



Cold tolerance of *Trissolcus japonicus* and *T. cultratus*, potential biological control agents of *Halyomorpha halys*, the brown marmorated stink bug



Erica Nystrom Santacruz^a, Robert Venette^b, Christine Dieckhoff^c, Kim Hoelmer^c, Robert L. Koch^{a,*}

^a Department of Entomology, University of Minnesota, 1980 Folwell Avenue, St. Paul, MN 55108, USA

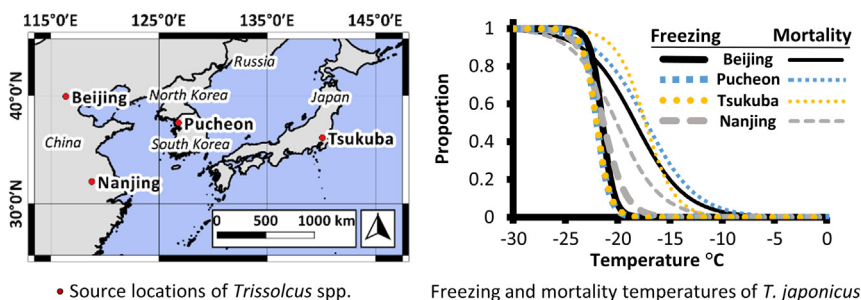
^b USDA-FS, Northern Research Station, 1990 Folwell Avenue, St. Paul, MN 55108, USA

^c USDA-ARS, Beneficial Insect Introduction Research Laboratory, 501 South Chapel St., Newark, DE 19713, USA

HIGHLIGHTS

- 50% of each of the populations of both species died between -17.4 and -20.0 °C.
- 50% of all four populations of *T. japonicus* froze between -21.4 and -22.0 °C.
- *Trissolcus japonicus* is chill-intolerant; populations die before freezing.
- Both *Trissolcus* spp. appeared more cold tolerant than the host, *Halyomorpha halys*.
- *Trissolcus japonicus* does not respond to the same overwintering cues as *H. halys*.

GRAPHICAL ABSTRACT



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ABSTRACT

Halyomorpha halys (Stål) (Hemiptera: Pentatomidae) is native to Asia and has become a severe agricultural and nuisance pest in the U.S. Therefore, foreign exploration was conducted in Asia to identify potential classical biological control agents. Several *Trissolcus* spp. (Hymenoptera: Scelionidae) parasitize *H. halys* eggs in Asia and are being evaluated for potential release in the U.S. Since *H. halys* has invaded regions that experience sub-zero winter temperatures, cold tolerance is important for evaluation of *Trissolcus* spp. Our study compared the cold tolerance of populations of *T. japonicus* and *T. cultratus*, in order to assess relative suitability of the populations for release. We used thermocouple thermometry to determine the supercooling point and lower lethal temperature after brief exposure to cold temperature for each population. In addition, we subjected adult *T. japonicus* to a short photoperiod and low temperature regime, which increases cold tolerance in *H. halys*, to observe whether these conditions cause a change in cold tolerance in the parasitoid. We found that populations of both species froze and survived at colder temperatures than those reported for *H. halys*. In addition, there were no ecologically relevant differences in the temperature at which freezing or survival occurred among populations of either species, indicating that these populations are equally cold tolerant and suitable for introduction. Finally, *T. japonicus* does not acclimate by increasing cold tolerance in response to conditions that increase cold tolerance in *H. halys*, suggesting that the above-mentioned measures of cold tolerance are ecologically relevant.

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* Corresponding author.

E-mail address: koch0125@umn.edu (R.L. Koch).

1. Introduction

Halyomorpha halys (Stål) (Hemiptera: Pentatomidae), the brown marmorated stink bug, is native to China, Japan, Korea, and Taiwan (Rider et al., 2002). In North America, this invasive species was first detected in Pennsylvania in 1996, and by June 2016 had spread to 43 states in the U.S., and two Canadian provinces (Hoebeker and Carter, 2003; Northeastern IPM Center, 2016). *Halyomorpha halys* has also invaded several European countries and has been intercepted in New Zealand (Harris, 2010; Haye et al., 2014). This highly polyphagous pest feeds on >100 species of plants in 45 families including fruits, vegetables, field crops, and ornamental plants in both native and invaded habitats (Haye et al., 2014; Hoebeker and Carter, 2003; Lee et al., 2013), and is considered a nuisance when aggregating en masse while overwintering inside man-made structures (Hoebeker and Carter, 2003; Inkley, 2012).

Some native North American insects have been identified as predators or parasitoids of *H. halys*, but are generally inconsistent in suppressing *H. halys* populations (Herlihy et al., 2016; Jones et al., 2014; Ogburn et al., 2016). As part of a classical biological control program for *H. halys*, foreign exploration has been conducted in Asia since 2007 and four species of egg parasitoids in the genus *Trissolcus* (Hymenoptera: Scelionidae) have been identified as potential biological control agents. These species are being evaluated for potential release against *H. halys* in the U.S. (Talamas et al., 2013). One of these species, *T. japonicus* (Ashmead), appears promising due to a short developmental time (10.5 d at 25 °C), multiple generations per year, highly female-biased sex ratio, and parasitism rates of 50–80% on *H. halys* in the native Asian range (Yang et al., 2009). A second candidate species, *T. cultratus* (Mayr), is also under consideration, though parasitism rates are lower than those of *T. japonicus* (Haye et al., 2015).

Low temperature is a limiting factor in the geographic distribution of many parasitoids (Hance et al., 2007); thus, understanding the cold tolerance of a potential biological control agent can help estimate its capacity to survive and establish in novel areas (Daane et al., 2012; Hanson et al., 2013). In fact, successful classical biological control programs ensure that potential biological control agents and the targeted pests share similar responses to temperature (Goolsby et al., 2005; Hoelmer and Kirk, 2005; Ulrichs and Hopper, 2008). Responses to temperature can vary among populations of the same species (Cira et al., 2016; Feng et al., 2014; Rochefort et al., 2011), so it is prudent to compare geographically-distinct populations to identify which population may be better suited for introduction (e.g., Mausel et al., 2011). Furthermore, because classical biological control programs depend on rearing individuals under laboratory settings for release, it is important to ensure that loss of overwintering potential over time due to exposure to long periods of non-diapause laboratory conditions does not occur (e.g., Garipey et al., 2015).

Commonly-used indices for quantifying cold tolerance in insects include the supercooling point (i.e., the lowest temperature reached prior to the release of latent heat of fusion, observed as an exotherm) and lower lethal temperature (i.e., the temperature at which brief exposure causes a desired level of mortality, often 50 or 90%) (Sinclair et al., 2015). The relationship between freezing and mortality in insects can be used to determine one of three basic cold tolerance categories: chill-intolerance (i.e., death from cold without internal ice formation), freeze intolerance (i.e., death coincides with internal ice formation), or freeze tolerance (i.e. survival of freezing) (Lee, 2010). However, during the onset of fall and through the winter, these strategies may change as insects acclimatize to decreasing temperatures and photoperiods (Hefty, 2016). Acclimatization can result in an increase in cold tolerance over the course of a season (Crosthwaite et al., 2011; Feng et al., 2016; Udaka and Sinclair, 2014). Therefore, it is important to try

to quantify indices of cold tolerance after prolonged exposure to cold temperatures and shorter photoperiods to see if there are any changes over time.

Halyomorpha halys has invaded northern temperate regions (Garipey et al., 2014; Koch, 2014) which experience winter temperatures below 0 °C. In fact, adult *H. halys* collected from Minnesota were found to be chill intolerant and to vary seasonally in their cold tolerance (Cira et al., 2016). In response to colder temperatures and shorter photoperiods, *H. halys* will enter a reproductive diapause (Niva and Takeda, 2002). In contrast, little is known about the cold tolerance or overwintering behavior of *T. japonicus* or *T. cultratus*, except that *T. japonicus* overwinters as an adult (Yang et al., 2009), presumably below bark (Hirose et al., personal communication). Related parasitoids are known to overwinter as adults (Fathi et al., 2011; Foerster and Nakama, 2002; James, 1988; Ryan et al., 1981) and below bark (Fathi et al., 2011; Safavi, 1968). No information exists on subzero temperature limits for *T. japonicus* and *T. cultratus*, nor the extent of cold tolerance variation among populations of each species.

Adventive populations of *T. japonicus* were recently found in the wild in North America (Milnes et al., 2016; Talamas et al., 2015), so there is a need to determine whether *T. japonicus* can survive cold winter temperatures. To assist in assessing the establishment potential of *T. japonicus* and *T. cultratus* in North America we measured supercooling points and lower lethal temperatures for unacclimated, geographically-distinct populations of these species (e.g., Hanson et al., 2013). In addition, we subjected adult *T. japonicus* to conditions that increase cold tolerance in *H. halys* in order to observe whether this induces a change in cold tolerance in the parasitoid.

