

Evolution of increased cold tolerance during range expansion of the elongate hemlock scale *Fiorinia externa* Ferris (Hemiptera: Diaspididae)

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Abstract. 1. Alien species often face novel challenges to their spread and population growth. One critical hurdle often involves an organism's ability to tolerate environmental extremes characteristic of their invaded range. Although abiotic factors often determine range limits, there is less evidence for local adaptation in invasive organisms whose initial arrival and rapid population growth is separated by a lengthy lag period.

2. The invasive elongate hemlock scale *Fiorinia externa* feeds on Eastern hemlock, *Tsuga canadensis*, on the east coast of North America. Following its 1908 arrival, it remained localised until entering a period of rapid northward range expansion in the 1970s.

3. The present study tested the survival of overwintering *F. externa* populations from four sites in the north and south of the invaded range ($n = 8$ sites total) when exposed to $-15\text{ }^{\circ}\text{C}$ for 0–36 h.

4. This experiment was repeated on *F. externa* offspring that had been reared in a common-garden environment in order to control for parental effects.

5. Northern populations were more tolerant of exposure to cold temperatures than were southern populations. This held true in both the source-population experiment and common-garden experiment. The common-garden experiment demonstrates that this difference has a genetic basis and may be the consequence of local adaptation to lower winter temperatures.

6. The results provide evidence for local adaptation to extreme temperatures in *F. externa*. This provides one possible explanation for the lag period between the arrival of this species and its eventual northward range expansion.

Key words. *Adelges tsugae*, cold tolerance, evolution, *Fiorinia externa*, invasive species, local adaptation, range limits, *Tsuga canadensis*.

Introduction

Substantial time lags often separate the establishment of an alien species and when it begins to be considered 'invasive'. Such lag periods may reflect the importance of evolutionary changes such as local adaptation or the purging of genetic load in facilitating species invasions (reviewed in Sakai *et al.*, 2001; Richardson & Pysek, 2006). As a result, understanding sudden range expansions of naturalised species (*sensu* Richardson & Pysek, 2006) may require addressing the complex interplay of

ecological and evolutionary factors occurring when species enter a novel environment (Lee, 2002; Lambrinos, 2004; Huey *et al.*, 2005; Chown *et al.*, 2007). In particular, spatially separated populations of the invading species can experience very different selective forces that may lead to genetic divergence and local adaptation (Huey *et al.*, 2000; Garcia-Ramos & Rodriguez, 2002; Balanya *et al.*, 2006). Such local adaptation can increase survival in initially inhospitable environments and may eventually facilitate range expansion into novel environments (Peck & Welch, 2004; Hastings *et al.*, 2005; Huey *et al.*, 2005).

Tolerance of extreme temperatures is commonly invoked as a major factor limiting species' range expansion (reviewed in Chen & Kang, 2005; Chown & Terblanche, 2007). In regions that experience wide seasonal fluctuations in temperature, the

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ability to tolerate low winter temperatures may be especially important (Gouli *et al.*, 2000; Parker *et al.*, 2002; Klok *et al.*, 2003; Paradis & Elkinton, 2005; Calosi *et al.*, 2008). If there is genetic variation in cold tolerance, invasive species may adapt to colder winter temperatures and their effective range may increase over time (but see Blows & Hoffmann, 2005 for the potential impacts of limited genetic variance).

Although evolution has been repeatedly invoked to explain range expansion in invading species, rigorously demonstrating that evolutionary changes facilitate range expansion requires evidence (i) for adaptation to novel environmental conditions; and (ii) that these adaptive changes are responsible for the expansion into novel environments. For instance, the hemlock woolly adelgid (HWA) *Adelges tsugae* Annand (Hemiptera: Adelgidae), an invasive hemipteran feeding on eastern hemlock [*Tsuga canadensis* (L.) Carr.] on the east coast of the U.S.A., is limited in the northern portion of its range by overwintering mortality (Parker *et al.*, 2002; Paradis & Elkinton, 2005). Recent work (Butin *et al.*, 2005) used a common-garden experiment to demonstrate that northern HWA populations are more tolerant of low winter temperatures than are populations from the southern portion of its invaded range. While genetic changes in cold tolerance cannot be definitively linked to the adelgid's range expansion, this is one of a series of recent studies examining adaptive divergence in traits associated with current range limits in an alien species (e.g. Gilchrist & Huey, 2004; Lee *et al.*, 2007; Urban *et al.*, 2007).

Although the HWA has received much of the blame for hemlock decline, a second invasive species, the elongate hemlock scale (EHS) *Fiorinia externa* Ferris (Hemiptera: Diaspididae), has also been cited as a contributing factor (Danoff-Burg & Bird, 2002; McClure, 2002). This invasive scale reproduces sexually and is univoltine in the northern portion of its invaded range, where it feeds almost exclusively on *T. canadensis* (McClure, 1978, 1986). Although both species originated in Asia, neither the factors determining EHS range limits nor the interactions between these two species have been studied in the native range (but see McClure, 1983, 1986 for research addressing EHS performance on native versus novel host plants). Although HWA is a fairly recent invader (first found in Richmond, Virginia in the 1950s; McClure & Cheah, 1999), EHS arrived in New York City in 1908 (Sasscer, 1912). Despite its early arrival, EHS remained largely restricted to the site of the initial invasion until the mid-1970s, when it began to expand its range northward (McClure, 1977). Although the threat posed by HWA has distracted attention from EHS, this insect's northward range expansion has accelerated over the past several decades (Preisser *et al.*, 2008). Although it appears to have coincided with HWA's invasion of this same northern region, the causes of EHS range expansion are unknown.

The present study assesses whether local adaptation to low temperatures has occurred in northern populations in the invaded range of the EHS. If northern and southern populations have diverged with respect to cold tolerance, then adaptive changes in cold tolerance (or another associated trait) may be responsible for the northern range expansion. Our research suggests that overwintering survival may affect the range expansion of this insect, and that local adaptation to extreme abiotic conditions plays a role in the continuing northward advance of this species.

Methods

Source population climate

We initially identified areas in the southern (Maryland; mean latitude 39°N) and northern (Connecticut; mean latitude 42°N) portion of the invaded range. Rather than using state-level data that might obscure ecologically important local climatic variation (Bale, 1991; Sinclair, 2001), we analysed daily temperature data for the period 1950–2005 from the individual weather station closest to each set of sampling sites (see below for details; Table 1). The Maryland data came from a NOAA weather station in Beltsville, Maryland, and our Connecticut data came from Bradley Airport in Windsor Locks, Connecticut.

In the southern portion of the range, Maryland experiences relatively mild winters. From 1950–2005, mean monthly winter (November–March) temperatures did not fall below 0 °C (Fig. 1a; NOAA, 2008). In contrast, northern Connecticut experiences relatively harsh winters, with mean monthly winter temperatures dropping below 0 °C from December through to February. Connecticut also experiences a greater number of unusually cold days than does Maryland. Over 1950–2005, Maryland averaged 16 fewer days per winter in which the temperature dropped below 0 °C (~15% less than in Connecticut; Fig. 1b), and seven fewer days per winter in which the temperature dropped below –15 °C (~74% less than in Connecticut; NOAA, 2008).

Cold tolerance source-population experiment

In early March 2005, we identified four sites in both the northern and southern portion of the invaded range. The four northern sites were located in East Granby, Somers, and Simsbury (Connecticut), and Longmeadow (Massachusetts). The four southern sites were located in Potomac, Bethesda, Silver Spring, and Maryland City, Maryland (Table 1). All of

Table 1. Sites of source populations.

Location	Site	Latitude	Longitude
Southern source populations			
8800 Clewerwall Rd, Potomac, Maryland	1	38.993	–77.271
Dudley Lane, Bethesda, Maryland	2	39.009	–77.102
9520 Crosby Rd, Silver Spring, Maryland	3	39.008	–77.026
Welch's Ct., Maryland City, Maryland	4	39.089	–76.776
Northern source populations			
5 Seneca Rd, E. Granby, Connecticut	1	41.951	–72.383
Sodom Rd, Somers, Connecticut	2	41.966	–72.399
Simsbury House on Rte 202, Simsbury, Connecticut	3	41.890	–72.804
Forest Park, Longmeadow, Massachusetts	4	42.076	–72.574

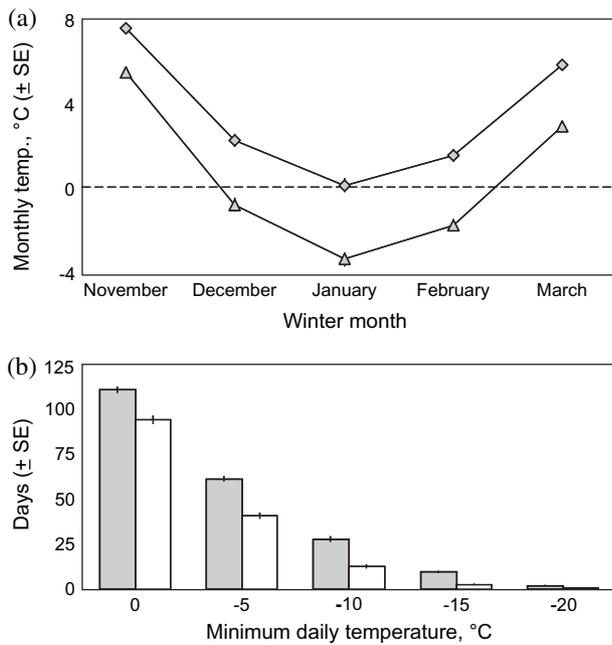


Fig. 1. Winter climate data, 1950–2005, from Maryland (southern EHS populations) and Connecticut (northern EHS populations). (a) Mean monthly winter temperatures in Maryland (◇) and Connecticut (△). (b) Mean days per winter in Maryland (open bars) and Connecticut (filled bars) for which the minimum daily temperature was ≤ 0 °C, -5 °C, -10 °C, -15 °C, and -20 °C.

the sites were separated from each other by at least 5 km. Before sampling, we checked with local property owners to confirm that none of the sites had been treated for pest suppression. At each site, multiple branches were removed from a single mature EHS-infested eastern hemlock and taken to the laboratory.

The initial cold tolerance experiment was started 2 days after bringing the cut branches from both the northern and southern sites back into the laboratory. The experiment was designed to test whether northern versus southern EHS populations would respond differently to exposure to -15 °C for a period ranging from 0–36 h. We chose -15 °C as the temperature threshold for the low temperature treatment since experiments on the HWA, another invasive hemlock-feeding hemipteran, have shown that prolonged exposure to this temperature causes significant but less than complete mortality (e.g. Parker *et al.*, 1999; Parker *et al.*, 2002; Skinner *et al.*, 2003; Butin *et al.*, 2005). In addition, temperatures of -15 °C closely approximate the coldest temperatures recorded in both the northern and southern areas of the study (Fig. 1b). There were a total of seven treatments: no exposure to cold temperatures (control), exposure to -15 °C for 6, 12, 18, 24, 30, and 36 h. EHS-infested branches from each site were placed in a water-filled aquapic (Diamond Line Containers, Akron, Ohio), exposing approximately 15 cm of EHS-infested foliage per branch, and subjected to each treatment. Data from the branches was used to calculate a mean response for each site \times treatment combination. A shortage of EHS-infested branches from some sites meant that the number of

branches used to calculate the response for each site \times treatment combination varied from one to nine, with a total of 153 branches in the entire experiment. However, all of the northern and two of the southern sites had at least three branches in each of the control, 12, 24, and 36 h low-temperature treatments. Control branches were kept in water-filled containers in a 5 °C growth chamber (70% RH and a LD 10:14 h cycle); other branches were similarly placed in the growth chamber after each treatment.

Two weeks after the final treatment had finished, scale survival was assessed. This delay was necessary because armoured diaspidid scales like EHS adhere tightly to plant material. As a result, dead scales desiccate slowly and recently killed individuals are virtually impossible to distinguish from live specimens. Starting at the newest new-growth hemlock needles (identifiable by their colour and position at the terminal end of branch tips), the live/dead status of up to 50 adult EHS branch⁻¹ site⁻¹ treatment⁻¹ was determined. Although we did not assess the few early-instar larvae we encountered on branches from southern sites [EHS produces a partial second generation each year in the southern portion of its invaded range (K. Abell, unpublished data)], we may not have been able to distinguish between later-instar and adult EHS. This data was used to calculate the proportion of scale surviving for each branch in each site \times treatment combination; the results were averaged to obtain a mean proportion surviving per site ($n = 8$) for each of the seven treatments (a total of 56 data points).

Cold tolerance common-garden experiment

The study addressed the possible influence of parental and environmental effects in the 2005 cold tolerance experiment by raising EHS collected from the same tree at each of the eight sites (four northern, four southern) in a common-garden environment in the Cadwell Forest, a research forest managed by the University of Massachusetts and located in Petersham, Massachusetts. Since scale oviposition and crawler production occurs earlier in the south than in the north, EHS-infested foliage were collected for a second time from the four southern sites in late April and from the four northern sites in mid-June 2005. These dates were timed to reflect the beginning of crawler emergence in each population. Infested foliage from each site was placed in water-filled aquapics and wrapped loosely around foliage on mature eastern hemlocks that had previously been inspected for EHS and found to be uninfested. Once the infested foliage was attached to the branches, each branch was enclosed in a 60- by 30-cm mesh sleeve (1 mm² mesh size; Kleantest Products, Milwaukee, Wisconsin). The mesh provided ventilation while preventing cross-colonisation by crawlers from different sites. Within each mesh bag, the crawlers emerging from the infested foliage settled on the uninfested branches and matured over the summer and fall. The newly infested foliage was collected from the mature trees in early January 2006 and moved into a 0 °C cold room. This step was taken as a precaution in response to forecasts of an extended period of unusually cold weather (night-time temperatures of between -13 °C and -18 °C) throughout north-central Massachusetts. Branches were kept hydrated in water-filled containers.

The 2006 cold tolerance experiment started in mid-February and was similar to the 2005 experiment, except that we tested offspring of the northern and southern populations that had been reared in a common-garden environment. To increase the number of branches used to calculate a mean response for each site \times treatment combination, four treatments were used: a control and exposure to -15°C for 12, 24, or 36 h. Although low crawler settlement in one of the southern sites restricted us to three or four branches per treatment for that site, there were five or six branches for each of the remaining site \times treatment combinations. Although we observed neither crawlers nor early-instar larvae on any of the branches, we may not have been able to distinguish between later-instar and adult EHS. The experiment was carried out as in the 2005 experiment, although EHS density (number of EHS per 50 needles) was also measured on each branch in the control treatment for every site, in order to assess whether differences in EHS survival were correlated with density.

Data analysis

Cold tolerance experiments. In both the 2005 and 2006 cold tolerance experiments, ANOVA in JMP v.6.0.2 (SAS, 2004) was used to test for the main effects of location (northern, southern) and treatment (number of hours at -15°C), and for an interaction between treatment and location. The dependent variable was the mean proportion of surviving EHS per site per treatment (56 data points in 2005, 32 data points in 2006). Data were arcsine sqrt transformed before analysis. If the initial ANOVA revealed a significant treatment \times location interaction, separate ANOVAs were run on the data from each location testing for a main effect of treatment. All data were checked for normality and homogeneity of variances prior to the analyses.

Data on EHS density per 50 needles (collected only during the 2006 experiment) was treated as above. The relationship between EHS density and overwintering survival was analysed using regression.

Results

Cold tolerance source-population experiment (Fig. 2a)

A total of 7887 EHS was examined in the 2005 experiment. There was a significant effect of treatment ($F_{6,42} = 2.75$, $P = 0.024$). Although the main effect of location was not significant ($F_{1,42} = 1.06$, $P = 0.309$), there was a significant location \times treatment interaction ($F_{6,42} = 2.88$, $P = 0.019$). Data were normally distributed (Shapiro–Wilk's $W = 0.99$, $P = 0.99$) and variances were homogeneous both between location (O'Brien's $F_{1,54} = 1.09$, $P = 0.300$) and between treatments (O'Brien's $F_{6,49} = 1.82$, $P = 0.115$). When data from the two locations was analysed separately, there was a significant effect of treatment on southern but not northern populations (southern: $F_{6,21} = 4.55$, $P = 0.004$; northern: $F_{6,21} = 1.31$, $P = 0.296$).

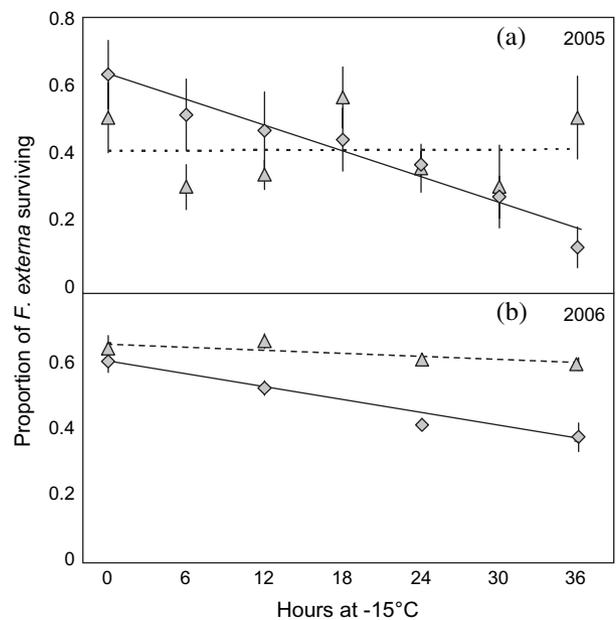


Fig. 2. Mean \pm SE proportion of *Fiorinia externa* surviving by location (◆, mean of southern populations; ▲, mean of northern populations), from the source-population experiment (a, '2005') and common-garden experiment (b, '2006'), when exposed to -15°C for 0–36 h. Solid line provides the best-fit regression for southern populations (2005: $y = -0.0125x + 0.6269$, $R^2 = 0.522$; 2006: $y = -0.0065x + 0.5855$, $R^2 = 0.742$). Dashed line provides the best-fit regression for northern populations (2005: $y = 0.0001x + 0.4073$, $R^2 = 0.291$; 2006: $y = -0.0016x + 0.6425$, $R^2 = 0.302$).

Cold tolerance common-garden experiment (Fig. 2b)

A total of 7355 EHS were counted in the 2006 experiment. The results of the 2006 common-garden cold tolerance experiment were qualitatively similar to those of the 2005 experiment (Fig. 2b). Southern populations had lower survival than northern populations in the 2006 control treatment. However, the population-specific responses to increased exposure to low temperatures were similar in both experiments. The proportion of surviving EHS in the control treatment for southern versus northern populations was 0.58 ± 0.022 (SE) and 0.62 ± 0.024 . In the 36-h treatment, the respective proportions were 0.38 ± 0.021 and 0.58 ± 0.018 . There was a significant main effect of treatment ($F_{3,24} = 10.42$, $P < 0.001$). Although the main effect of location was not significant ($F_{1,24} = 1.13$, $P = 0.300$), there was a significant location \times treatment interaction ($F_{3,24} = 3.827$, $P = 0.023$). Data were normally distributed (Shapiro–Wilk's $W = 0.94$, $P = 0.083$) and variances were homogeneous both between location (O'Brien's $F_{1,30} = 3.72$, $P = 0.063$) and between treatments (O'Brien's $F_{3,28} = 1.45$, $P = 0.251$). When the two locations were analysed separately, there was a significant effect of treatment on southern but not northern populations (southern: $F_{3,12} = 9.96$, $P = 0.001$; northern: $F_{3,12} = 1.75$, $P = 0.209$). There was no significant relationship between EHS density and overwintering survival either between sites ($F_{1,6} = 0.58$, $P = 0.48$) or within site for any of the eight sites (all $P > 0.05$).

Discussion

Tolerance of novel abiotic conditions is thought to play an important role in determining the success or failure of species colonising new environments (Baker, 1965; Sakai *et al.*, 2001; Lee *et al.*, 2003; Richardson & Pysek, 2006; Chown *et al.*, 2007). In the short term, a species' establishment is largely a function of the correspondence between the abiotic conditions in their native and novel environments (although interspecific interactions may also affect a species' tolerance of extreme conditions, e.g. Bertness & Hacker, 1994). Over time, however, local adaptation can lead to these 'boundary' populations overcoming the challenges posed by novel environments (Lee, 2002; Gilchrist & Huey, 2004; Lambrinos, 2004; Peck & Welch, 2004). As a result, the rapid spread of invasive species across landscapes provides a unique opportunity to observe how ecological and evolutionary dynamics contribute to the expansion of range boundaries in real time (Hastings *et al.*, 2005; Johnson *et al.*, 2006; Sax *et al.*, 2007).

The present study provides evidence that EHS populations from areas with varying winter conditions (Fig. 1) have diverged with respect to cold tolerance (Fig. 2). This is especially likely since the northern and southern locations differ substantially in the number of extended low-temperature periods. Between 1950 and 2005, the nearest weather station to the study sites in Connecticut averaged more than four times as many 2-day periods per year in which the minimum temperature dropped below -15°C each day than did the weather station in Maryland [4.84 ± 0.52 (SE) vs 1.11 ± 0.22 ; NOAA, 2008]. As a result, northern EHS populations must survive conditions similar to those employed in our research far more often than southern EHS populations. By rearing the offspring of both northern and southern EHS populations together in a common-garden environment before testing their response to cold temperatures, we remove the possibility that differing environmental conditions or differences in phenology experienced by the parental generation prior to the test were responsible for the observed effects. The effects observed are, therefore, most likely genetic, and the outcome of the common-garden experiment suggests that local adaptation of northern populations to low winter temperatures is at least partly responsible for the observed differences in population-level responses. The relatively benign environmental conditions experienced by southern scale populations are also the most likely explanation for the crossed reaction norms seen in Fig. 2a. While the least cold-tolerant scales from northern populations would already have died prior to the beginning of the experiment, many of these same individuals might have survived in the south. This would lead to a higher % survival for southern populations in the absence of cold temperatures and a large decrease in % survival in the presence of cold temperatures. This would explain the crossed reaction norms.

Although both the source-population and common-garden experiments produced qualitatively similar results, southern EHS populations in the source-population experiment showed a stronger response to low temperatures than they did in the common-garden experiment. One potential explanation for this fact is that the cold temperatures experienced by both northern and southern populations in the common-garden experiment,

prior to the removal of the hemlock branches on which they were feeding in early January 2006, killed off some of the least cold-tolerant southern individuals prior to the experiment. While the removal of the hemlock branches in early January was carried out as a precaution against a period of extreme cold, November–December 2005 temperatures were also lower in Massachusetts (42° latitude) than in Maryland (39° latitude). By removing the least cold-tolerant individuals, this source of error should reduce the power of the experiment to detect north–south differences and increase the likelihood of a type II error (i.e. failing to detect a real between-population difference).

Another potential source of error involves the fact that while northern EHS are univoltine (McClure, 1986), this species produces a partial second generation over the same time period in the southern portion of its invaded range (K. Abell, unpublished data). We occasionally encountered early-instar larvae on branches from the southern sites during the source-population experiment, suggesting that southern EHS in the source-population experiment may have included middle-instar individuals as well as adults. There were no early-instar individuals present on branches from the common-garden experiment. However, since individuals from different populations may grow/develop at different rates, we cannot exclude the possibility that some middle-instar individuals were present. This is an issue because cold tolerance can vary with age, developmental state, and sometimes between years (reviewed in Block, 1991). This is especially problematic since larvae are more tolerant of low temperatures than adults in some species (Rossolimo, 1997, also see Bale, 1991), while adults are hardier than early-instar larvae in other species (Cho *et al.*, 2007; Jensen *et al.*, 2007). Since we are unaware of any other published research on cold tolerance in *F. externa* (or, indeed, on any species of *Fiorinia* scale), we do not know whether stage-dependent cold tolerance actually affected the results and, if so, what impact it might have had. However, we raise this point as a cautionary note.

Increased cold tolerance may be one factor explaining the relatively recent advent of northward range expansion by EHS. As many founding populations consist of relatively few individuals and come from a single discrete source population, the resulting populations may possess relatively low genetic diversity (Sakai *et al.*, 2001; Tsutsui *et al.*, 2001; Lee, 2002; Ingram & Gordon, 2003). In our case, selection for cold-tolerant genotypes in northern boundary populations (Fig. 1) could eventually yield EHS capable of surviving in colder climes (Fig. 2). This is especially likely in light of the fact that northern populations of the later-arriving HWA, an organism that also feeds and overwinters on eastern hemlock, also exhibit increased cold tolerance (Butin *et al.*, 2005). Since HWA is obligately parthenogenetic in its invaded range (McClure, 1989), adaptations in this species can only arise via natural selection acting upon randomly occurring mutations. In contrast, sexual reproduction in EHS allows the rapid generation of new allelic combinations and should increase the rate of adaptive change. Interestingly, northern EHS populations exposed to -15°C for 36 h had a higher survivorship than did northern HWA assessed under nearly identical experimental conditions [$58.1 \pm 1.9\%$ (SE) and $41.1 \pm 7.1\%$, respectively; Butin *et al.*, 2005]. Although acute exposure to low temperatures is one of a myriad of ways to

assess temperature tolerance (Block, 1991; Sinclair & Roberts, 2005), the fact that HWA currently occurs north of the northernmost EHS populations in Massachusetts (Preisser *et al.*, 2008) suggests that EHS may continue to expand its range northward until stopped by more extreme climatic conditions and/or evolutionary constraints (Hoffmann *et al.*, 2003). Since both HWA and EHS share the same native host plant, interactions between these insects may also prove important (Preisser & Elkinton, in press).

Although invasive species are often treated as genetically homogeneous units, adaptive changes affecting local populations can alter the potential for and rate of range expansion (Lambrinos, 2004; Peck & Welch, 2004; Huey *et al.*, 2005; Lee *et al.*, 2007; Sax *et al.*, 2007). Understanding the interplay between ecological and evolutionary processes in the case of EHS and other similar biological invasions is critical, especially in light of the fact that the loss of hemlock in eastern North America will yield long-term changes in forest structure and composition (Orwig & Foster, 1998; Orwig *et al.*, 2002) and regional carbon fluxes (Albani & Moorcroft, 2004).

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