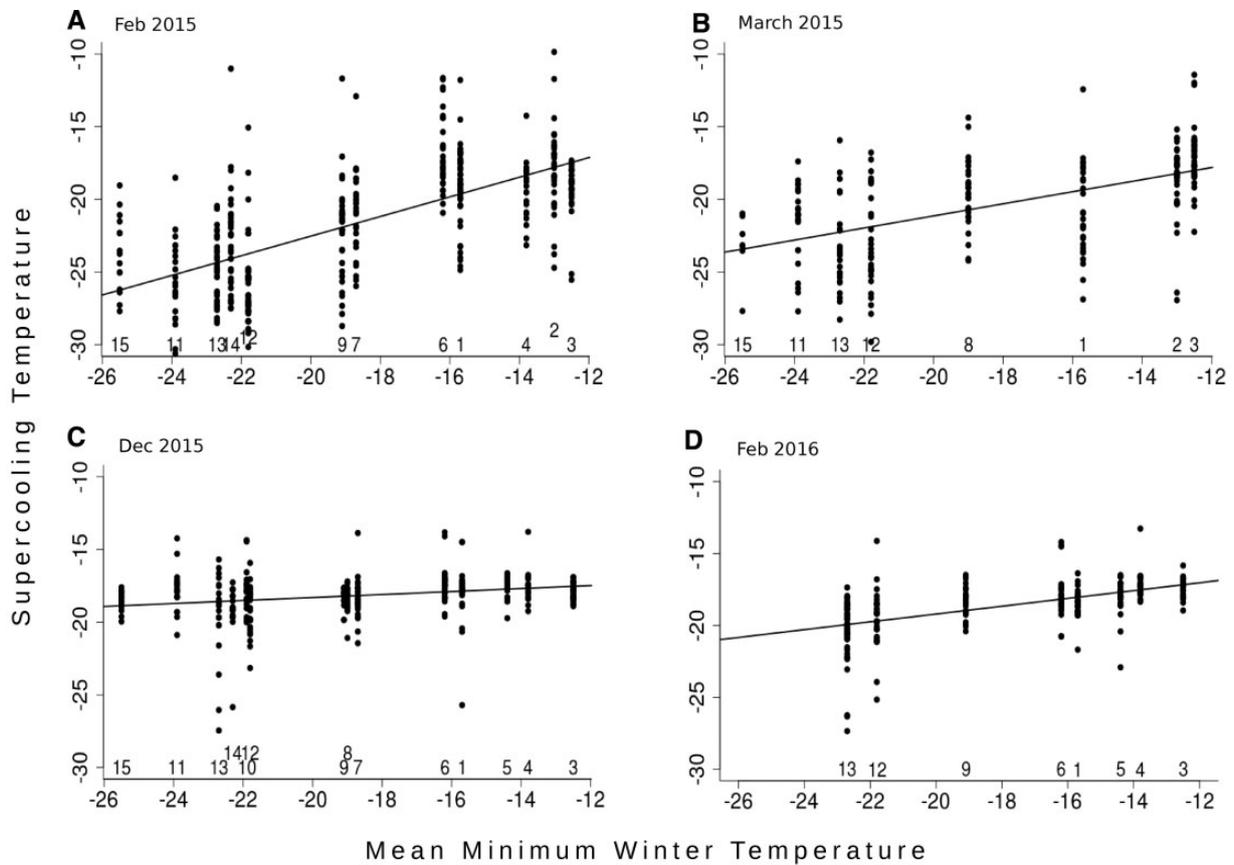


Fig. 3. Supercooling points of hemlock woolly adelgids collected from 15 northern, coastal, and southern locations (Table 1) in (A) February 2015, (B) March 2015, (C) December 2015, and (D) in February 2016 plotted versus mean minimum winter temperature (coldest temperature of the winter) over the past 10 yr (2006–

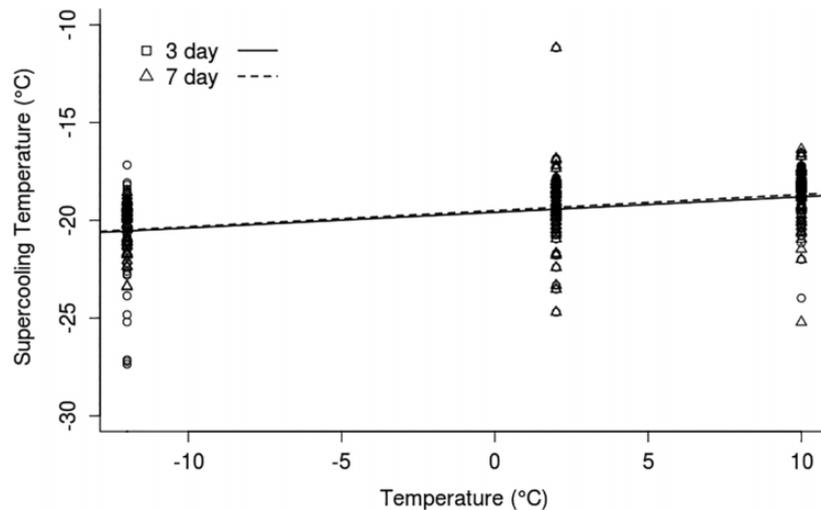


Fig. 4. Supercooling point of adelgids collected from Amherst, MA, on 20 January 2016 and held at -12°C , 2°C , and 10°C for 3 and 7 d.

On 14 February 2016, a sudden cold event swept the northeastern United States. As stated above, temperatures prior to that time had been unusually mild and northern adelgids were much less cold hardy (Figs. 2A and 3D; Table 2), than they had been the previous winter. Temperatures at our research sites near Amherst, MA, dropped to -24.4°C . These temperatures were below the supercooling points of nearly all adelgids from this region we had just tested

(Fig. 1C). We were thus not surprised to discover that nearly 100% of these adelgids from our research sites in western Massachusetts were dead. In contrast, overwintering mortality in these populations was only 10–20%, when measured the previous week prior to the cold event. We examined adelgids from other locations in New England, including coastal sites in Massachusetts, Rhode Island, and Connecticut, and also at our two sites in New York. Nearly 100%

of them were dead as well. The implications of this event are that sudden declines in temperature following periods of warmer temperatures can cause high adelgid mortality. These adelgids can adapt to cold temperatures, if they are given sufficient time to do so. What that timeframe is should be investigated in future experiments.

These observations are consistent with the findings of Paradis et al. (2008), who reported that overwintering, adelgid mortality was more highly correlated with variation in temperature than with the absolute minimum temperature across a range of sites and years (2004–2007) in Massachusetts. Before we conducted our study, we knew from the work of Skinner et al. (2003) that adelgid cold hardiness would vary from month to month over the winter. We assumed, however, that these values would be the same from one winter to the next in any population. Now we know that is not true. It depends on prior exposure to cold temperatures, which varies from winter to winter. The ability of this insect to survive winter temperatures is a function of previous exposure to cold, which builds upon the north–south genetic differences in cold hardiness.

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