2. Materials and methods

2.1. Parasitoid acquisition and rearing

Trissolcus japonicus and *T. cultratus* populations were collected from four locations in Asia (Beijing and Nanjing, China; Pucheon, South Korea; and Tsukuba, Japan) between 2007 and 2012 (Table 1). Populations were reared at 20 °C, 16:8 h L:D, and 60–65% RH, and provided honey for nutrition and laboratory-reared *H. halys* eggs for reproduction at the U.S. Department of Agriculture (USDA), Agricultural Research Service, Beneficial Insects Introduction Research Unit, in Newark, Delaware. Separate egg masses of *H. halys* were parasitized by four populations of *T. japonicus* or two populations of *T. cultratus* with four parasitized egg masses of each population shipped overnight under USDA PPQ permit P526-14-01139 to a Biosecurity-Level-2 containment facility in Saint Paul, Minnesota during July 2014, and February and May 2015 for experiments (Table 1). At the Saint Paul location, some individuals of each species were provided with laboratory-reared *H. halys* eggs masses and reared under the conditions described above unless otherwise specified.

2.2. Cold tolerance experiments

The cold tolerance of adult *T. japonicus* and *T. cultratus* was evaluated separately in August 2014, and March and July 2015. The supercooling points and lower lethal temperatures of *T. japonicus*, and the lower lethal temperatures of *T. cultratus* were examined. Due to the limited number of individuals, supercooling points for *T. cultratus* were not measured. In order to facilitate comparison to the host *H. halys*, the cold tolerance testing methods generally followed those employed by Cira et al. (2016) for *H. halys*, with the substitution of a smaller thermocouple (e.g., Stephens et al., 2015). Recently emerged (i.e., 12–36 h old)

Table 1Source information for populations of *T. japonicus* and *T. cultratus* used to measure lower lethal temperatures (LLTemp) and supercooling points (SCP).

Experiment	Population or generation	Experimental date	Latitude (°N)	Longitude (°E)	JALT [†] (°C)	Collection year	Shipment date	No. SCP tested	No. LLTemp tested (n/exposure temperature)
Supercooling point and lower lethal temperature	<i>T. japonicus</i>								
	Beijing, China	Aug.–Oct. 2014	39.91	116.39	−9.4	2009	Jul. 2014	22	88 (15–20)
	Pucheon, South Korea	Aug.–Oct. 2014	37.89	127.20	−6.1 [‡]	2009	Jul. 2014	22	111 (20–25)
	Tsukuba, Japan	Aug.–Oct. 2014	36.03	140.07	2.5 [‡]	2012	Jul. 2014	18	68 (12–15)
	Nanjing, China	Aug.–Oct. 2014	32.05	118.77	−1.6	2009	Jul. 2014	14	74 (14–15)
Lower lethal temperature	<i>T. cultratus</i>								
	Tsukuba 2007, Japan	Feb.–Mar. 2015	36.03	140.07	2.5 [‡]	2007	Feb. 2015	–	80 (16)
	Tsukuba 2012, Japan	Feb.–Mar. 2015	36.03	140.07	2.5 [‡]	2012	Feb. 2015	–	100 (20)
Cold acclimation	<i>T. japonicus</i> [*]								
	Summer, P	Jul.–Oct. 2015	39.91	116.39	−9.4	2009	May 2015	–	78 (6)
	Summer, F ₁	Jul.–Oct. 2015	39.91	116.39	−9.4	2009	May 2015	–	78 (6)
	Summer, F ₂	Jul.–Oct. 2015	39.91	116.39	−9.4	2009	May 2015	–	65 (5)
	Summer, F ₃	Jul.–Oct. 2015	39.91	116.39	−9.4	2009	May 2015	–	78 (6)
	Fall, F ₁	Jul.–Oct. 2015	39.91	116.39	−9.4	2009	May 2015	–	56 (4–5)
	Fall, F ₂	Jul.–Oct. 2015	39.91	116.39	−9.4	2009	May 2015	–	65 (5)
	Fall, F ₃	Jul.–Oct. 2015	39.91	116.39	−9.4	2009	May 2015	–	65 (5)

^{*} Experiment included individuals from the Beijing, China population.[†] JALT January average lowest temperature (30-year period). Data from the World Meteorological Organization, 2016.[‡] Due to the lack of climatological information available for Pucheon, South Korea, and Tsukuba, Japan, a substitution was made with information from the nearest cities: Seoul, South Korea (20 km) and Tokyo, Japan (70 km).

adult parasitoids were used in all experiments, and the sex of each individual was determined after death by examination of the antennae (Yang et al., 2009). All statistics were performed using R (R Core Development Team, 2015; RStudio Team, 2015), and all commands originate from the base package unless otherwise specified.

2.2.1. Supercooling point determination of *T. japonicus*

Methods for contact thermocouple thermometry followed Stephens et al. (2015). Adult *T. japonicus* were placed individually inside 1.5-ml microcentrifuge tubes. Each tube was sealed with a milled plastic dowel that had been fitted with an o-ring. A 0.127-mm copper-constantan thermocouple was threaded through a hole in the center of the dowel. Thermocouples with insects were placed inside 20 × 20 × 20 cm polystyrene cubes calibrated to cool at a rate of 1 °C per minute when placed inside an ultralow freezer at −80 °C (Carrillo et al., 2004). Cubes were cooled in groups (i.e., blocks) of 4–16 cubes and the temperatures of the insects were recorded every second by use of an analog data acquisition unit (USB-TC, Measurement Computing Core, Norton, MA), and visualized in real time on a computer using TracerDAQ Pro (Measurement Computing Core, Norton MA). For each individual, the supercooling point was recorded as the lowest temperature reached before the release of latent heat of fusion (i.e., an exotherm) (Carrillo et al., 2004). For each population, 14–22 exotherms were detected (Table 1).

Regression analysis was used to compare the distributions of supercooling points among populations of *T. japonicus* because such distributions are often not normal (Sinclair et al., 2015). Logistic regression was used to compare the cumulative proportion of individuals that began to freeze (i.e., an event) by a particular temperature among populations of *T. japonicus* (e.g., Stephens et al., 2015). For this analysis a generalized linear model was created using the glm() command with the probability of observing freezing as the response variable, and population, temperature, sex, and their interaction as explanatory variables. There was no effect of sex ($Z = -0.15$, $P = 0.88$), so this variable was dropped, and the data were pooled in the reduced model. To test for differences among populations in the temperature at which freezing occurred, intercepts (b_0) and slopes (b_1) among the four populations of

T. japonicus were compared by changing the model reference population, and a Bonferroni adjustment (critical $\alpha = 0.008$ per comparison) was used to maintain an overall $\alpha = 0.05$. The dose.p() command from the MASS package (Ripley, 2015) followed the general equation: $P(\text{Insect freezes}) = 1/[1 + e^{-(b_0 + b_1 \cdot \text{temperature})}]$, to identify the 50th percentile freezing temperature (i.e., $P(\text{Insect freezes}) = 0.5$) ($\pm$ SE) for each population.

2.2.2. Lower lethal temperature determination of *T. japonicus* and *T. cultratus*

To determine the lower lethal temperature of *T. japonicus* and *T. cultratus*, two experiments were performed (one for each species), and both experiments followed a randomized block design. For each population of a given species, individual adults were cooled to an assigned exposure temperature following the same protocol as the supercooling point experiments, except that individuals were removed from the freezer and cubes upon reaching the assigned exposure temperatures. Individuals were assigned randomly to exposure temperatures of −23, −21, −19, −17 °C, or a control of 25 °C. Previously-collected data suggested that mortality varied from 0 to 100% in this range. The number of individuals randomly assigned to each exposure temperature ranged from 12 to 22 individuals for each population of both species (Table 1). Up to 20 individuals were cooled in a block, for a total of 18 blocks for *T. japonicus* and 12 blocks for *T. cultratus*. Due to the limited supply of individuals and the inability to synchronize emergence, it was not possible to represent each population and exposure temperature equally within a block; however, each population represented in a block included one control. Survivorship of each individual was visually determined 30 min after returning to room temperature and survival was defined as the parasitoid walking within the centrifuge tube. To determine the effect of low temperature exposure on longevity, individuals from the lower lethal temperature experiments were transferred in their centrifuge tubes to a growth chamber at 23 °C, 16:8 h L:D, and 60–65% RH, and were maintained without food and monitored every 1–3 d until death.

To compare cumulative mortality as a function of exposure temperature among populations of each species, generalized linear models were prepared as described for the supercooling point analysis. However, for lower lethal temperature, an event was

defined as whether an insect died by the exposure temperature. As before, sex was dropped from both models because the effect was not significant (*T. japonicus*: $Z = -1.21$, $P = 0.22$, and *T. cultratus*: $Z = -0.74$, $P = 0.46$), and the data were pooled in the reduced models. Testing for differences in the temperature at which mortality occurs among populations and identification of the 50th percentile mortality temperature ($\pm$ SE) for each population were determined as outlined for the supercooling point analysis.

To compare the effect of low temperature exposure on longevity among populations of each species, a parametric survival regression model was created for each species by using the `survreg()` command from the Survival package (Therneau, 2015). For these models, the response variable was the number of days an individual was alive, and individuals that died between monitored dates were censored at the last day that they were seen alive. The explanatory variables for each model consisted of population, temperature, and their interaction. Models were reduced with a maximum likelihood ratio test (χ^2) through use of the `anova()` command. The `predict()` command was used to identify the median number of days until death ($\pm 95\%$ CI) (i.e. longevity) for each combination of population and temperature. Tukey pairwise comparisons within a population were made using the `glht()` command from the multcomp package (Hothorn et al., 2015). All reported p -values were adjusted at $\alpha = 0.05$.

2.2.3. Cold-tolerance strategy analysis of *T. japonicus*

To determine cold-tolerance strategies for the four populations of *T. japonicus*, cumulative mortality and cumulative freezing as a function of temperature were compared within a population as per Stephens et al. (2015). The cumulative mortality curve describes the total proportion of individuals that would be expected to die after brief exposure to a specified temperature; death may have occurred at a warmer temperature than the specified temperature. Similarly, the cumulative supercooling point curve describes the total proportion of individuals that would be expected to start freezing at a temperature greater than or equal to the specified temperature. This comparison was performed by creating generalized linear models as described for the supercooling point analysis for each population, but in this case the probability of observing an event (i.e., freezing or dying) as the response variable, and treatment (i.e., supercooling point or lower lethal temperature), temperature, and their interaction, as explanatory variables.

2.2.4. Cold acclimation experiment for *T. japonicus*

An experiment was performed to determine if temperature and photoperiod conditions cooler than those known to increase cold tolerance in *H. halys* (e.g., 20 °C, 12:12 h L:D, T. Cira, personal communication) could enhance the cold tolerance of *T. japonicus*. Adult parasitoids from the Beijing, China population were received from Newark in May 2015 and maintained at summer conditions (23 °C, 16:8 h L:D, and 60–65% RH), for two generations. Recently emerged (i.e., 12–36 h old) adult males and females were transferred into eight separate (9 dram) plastic vials (1–3 per sex, per vial), provisioned with honey, and allowed to mate for 24 h in the summer conditions. After 24 h, males were removed and the vials were evenly split between two sets of rearing conditions: summer or fall (18 °C, 12:12 h L:D, and 60–65% RH). Each vial was provisioned with a thawed *H. halys* egg mass (~25–28 eggs per egg mass) prepared following Haye et al. (2015). *Halyomorpha halys* egg masses less than 24 h old were collected from a University of Minnesota (St. Paul, MN) laboratory colony (maintained at 23 °C, 16:8 h L:D, and 60–65% RH), and stored in a –80 °C freezer for up to four weeks until needed. The egg masses were thawed for 30 min at room temperature before use. After 48 h, females were removed from the vials and the egg masses (containing first

generation, or F_1) were maintained in their respective rearing conditions until emergence. Upon emergence, the mating and oviposition process outlined above was repeated for two additional generations (F_2 and F_3), though individuals remained in their respective rearing conditions.

Each generation of adult female parasitoids produced under the two rearing conditions were used in lower lethal temperature experiments similar to those described for the lower lethal temperature determination. However, exposure temperatures for these lower lethal temperature measurements spanned every degree between –25 and –14 °C, and a control of 25 °C, and the number of individuals randomly assigned to each exposure temperature ranged from 4 to 6 individuals for each generation of each rearing condition (Table 1). Again, up to 20 individuals were cooled in a block, for a total of 40 blocks.

Like the lower lethal temperature analyses, generalized linear models were created to compare cumulative mortality as a function of exposure temperatures between rearing conditions of the same generation, and among generations of each rearing condition. Three models were created to compare rearing conditions (summer or fall) within a generation (F_1 , F_2 , or F_3), where the explanatory variables included temperature, rearing condition, and their interaction. Two models were created to compare generations (P , F_1 , F_2 , and F_3) within each rearing condition (summer or fall), where the explanatory variables included temperature, generation, and the interaction. For the latter two models, testing for differences in the temperature at which mortality occurs among generations and identification of the 50th percentile mortality temperature ($\pm$ SE) for each generation was determined as outlined for the supercooling point analysis.

3. Results

3.1. Supercooling point of *T. japonicus*

Among all populations of *T. japonicus*, supercooling points ranged from –23.67 to –16.73 °C. The temperatures that caused 50% of the population to begin to freeze ($\pm$ SE) ranged from –22.04 $\pm$ 0.07 (Pucheon) to –21.43 $\pm$ 0.19 °C (Nanjing) (Table 2). The distributions of supercooling points were not the same for each population. From the logistic regressions of cumulative percent of individuals freezing (i.e., supercooling) as a function of temperature for *T. japonicus*, the intercept (b_0) and slope (b_1) for Nanjing were significantly higher than the intercepts and slopes of Beijing and Pucheon, but not significantly different from those of Tsukuba (critical $\alpha = 0.008$) (Table 2, Supplemental Table 1). In general, more individuals from Nanjing began to freeze at warmer temperatures than individuals from Beijing or Pucheon, but not Tsukuba, and the change in the rate at which individuals began to freeze as temperatures declined was significantly greater for individuals from Nanjing than from Beijing or Pucheon, but not Tsukuba (Fig. 1A–D).

3.2. Lower lethal temperature of *T. japonicus* and *T. cultratus*

For *T. japonicus*, the 50th percentile mortality temperature ($\pm$ SE) was lowest for the southernmost population of Nanjing (–20.03 $\pm$ 0.61 °C) and highest for the second northernmost population of Pucheon (–17.36 $\pm$ 0.84 °C) (Table 2). The 50th percentile mortality temperature ($\pm$ SE) for Tsukuba 2007 and Tsukuba 2012 of *T. cultratus* was –19.88 $\pm$ 0.46 °C and –19.86 $\pm$ 0.45 °C, respectively. In general, while mortality increased as individuals were exposed to decreasing temperature, the likelihood of mortality did not differ significantly among populations within either species (Fig. 1A–F). Logistic regressions describing mortality as a function

Table 2

Coefficients ($\pm$ SE) for logistic regression models to describe the cumulative frequency of freezing (from supercooling point measures) and the cumulative extent of mortality from brief cold exposure (from lower lethal temperature treatments) for populations of *Trissolcus japonicus*.

Population	df	Intercept (b_0)				Slope (b_1)				50th percentile	
		Freezing	Mortality	Z	P	Freezing	Mortality	Z	P	Freezing	Mortality
Beijing	106	$-37.88 \pm 3.85_a$	$-7.71 \pm 2.64_a$	-6.46	<0.001	$-1.76 \pm 0.18_a$	$-0.42 \pm 0.13_a$	-6.02	<0.001	-21.50 ± 0.06	-18.56 ± 0.72
Pucheon	129	$-35.71 \pm 3.41_a$	$-7.26 \pm 2.50_a$	-6.74	<0.001	$-1.62 \pm 0.15_a$	$-0.42 \pm 0.13_a$	-5.99	<0.001	-22.04 ± 0.07	-17.36 ± 0.84
Tsukuba	82	$-33.54 \pm 4.40_{ab}$	$-12.34 \pm 4.32_a$	-3.44	<0.001	$-1.52 \pm 0.20_{ab}$	$-0.71 \pm 0.23_a$	-2.66	0.01	-22.00 ± 0.09	-17.41 ± 0.64
Nanjing	84	$-19.71 \pm 3.27_b$	$-9.67 \pm 2.92_a$	-2.60	0.02	$-0.92 \pm 0.15_b$	$-0.48 \pm 0.15_a$	-2.09	0.04	-21.43 ± 0.19	-20.03 ± 0.61

Models follow the general equation: $P(\text{Insect freezes or dies}) = 1/[1 + e^{-(b_0 + b_1 \cdot \text{temperature})}]$.

Within rows (i.e., within populations), intercepts and slopes from the models for freezing were all significantly different from the models for mortality at $\alpha = 0.05$.

Within a column, different letters indicate significant differences for intercepts or slopes among population freezing (df = 68) or mortality (df = 333) treatments at an adjusted $\alpha = 0.008$ to maintain an overall $\alpha = 0.05$.

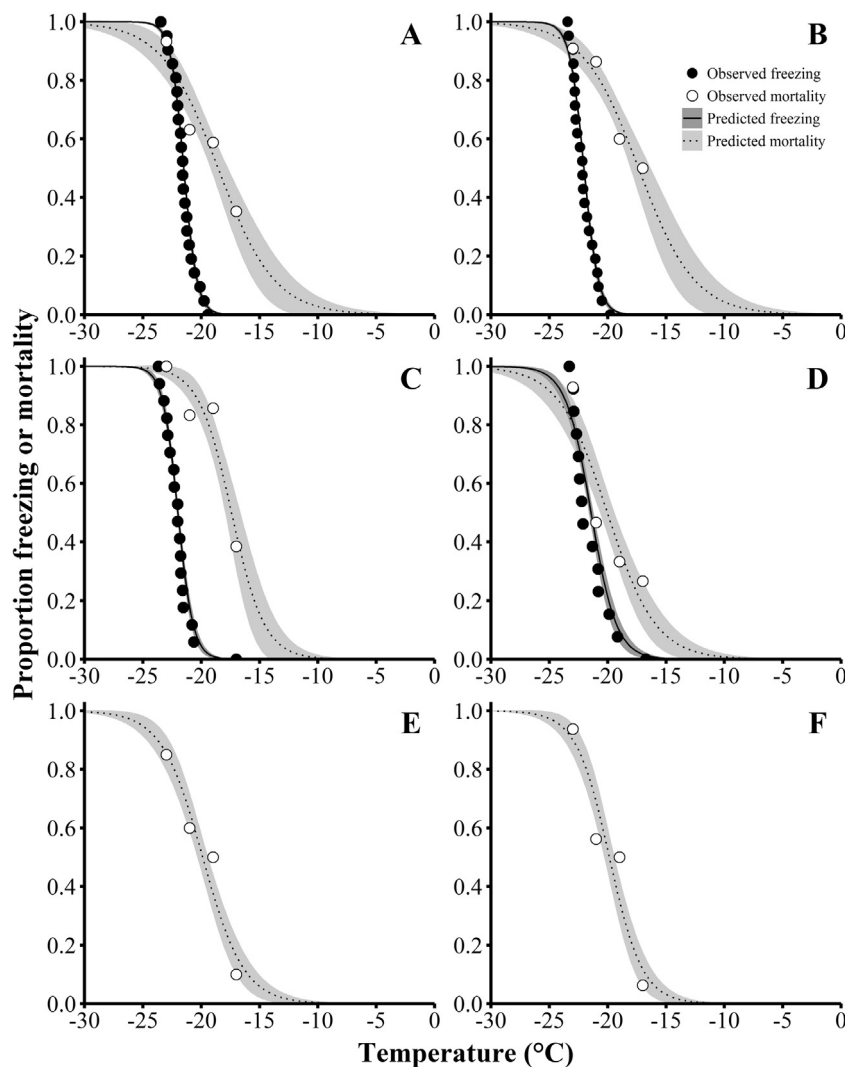


Fig. 1. Observed and predicted ($\pm$ SE band) cumulative freezing and/or mortality for populations of *Trissolcus japonicus* (A: Beijing, China; B: Pucheon, South Korea; C: Tsukuba, Japan; D: Nanjing, China) and *T. cultratus* (E: Tsukuba 2007, Japan; F: Tsukuba 2012, Japan).

of temperature for *T. japonicus* did not have significantly different intercepts or slopes among populations (Table 2, Supplemental Table 1). Similarly, the intercepts (-11.28 ± 2.67 and -13.98 ± 3.39) and the slopes (-0.57 ± 0.13 , and -0.70 ± 0.17) for the Tsukuba 2007 and Tsukuba 2012 populations of *T. cultratus* did not differ significantly between collection years ($Z = -0.63$, $P = 0.53$ and $Z = -0.63$, $P = 0.53$, respectively).

For *T. japonicus* and *T. cultratus*, longevity for each population was generally lower after exposure to low temperature (i.e., -23 and -21 °C), than to the control temperature (i.e., 25 °C) (Fig. 2). