

## Cold Tolerance of *Pityophthorus juglandis* (Coleoptera: Scolytidae) From Northern California

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### Abstract

Winter survivorship of insects is determined by a combination of physiological, behavioral, and microhabitat characteristics. We characterized the cold tolerance of the walnut twig beetle, *Pityophthorus juglandis* Blackman, a domestic alien invasive bark beetle that vectors a phytopathogenic fungus. The beetle and fungus cause thousand cankers disease in species of *Juglans* and *Pterocarya*. The disease is spreading in the United States of America (USA) and Italy. Contact thermocouple thermometry was used to measure the supercooling points of adults and larvae and lower lethal temperatures of adults from a population from northern California. Supercooling points ranged from  $-12.2^{\circ}\text{C}$  to  $-25.0^{\circ}\text{C}$  for adults and  $-13.6^{\circ}\text{C}$  to  $-23.5^{\circ}\text{C}$  for larvae; lower lethal temperatures of adults ranged from  $-14^{\circ}\text{C}$  to  $-23^{\circ}\text{C}$ . We found seasonal changes in adult supercooling points in fall, winter, and spring. The supercooling point for males was  $0.5^{\circ}\text{C}$  colder than for females over all months and  $1^{\circ}\text{C}$  colder in the winter than in other seasons. The cold-tolerance strategy shifted in *P. juglandis* adults from freeze intolerance (December 2013 and January 2014) to partial freeze tolerance (February 2014). An intermediate level of cold tolerance with a plastic response to cold partially explains survival of *P. juglandis* outside of its native range in the southwestern USA. In addition, we characterized the relationship between minimum air temperatures and minimum phloem temperatures in two *Juglans* spp. in northern California and Colorado and characterized portions of the native geographic range of eastern black walnut, *J. nigra* L., that may be too cold currently for this insect to persist.

**Key words:** walnut twig beetle, thousand cankers disease, supercooling point, lower-lethal temperature, plasticity

The walnut twig beetle, *Pityophthorus juglandis* Blackman (Coleoptera: Scolytidae, *sensu* Bright 2014), is a domestic alien invasive bark beetle that vectors a phytopathogenic fungus, *Geosmithia morbida* Kolařík et al. (Ascomycota, Hypocreales) in *Juglans* spp. and *Pterocarya* spp. (Kolařík et al. 2011, Hishinuma et al. 2016). *Pityophthorus juglandis* and *G. morbida* are native to the southwestern United States of America (USA) and appear to have coevolved in a native host, Arizona walnut, *Juglans major* (Torrey) Heller (Zerillo et al. 2014, Rugman-Jones et al. 2015). This insect-pathogen complex is the primary cause of thousand cankers disease, first described in Colorado (Tisserat et al. 2009). The disease is named for the large number of small cankers that develop at or near the point where beetles penetrate the outer bark. The abundance of the cankers is a consequence of the aggregation behavior of the beetles on host trees. Coalescence of these cankers on the main stem and intensive feeding by *P. juglandis* adults and larvae can cause tree death (Seybold et al. 2013b). The typical decline pattern of an originally healthy tree is a slowly developing die back of the crown. As a

result, thousand cankers disease threatens native *Juglans* species across the USA in forests (Graves et al. 2011, Wiggins et al. 2014), urban landscapes (Tisserat et al. 2011), and orchards (Flint et al. 2010, Yaghmour et al. 2014). Widespread tree mortality due to thousand cankers disease would lead to significant economic losses in the timber and nut industries (Newton and Fowler 2009) and threaten genetic resources of *Juglans* spp. in the USA (Leslie et al. 2010).

The current distribution of *P. juglandis* in the USA includes >120 counties, with individual collection sites located primarily in urban or peri-urban areas (Seybold et al. 2016). *Pityophthorus juglandis* also has been collected from forested areas in Arizona, California, and New Mexico (Flint et al. 2010, Graves et al. 2011, Rugman-Jones et al. 2015). One of the first finds of *P. juglandis* in a forested area in the eastern USA was near a campground in Great Smoky Mountains National Park in North Carolina (Hadziabdic et al. 2014). The current north-south range of *P. juglandis* extends from  $47^{\circ} 43' \text{N}$  (Kootenai Co., ID) to  $31^{\circ} 24' \text{N}$  (Cochise Co., AZ;

Seybold et al. 2012a). The beetle occurs in Mexico (Bright 1981), but has not been detected in Canada to date (Seybold et al. 2016). *Pityophthorus juglandis* appears to have two genetic lineages (L1 and L2; Rugman-Jones et al. 2015). Individuals from the L1 lineage are found primarily west of the Rocky Mountains and in all populations east of the Mississippi River, whereas the L2 lineage is limited to New Mexico, Colorado, Utah, and southern Idaho. Both lineages co-occur in populations from Sierra County, New Mexico, and the Wasatch and Front Ranges of the Rocky Mountains (Rugman-Jones et al. 2015). In addition, the L1 lineage of *P. juglandis* has been found in northern Italy in *J. nigra* and *J. regia* timber plantations and residential gardens, respectively (Montecchio and Faccoli 2014; Montecchio et al. 2014, 2016; Faccoli et al. 2016).

The effect of cold on bark and ambrosia beetles is important for modeling potential spread (e.g., Hansen and Sømme 1994, Koch and Smith 2008) and population dynamics (e.g., Trân et al. 2007). Plasticity in cold tolerance, such as seasonal changes in the temperatures at which they freeze or die, of invasive bark and ambrosia beetles can explain how they establish or spread successfully in the introduced range (Migeon et al. 2015; or, conversely, be limited by a lack of plasticity, c.f., Sobek-Swant et al. 2012). Historically, cold temperature mortality has been observed in many species of bark beetles when air temperatures reach extreme lows (Chandler 1966, Berryman 1970, Frye et al. 1974, Ragenovich 1980). As climate change continues to shift winter minimum temperatures, beetle outbreaks at higher elevations and latitudes that will negatively impact North American forests become more likely (Venette et al. 2009, Bentz et al. 2010, Sambaraju et al. 2012).

Relatively little is known about the overwintering ecology of *P. juglandis*. "Winter" for these insects likely begins when temperatures remain below the flight threshold (17–18°C; Seybold et al. 2012b, Chen and Seybold 2014) or the developmental threshold [an as-yet unidentified temperature between 0–10°C; see Seybold and Downing 2009 for a discussion of this concept with the Mediterranean pine engraver, *Orthotomicus erosus* (Wollaston)]. An overwintering diapause for *P. juglandis* is unlikely because flight has been observed in northern CA during all seasons when temperatures exceed the flight threshold (Seybold et al. 2012b, Chen and Seybold 2014). The insects do not appear to overwinter at the base of a tree, in aggregation, or in the litter layer of the forest or orchard floor as do other bark and ambrosia beetles (Kinghorn and Chapman 1959, Annala 1969, Weber and McPherson 1983, Lombardero et al. 2000). Live *P. juglandis* larvae, pupae, and adults have been observed under the bark of northern California black walnut, *Juglans hindsii* (Jeps.) Jeps. ex R.E. Sm., during the winter in Alameda County, CA (P. L. Dallara, University of California, Davis, personal communication), and both larvae and adults have been found during winter months in Colorado (Luna et al. 2013) and Tennessee (Nix 2013). Luna et al. (2013) described the cold tolerance of *P. juglandis* from the Front Range in Colorado and reported that –23°C is lethal to ~99% of *P. juglandis* adults. They also suggested that larvae and adults are intolerant of freezing. Their test insects were likely composed of the L2 lineage or a hybrid of L1 and L2 individuals (Rugman-Jones et al. 2015). Little is known about the cold tolerance of the L1 lineage.

We are interested in the effects of low temperatures on larvae and adults of northern Californian *P. juglandis*, representative of the L1 lineage that has invaded the eastern USA and Italy, to determine if and where cold temperatures might limit spread. Laboratory cold-tolerance measurements for insects include supercooling point (i.e., the temperature at which insect body fluids begin to freeze; Lee 2010), lower lethal temperature (i.e., changes in mortality after

instantaneous exposure to subzero temperatures), and lower lethal time (i.e., changes in mortality over time during exposure to constant, low temperatures; Salt 1950). Functions that characterize the probability of freezing and the probability of dying at a particular temperature can be compared in the laboratory to determine cold-tolerance strategy (Slabber et al. 2007, Hanson et al. 2013). If mortality occurs before individuals begin to freeze, the population is chill intolerant, although technically this is not a cold-tolerance strategy. If mortality coincides with the onset of freezing, the population is chill tolerant/freeze intolerant. If mortality occurs after equilibrium ice formation, the population is freeze tolerant (Lee 2010). The extent of cold-induced mortality in the field can also be affected by an insect's behavior and its microhabitat (Lombardero et al. 2000).

The objective of our study is to measure potential seasonal changes in the lower lethal temperature of L1 adults and in the supercooling points of larvae and adults. For both life stages, we hypothesize that supercooling points change seasonally. We also hypothesize that supercooling points of adults may differ by sex. We predict that larval and adult supercooling points decrease in the fall, remain low in the winter, and increase in the spring. Finally, we hypothesize that L1 adults are chill tolerant and freeze intolerant.

## Materials and Methods

### Insects

Branches from mature trees of a black walnut hybrid, *Juglans hindsii* X (*J. nigra* X *J. hindsii/J. californica*), were collected monthly (June 2012 to May 2014) in a commercial seed orchard located 5 km south of Yuba City, CA (Sutter Co., 39° 03.681' N, 121° 36.818' W, 19.2-m elevation). *Pityophthorus juglandis* was generally in flight at this site during all months of the year except December and January when it flew sporadically when air temperatures warmed (S.J.S., unpublished data). Branches with entrance and emergence holes or sap staining on the bark surface were cut from trees and shipped overnight to a biosafety level-2 facility in St. Paul, MN, under the terms and conditions specified in Permits P526P-12-01650 and P526P-12-02498 from the U.S. Department of Agriculture, Animal and Plant Health Inspection Service. Adults and larvae were excised from branches on the day of arrival by using a #22 blade X-ACTO knife (Elmer's Products, Inc., Westerville, OH) and a fine paintbrush. Insects were held in sealed Petri dishes on moist Kimwipes (Kimberly Clark, Roswell, GA) at room temperature (~22°C) for 48 h to allow for gut evacuation. Preliminary starvation assays indicated that gut contents could elevate supercooling points (Supp. Fig. 1 [online only]).

### Cold-Tolerance Assays

To ensure that adult and larval *P. juglandis* were undamaged after removal and starvation, we measured the cold tolerance of individuals that could walk normally when placed on a Kimwipe or, if in the larval stage, were white and moved when probed. For both life stages, we removed external frass or phoretic mites with a fine paint brush under a dissecting microscope (30–60×) to avoid external ice-nucleation. The sex of each adult was determined based on morphological features described in Bright (1981) and Seybold et al. (2013a). All cold-tolerance trials were completed within 7 d after infested branches arrived in St. Paul. A preliminary analysis showed that supercooling points of adults extracted from bark in November 2013 did not change significantly after a week at room temperature (i.e., there was no evidence of de-acclimation; Supp. Fig. 2 [online only]).

### Supercooling Point Determination

The supercooling points of adult *P. juglandis* were measured once per month from December 2012–March 2013, and May 2013–May 2014. Larval supercooling points were measured November 2012–February 2013, June 2013, December 2013, and February–May 2014. For both life stages, between 4 to 43 individuals, most typically about 25, were tested per month. Females and males were sampled arbitrarily from cut branches. After starvation, one active insect was placed in a 5- by 7-mm gelatin capsule (Capsuline, Pompano Beach, FL) and secured to a copper-constantan cradle thermocouple with a small amount of high vacuum grease (Dow Corning, Auburn, MI) per Hanson and Venette (2013). Females and males were placed on separate thermocouples in random order. Each thermocouple was placed in a calibrated polystyrene cube and cooled at  $\sim 1^{\circ}\text{C min}^{-1}$  when placed in a  $-80^{\circ}\text{C}$  freezer (Carrillo et al. 2004). Eight thermocouples were attached to each of two multi-channel data loggers (USB-TC, Measurement Computing, Norton, MA) for a total of 16 thermocouples. Temperatures were recorded once per second. The supercooling point was recorded as the lowest temperature before a spontaneous release of heat (i.e., an exotherm) due to the formation of ice within the body.

### Supercooling Point Statistical Analyses

All analyses were done in R, v2.15.1 (R Core Team 2013). Analytical assumptions of homoscedasticity and normality of errors were assessed by graphical inspection of residual plots. We tested the effects of sex, sampling period (i.e., month and year), and the interaction between sex and sampling period on adult supercooling points with ANOVA. We also used ANOVA to examine the effect of sampling period on larval supercooling points. Differences among the mean supercooling points of adults and larvae on the same collection date were analyzed by using a two-sample independent *t*-test with a Bonferroni correction for multiple mean comparisons (critical  $\alpha = 0.006$  to maintain an overall  $\alpha = 0.05$ ).

To examine seasonal changes in adult and larval supercooling points, each climatological season was analyzed separately. We used ANCOVA to examine the effect of month, included as a continuous variable, on supercooling point. A term for sex (adult data) was included as a categorical variable in each model. If sex was not significant ( $P \geq 0.05$ ), we removed it, and refit the model as a linear regression. Simple linear regression or mixed-effects models were used to evaluate changes in supercooling point over time within each season, and in each case, month was treated as a fixed effect and year (when applicable) was treated as a random effect. Supercooling points of adults in summer and fall and larvae in spring were analyzed by using simple linear regression models. A square root transformation was applied before analysis of supercooling points for adults in spring to satisfy assumptions of homoscedasticity and normality of errors; these data were analyzed with a linear mixed effects model. Supercooling points for adults in winter were analyzed with a polynomial mixed effects model because exploratory data analysis indicated a curvilinear trend in supercooling points through time that could be described by a quadratic function. Larval supercooling points in winter were analyzed with a linear mixed effects model. R packages lme4 and lmerTest were used to fit mixed-effects models (Bates et al. 2013, Kuznetsova et al. 2013).

### Lower Lethal Temperature (LLT) Determination

The lower lethal temperature of adults was measured once per month from June–December 2013 and January–May 2014. The

experiment followed a randomized complete block design. One adult was placed in a gel capsule, onto a thermocouple, and into a polystyrene cube as described for supercooling point determinations. Each insect was assigned randomly to one of five treatment temperatures:  $21^{\circ}\text{C}$ ,  $-5^{\circ}\text{C}$ ,  $-15^{\circ}\text{C}$ ,  $-20^{\circ}\text{C}$ , and  $-25^{\circ}\text{C}$ . This method allowed up to four insects to be exposed to each treatment temperature per block. An insect was removed from the freezer after a brief (<10 s) exposure to the treatment temperature per Bale et al. (1988), Pullin and Bale (1989), and Lencioni et al. (2015), regardless of whether an exotherm was detected. Individuals were allowed to warm to room temperature while in the gel capsule. Survival was determined 1 h after cold exposure. An individual was considered alive if it moved when probed with a fine paint brush. The number of insects that were available in a month affected the number of blocks (between two and six, inclusive) in the experiment.

### Lower Lethal Temperature Statistical Analysis

We examined the relationship between brief exposures to cold and mortality using logistic regression per Hanson et al. (2013) and Stephens et al. (2015). Modifications to glm.control in R, such as increasing the number of iterations to 50 in the parameter estimation routine, were made when fitted probabilities did not initially converge. From these functions, lower lethal temperatures that resulted in 50% (i.e., LLT<sub>50</sub>) or 90% mortality (LLT<sub>90</sub>) of individuals after acute cold exposure and associated standard errors of the mean (SEMs) were estimated each month by using the command dose.p() in the MASS package (Venables and Ripley 2002).

### Cold-Tolerance Strategy Statistical Analysis

We compared the cumulative proportion of individuals that began to freeze and the cumulative proportion that died as a function of exposure temperature in each month (June 2013–May 2014) using analysis of covariance (ANCOVA) logistic regression per Slabber et al. (2007) and Stephens et al. (2015). The first logistic model described the expected proportion of individuals that would have started to freeze by a specified temperature. This model was based on the distribution of supercooling points. The second model described the expected extent of mortality after acute exposure to a specified temperature. Mortality may have occurred at a temperature greater than or equal to the specified temperature. We incorporated terms for treatment (categorical variable for supercooling point or lower lethal temperature), temperature as a continuous variable, and an interaction between treatment and temperature. If model terms were not significant ( $P \geq 0.05$ ), they were removed, and the model was refit.

Using ANCOVA logistic regression, we tested for initial differences between the estimated proportion of individuals that froze or died at  $0^{\circ}\text{C}$  (i.e., a test of different intercepts) and differences between the rates of change in the proportion of individuals that froze or died with each degree change in temperature (i.e., a test of different slopes). The cold-tolerance strategy can be interpreted from significant differences found between the proportions of individuals that freeze and die at temperatures < $0^{\circ}\text{C}$  (Slabber et al. 2007). At such temperatures, if the proportion of individuals that die is significantly greater than those that froze, the individuals are chill-intolerant. If the proportion of individuals that began to freeze is not statistically different than the proportion that die, the individuals are freeze-intolerant. If the rate of freezing varies significantly from the rate of dying, above or below a cold temperature, then this pattern suggests a population with a mixed-strategy (i.e., freeze intolerance and partial freeze tolerance). If the proportion of individuals that die, at low temperatures, is

significantly less than those that froze, then this suggests a partial freeze tolerant or freeze tolerant population.

### Estimated Cold Mortality of *P. juglandis* in the USA

To characterize spatial variation in the potential extent of *P. juglandis* mortality due to cold in the conterminous USA, we first identified the month (February 2014) in which *P. juglandis* adults were most cold tolerant (i.e., had greatest survival during lower-lethal temperature trials; Fig. 3). We relied on USDA Plant Hardiness Zones (1976–2005) (USDA 2012) to provide estimates of the average extreme low air temperature across the conterminous USA. We estimated the range of mortality in each hardiness zone by substituting the upper and lower temperature limit for each zone into the logistic regression model for lower lethal temperature in February 2014. We rounded the estimate to the nearest five percent, and classified each zone based on whether the minimum overwintering mortality was forecasted to be <50%, 50–75%, 75–90%, or >90%. We used ArcMap 10.1 (ESRI, Redlands, CA) to map areas of predicted beetle mortality and U.S. counties with *P. juglandis* (Seybold et al. 2016). The forecasts assume that *P. juglandis* have achieved their most cold-tolerant condition before the winter extreme low actually occurred and that adults were exposed to those temperatures. The model does not account for other sources of winter mortality such as starvation or desiccation nor does it account for the effects of prolonged or repeated exposures to low temperatures. We further assume that all insects have voided their guts of ice nucleating agents. All of these assumptions are intended to provide a risk averse model (i.e., realized mortality should be greater than or equal to our forecasted mortality).

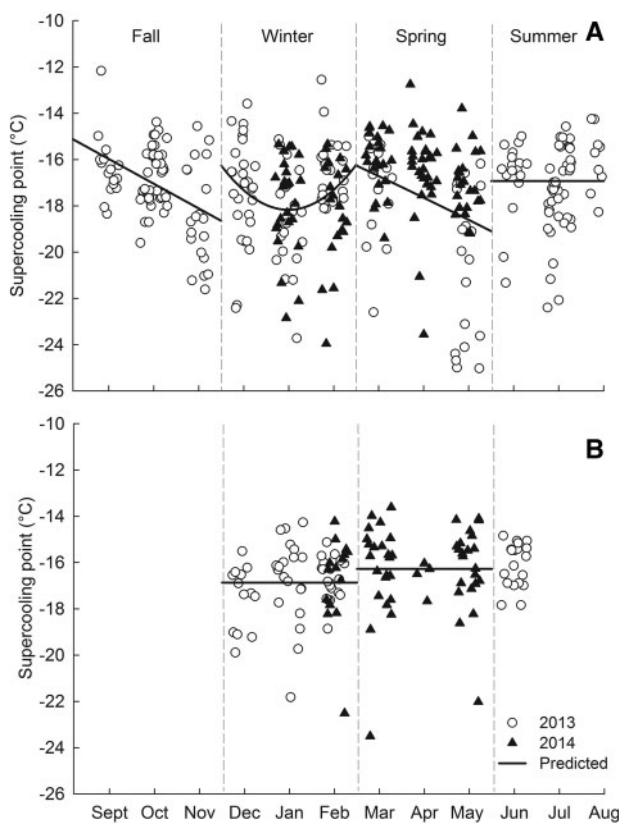
## Results

### Cold Tolerance

#### Adult Supercooling Point

On average over the entire study period, male *P. juglandis* had a mean supercooling point ( $\pm$  SEM) of  $-17.5 \pm 0.16^\circ\text{C}$ , which was half a degree lower than the mean supercooling point for females ( $F_{1,364} = 5.67, P = 0.0178, n = 398$ ). However, the difference in supercooling points between sexes changed depending on month collected ( $F_{16,364} = 2.25, P = 0.0040, n = 398$ ). Females, though not significantly so, had a higher mean supercooling point than males in December 2013, January 2014, and March 2014. Males had a higher mean supercooling point than females in March 2013 and February 2014, but the difference was not significant. Mean supercooling points for both sexes were similar in February 2013. Overall, supercooling points for adult *P. juglandis* differed across the 18 months of the study ( $F_{16,364} = 7.68, P < 0.001, n = 398$ ; Fig. 1A).

We found significant within-season changes in adult supercooling points collected in climatological fall, winter, and spring (Fig. 1A). In fall, adult supercooling points decreased with month ( $y = -14.9 - 1.1x$ , where  $y$  is supercooling point and  $x$  is month 1–3, where month 1 is September;  $F_{1,77} = 16.9, P < 0.001$ ). In winter, on average males had a slightly colder supercooling point ( $\approx 1^\circ\text{C}$ ) than females. During this period, supercooling points initially decreased then increased (males,  $y = 3.7 - 9x + x^2$ ; females,  $y = 4.7 - 9x + x^2$ ; month 1 in this model is December; for statistical results, see Supp. Table 1 [online only]). In spring, male and female supercooling points decreased with month ( $y = -3.3 - 0.11x$ ; month 1 in this model is March;  $F_{1,106} = 17.07, P < 0.001$ ). In the summer, we did not find a significant trend in supercooling points for either sex ( $F_{1,70} = 2.15, P = 0.15$ ).



**Fig. 1.** Supercooling point ( $^\circ\text{C}$ ) data of two *P. juglandis* life stages (adults-A and larvae-B) collected every 4 wk from December 2012 to May 2014 (data are jittered on x-axis). (A) Adult supercooling points significantly varied by month ( $n = 398$ ) and seasonal trends showed a decline in supercooling point from fall to winter (acclimation) but did not show an increase in supercooling points from winter to spring. (B) Larval supercooling points did not differ across months ( $n = 136$ ). No seasonal trend was found in winter or spring. In January 2013, the larval mean supercooling point was significantly lower than the adult mean supercooling point ( $P < 0.006$ ).

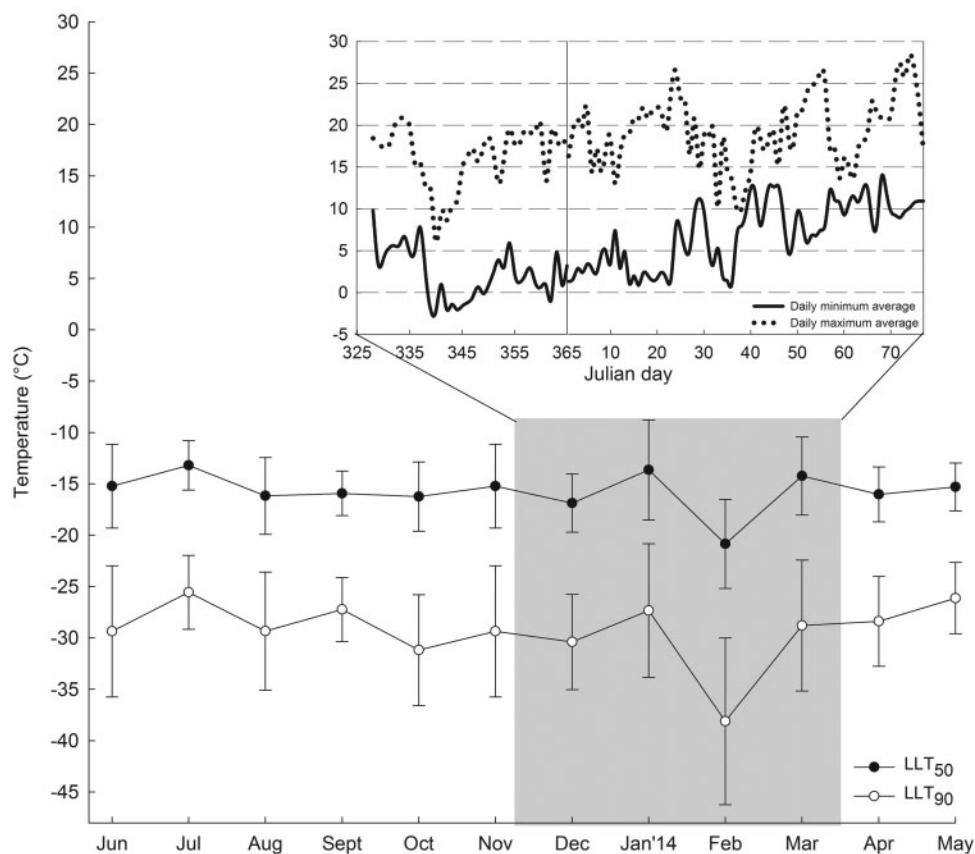
#### Larval Supercooling Point

Larval supercooling points did not differ across eight months ( $F_{7,125} = 1.21, P = 0.30, n = 136$ ), and the range of larval supercooling points ( $-13.6^\circ\text{C}$  to  $-23.5^\circ\text{C}$ ; Fig. 1B) was generally within the range of supercooling points that we measured for adults ( $-12.2^\circ\text{C}$  to  $-25.0^\circ\text{C}$ ; Fig. 1A). However, in January 2013, the mean supercooling point for larvae ( $-16.7 \pm 0.41^\circ\text{C}$ ) was significantly greater than that for adults ( $-18.7 \pm 0.51^\circ\text{C}, t = -3.1, df = 37, P = 0.004$ , critical  $\alpha = 0.006$ ). Mean supercooling points for adults and larvae were not significantly different in all other months ( $t < 0.55, df = 11–38, P \geq 0.05$ , critical  $\alpha = 0.006$ ). We found no significant seasonal trends for larvae in winter ( $F_{1,66} = 1.75, P = 0.19$ ) or spring ( $F_{1,47} = 0.02, P = 0.89$ ).

#### Adult Lower Lethal Temperature and Overwintering Strategy

We found that the LLT<sub>50</sub> and LLT<sub>90</sub> were relatively constant through the year (Fig. 2). The likelihood that adult *P. juglandis* would die increased as the temperature declined to  $-25^\circ\text{C}$  for all insects assayed, regardless of month (Fig. 3). From June 2013 to May 2014, all individuals began to freeze between  $-14^\circ\text{C}$  and  $-23^\circ\text{C}$ .

In June, August, November, December 2013 and January and April 2014, the cumulative frequency of individuals that froze (i.e., gave an exotherm) and the extent of mortality in *P. juglandis* adults at temperatures  $< 0^\circ\text{C}$  was not different (Table 1). All individuals



**Fig. 2.** Adult *P. juglandis* lower lethal temperature (LLT  $\pm$  SEM) measured once per month from June 2013 to May 2014. Inset shows minimum and maximum daily average phloem temperatures (means of north and south side across four trees) logged from Nov. 24, 2013 to Mar. 18, 2014 in northern California. LLT<sub>50</sub> is the temperature estimated to cause 50% mortality in a population and LLT<sub>90</sub> is the temperature estimated to cause 90% mortality.

survived cooling to near the supercooling point but did not survive freezing, a pattern consistent with freeze intolerance (Lee 2010).

In July, September, and October 2013 and February, March, and May 2014, freezing was not consistently associated with mortality (Fig. 3). In July 2013, as the temperature fell below  $-5^{\circ}\text{C}$ , the proportion of individuals that died was significantly and consistently greater than those that froze (Table 1). In this month, the population was chill-intolerant because mortality occurred before freezing occurred (Lee 2010).

In September and October 2013, at temperatures  $<0^{\circ}\text{C}$ , the rate of freezing was significantly different than the rate of mortality (Table 1). Above  $-15^{\circ}\text{C}$ , a significantly greater proportion of adults had died before freezing. Below  $-15^{\circ}\text{C}$ , of the remaining adults, all individuals survived freezing. This result suggests that *P. juglandis* adults in September and October were using a mixed-strategy of freeze intolerance and partial freeze tolerance. In this case, we suggest “partial freeze tolerance” because individuals were removed from treatment temperatures before whole-body freezing could have occurred. Our total sample of adults for this period consisted of 28% teneral and 72% fully sclerotized adults.

In February 2014, the proportion of individuals that died was significantly and consistently less than those that froze at low temperatures (Table 1). In this month, all individuals were able to survive partial freezing.

In March and May 2014, as during fall months in 2013, the rate of freezing was significantly different than the rate of mortality at temperatures  $<0^{\circ}\text{C}$ . Above  $-16^{\circ}\text{C}$  in March and  $-17^{\circ}\text{C}$  in May, a greater proportion of adults died before freezing (Table 1). Below  $-16^{\circ}\text{C}$  in March and  $-17^{\circ}\text{C}$  in May, all remaining adults survived

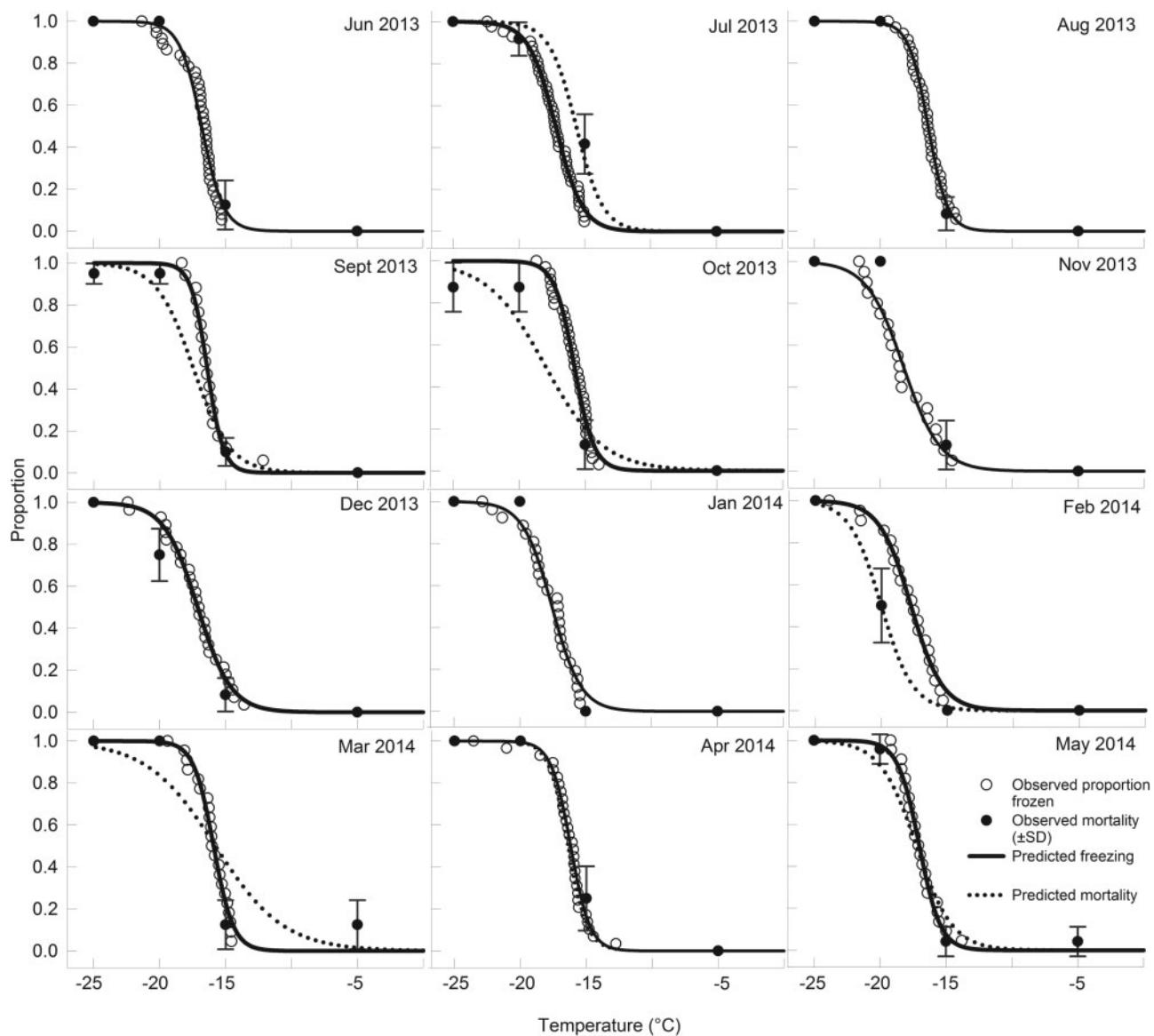
freezing. These results suggest a mixed cold-tolerance strategy, where individuals are chill-intolerant above  $-16^{\circ}\text{C}$  in March and  $-17^{\circ}\text{C}$  in May and freeze-intolerant below these temperatures, respectively. Our total sample for this period consisted of 4% teneral and 96% fully sclerotized adults.

#### Estimated Cold Mortality of *Pityophthorus juglandis* and Risk to *Juglans nigra* in the United States

We forecast differing degrees of winter mortality from acute exposure to low temperatures for the L1 lineage of *P. juglandis* across the USA (Fig. 4). If *P. juglandis* spreads westward in Pennsylvania and Maryland, the beetle would encounter colder areas where winter mortality could be 50–75%. Approximately half of the current range of *J. nigra* has winter temperatures under which *P. juglandis* might survive (i.e., forecasted winter mortality  $<50\%$ ) if it arrived. Substantially fewer infested counties have been reported in the zone with winter temperatures cold enough to cause 50–75% winter mortality than in the  $<50\%$  winter mortality zone. The majority of those counties occur in Colorado. In more northern latitudes, we forecast that low winter temperatures might cause at least 75–90% mortality annually if *P. juglandis* was introduced to these areas. However, in these areas, native stands of black walnut are rare (Fig. 4).

#### Discussion

Our study provides an extensive assessment of seasonal changes in cold-tolerance strategy and indicates notable plasticity in this trait.



**Fig. 3.** Comparison of proportions of frozen (pooled male and female) (○) and lower-lethal temperature mortality (● ± SD) calculated from adult *P. juglandis* once per month from June 2013 to May 2014. Adults appear to be mainly freeze-intolerant, but they did show mixed strategies in critical periods (fall and spring) and increased cold tolerance after a long period of cold (partial freeze tolerance in February).

Plastic responses to cold have been observed in other insects, where individuals or populations can shift from one cold-strategy to another depending on environmental cues (e.g., temperature, photoperiod, or moisture; Horwarth and Duman 1984, Kukal and Duman 1989, Brown et al. 2004, Danks 2005), thus lowering the risk of death by freezing in variable environments (see Voituron et al. 2002). Though adults from the L1 lineage of *P. juglandis* are freeze-intolerant for most of the year, a result that is consistent with Luna et al. (2013), we found evidence for chill intolerance and partial freeze tolerance at different times of the year (Table 2). Chill intolerance was detected in some or all adults in July 2013 and March and May 2014, a result that likely has little ecological relevance as temperatures in these months are unlikely to be sufficiently low to cause substantial mortality. In September and October 2013 and February 2014, some or all individuals had evidence of “partial freeze tolerance”; a greater proportion of individuals were beginning to freeze than were dying over

a range of temperatures. These survivors cannot be considered freeze tolerant in the classical sense because we did not attempt to quantify the extent of ice formation, nor did we attempt to evaluate mortality after holding adults for an extended period in a semifrozen state (Koch et al. 2004, Koštál et al. 2012). Nevertheless, the greatest degree of cold tolerance (i.e., lowest LLT<sub>50</sub> and LLT<sub>90</sub>) coincided with a period when the entire population exhibited partial freeze tolerance (i.e., February 2014). These alternative cold tolerance strategies typically occurred in fall and spring, climate periods known to have considerable annual variation. Some modest tolerance of freezing in late fall or early spring could be advantageous for adult *P. juglandis*.

To further study plasticity of *P. juglandis* cold tolerance, supercooling points and lower lethal temperature should be measured over two or more cold periods with beetles from the native range (Arizona, Mexico, and New Mexico). Similar studies should be conducted for populations of *P. juglandis* at the minimum temperature

**Table 1.** Coefficient estimates ( $\pm$  SEM) for logistic regression models to estimate the cumulative proportion of adult *P. juglandis* that were frozen or dead from  $-25^{\circ}\text{C}$  to  $-5^{\circ}\text{C}$ 

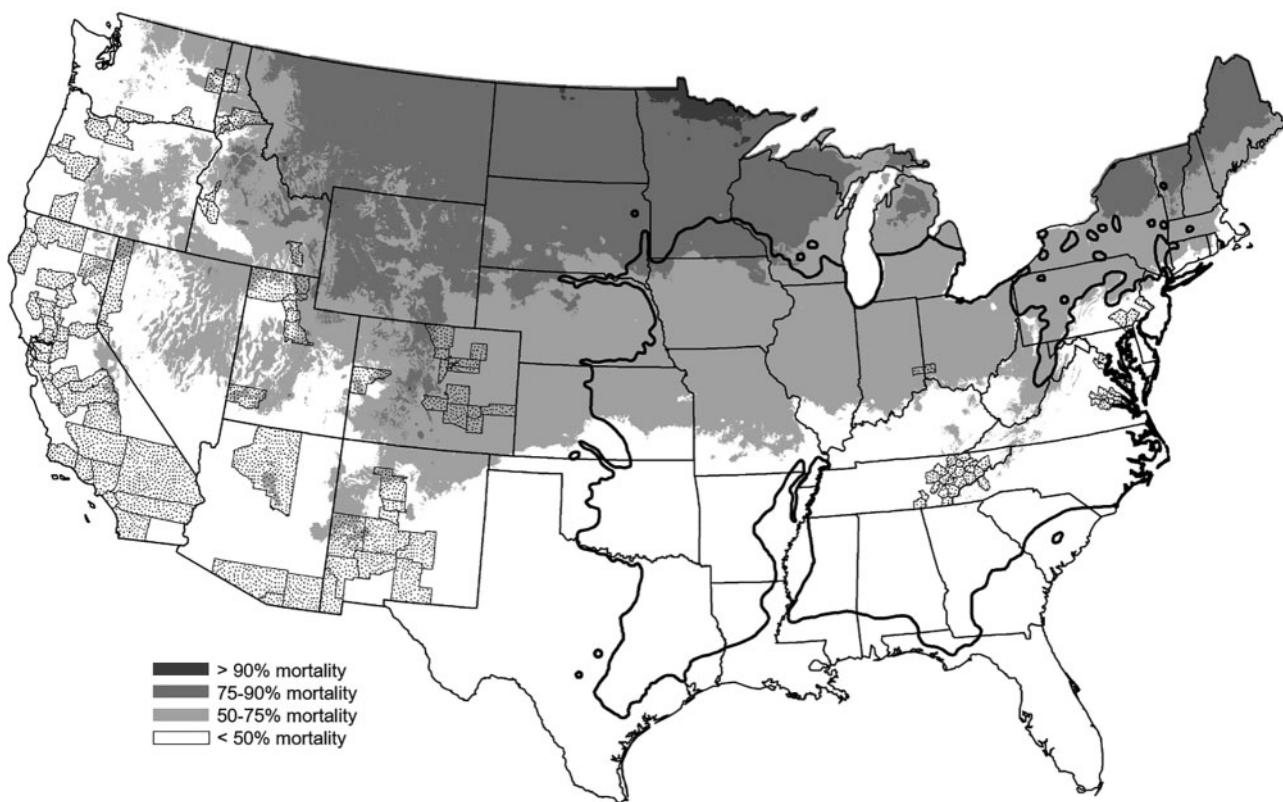
Period	<i>n</i>	Intercept	Temp	Treatment	Temp $\times$ Treatment
June 2013	45	$-20.22 \pm 1.33^{***}$	$-1.21 \pm 0.08^{***}$	—	—
July 2013	54	$-15.35 \pm 0.97^{***}$	$-0.98 \pm 0.05^{***}$	$-1.53 \pm 0.6^*$	—
August 2013	39	$-22.38 \pm -1.38^{***}$	$-1.37 \pm 0.08^{***}$	—	—
September 2013	37	$-12.52 \pm 2.96^{***}$	$-0.72 \pm 0.17^{***}$	$-14.27 \pm 4.84^{**}$	$-0.92 \pm 0.28^{**}$
October 2013	42	$-8.2 \pm 3.1^*$	$-0.45 \pm 0.17^*$	$-12.81 \pm 3.35^{***}$	$-0.87 \pm 0.18^{***}$
November 2013	42	$-13.13 \pm 1.3^{***}$	$-0.72 \pm 0.07^{***}$	—	—
December 2013	42	$-13.48 \pm 1.01^{***}$	$-0.79 \pm 0.06^{***}$	—	—
January 2014	42	$-15.91 \pm 1.28^{***}$	$-0.91 \pm 0.07^{***}$	—	—
February 2014	42	$-16.75 \pm 1.79^{***}$	$-0.84 \pm 0.08^{***}$	$1.88 \pm 0.65^*$	—
March 2014	30	$-6.08 \pm 2.47^*$	$-0.39 \pm 0.14^*$	$-15.06 \pm 3.22^{***}$	$-0.94 \pm 0.19^{***}$
April 2014	45	$-22 \pm 1.79^{***}$	$-1.36 \pm 0.11^{***}$	—	—
May 2014	46	$-12.59 \pm 2.73^{***}$	$-0.73 \pm 0.16^{***}$	$-8.54 \pm 3.42^*$	$-0.51 \pm 0.2^*$

The model followed the general equation  $P(\text{Insect dies or freezes}) = 1/[1 + \exp(-1^*(\text{intercept} + b_1^*\text{temperature} + b_2^*\text{treatment} + b_3^*\text{temperature} \times \text{treatment}))]$ , where treatment = 1 for frozen individuals (i.e., supercooling assays) and treatment = 0 for dead individuals (i.e., lower lethal temperature assays).

\*|z| > 2.46;  $P \leq 0.05$ .

\*\*|z| > 3.21;  $P \leq 0.001$ .

\*\*\*|z| > 4.61;  $P \leq 0.001$ .



**Fig. 4.** Estimated mortality of adult *P. juglandis* (L1 lineage) and native host range of *Juglans nigra* (<http://espt.cr.usgs.gov/data/little/>, accessed on 2 May 2017) in the conterminous United States. Perimeter of *J. nigra* distribution outlined in dark black. Stippled counties have collection records for *P. juglandis* (Seybold et al. 2016). We used February 2014 data to estimate mortality at each zone's upper temperature limit based on mean annual extreme minimum temperature (1976–2005).

extremes of its current range (e.g., northeastern Colorado, northern Idaho, and Washington). Moreover, care must be taken to determine the genetic lineage of individuals that are tested.

We found that supercooling points of adults and larvae from northern California were sufficiently low to avoid freezing in portions of that region. Both larvae and adults of *P. juglandis* appear to

be capable of overwintering, as both life stages were detected alive in winter. However, our study suggests that adults are the more cold-hardy overwintering stage because they have slightly lower supercooling points than larvae, and thus would be more likely to survive the winter if found in colder areas. Lower lethal temperature studies for larvae would be needed to confirm this notion. The

**Table 2.** Summary of cold tolerance strategy of adult *P. juglandis* by month from June 2013–May 2014 in northern California (Sutter Co.)

Month	Cold strategy
June 2013	Freeze intolerance
July 2013	Chill intolerance
Aug. 2013	Freeze intolerance
Sept. 2013	Mixed strategy (freeze intolerance/partial freeze tolerance)
Oct. 2013	Mixed strategy (freeze intolerance/partial freeze tolerance)
Nov. 2013	Freeze intolerance
Dec. 2013	Freeze intolerance
Jan. 2014	Freeze intolerance
Feb. 2014	Partial freeze tolerance
March 2014	Mixed strategy (chill intolerance/freeze intolerance)
April 2014	Freeze intolerance
May 2014	Mixed strategy (chill intolerance/freeze intolerance)

presence of immature and adult *P. juglandis* at the start of winter is a natural consequence of multiple, overlapping generations (Faccoli et al. 2016).

Prior to this study, little was known of the cold tolerance of the L1 lineage of *P. juglandis* that has spread from the southwestern to the eastern USA. The eastern USA contains the native ranges of butternut, an imperiled species (Parks et al. 2013), and eastern black walnut. As such, grave concerns have been expressed about the potential economic and ecological impacts of *P. juglandis* in the eastern USA if this insect and associated fungus continue to cause tree mortality as they have in the western USA (Seybold et al. 2016). Our studies provide the basis for simple mechanistic models to help forecast where cold temperatures might preclude this domestic invasive species from establishing or persisting through time. If establishment or persistence is not likely to occur in an area, impacts may be limited or non-existent.

By applying 30-yr-mean annual extreme low temperatures to the function that described the extent of mortality after acute cold exposure (i.e., lower lethal temperatures measured when *P. juglandis* was most cold tolerant), we found that cold is unlikely to have a substantial impact on populations of *P. juglandis* in the beetle's native range (i.e., portions of southern Arizona and New Mexico). Substantial cold mortality is also not expected in much of its Pacific Coast invaded range (i.e., in most of California and portions of Oregon and Washington). In eastern Colorado, however, where genetic lineages, L1 and L2, co-occur (Rugman-Jones et al. 2015), we forecast 50–75% annual mortality from cold. The distributions of L1 and L2 in Colorado are unknown and therefore it is difficult to predict if *P. juglandis* populations are persisting via increased cold-hardiness due to genetic differences or some other factor. In the eastern USA, typical mortality rates from acute cold exposure are forecasted to be low where populations of *P. juglandis* occur in Tennessee, Virginia, Pennsylvania, and Maryland. Overwintering mortality rates of 50–75% may slow population growth, but *P. juglandis* may persist and spread if it encounters suitable hosts in these areas.

Our model is derived from data that depend on a number of simplifications. We used a cooling rate ( $\sim 1^{\circ}\text{C}/\text{min}$ ) that is a common laboratory standard (Bale et al. 1988, Lencioni et al. 2015) but faster than typically found in nature (Sinclair 2001). Slower cooling rates may allow insects to rapidly cold harden, which has

been shown to reduce mortality (Teets and Denlinger 2013). Our model does not account for the effects of extended exposure to cold temperatures. Longer exposure times and repeated exposures have been shown to increase mortality (Berkvens et al. 2010, Morey et al. 2012, Obeyesekara et al. 2015). Finally, our model does not account for fluctuating winter temperatures that may permit some degree of recovery from cold stress during the winter (Koštál et al. 2007).

Forecasted cold mortality is also based on assumptions about the beetle, climate, host, landscape, and the interactions that occur among these factors. First, we used a starvation period before measuring lower lethal temperature to avoid underestimating the geographic risk of *P. juglandis* in the USA. The presence of food in the gut provides ice nucleation sites that permit the onset of freezing at higher temperatures than in insects that cease feeding or void their gut prior to overwintering (Sømme 1999). For our geographic forecast, we assume that adults achieve their maximum level of cold tolerance when extreme low temperatures occur. Second, the map is based on the average extreme minimum temperature from 1976–2005 (Daly et al. 2012). Many recent winters have been warmer than this average. Third, a suite of biotic interactions that may affect insect overwintering physiology are not included in our model. For example, temperatures beneath the bark of walnut are slightly warmer than air temperatures (Fig. 2 inset and Supp. Fig. 3 [online only]). Host and interactions between *G. morbida* and *P. juglandis* may also affect *P. juglandis* overwintering physiology.

The preponderance of occurrences of *P. juglandis* in areas forecasted typically to have <50% winter mortality provides a measure of validation for our method to forecast winter mortality rates from laboratory data, independent of the occurrences of *P. juglandis*. In many counties where *P. juglandis* has been reported and typical rates of 50–75% mortality have been forecasted, at least a portion of the same county is projected to have <50% mortality. The notable exceptions to this pattern are the occurrences of *P. juglandis* in the Front Range of Colorado, which likely includes members of the L2 lineage, and in adjacent counties in Indiana and Ohio. Anecdotal evidence provides some support for our general forecast for the Midwest as no catches of *P. juglandis* in pheromone-baited traps were detected during the 2014 growing season in Butler Co., OH (Daniel Kenny, Ohio Department of Agriculture, personal communication), after a colder than normal winter (Marinaro et al. 2015).

Federal, state, and local governments in the eastern USA can take advantage of preliminary information about *P. juglandis* cold mortality to understand its risk to *J. nigra* resources. Pest risk maps help managers make decisions under high uncertainty and knowledge gaps about an invasive insect's biology (Venette et al. 2010, Yemshanov et al. 2010). Further studies on field mortality and continued trapping and hand-collecting efforts across the current range of *P. juglandis* would verify if these limits generally confirm the accuracy of our forecasts. If arrival of *P. juglandis* coincides with winter months, *J. nigra* in the Midwest and Northeast may be less at risk than the southern areas of its range (Fig. 4). To further understand the risk of *P. juglandis* to *J. nigra* in the USA, future studies might include host effects on cold tolerance and reproduction among propagated cultivars.

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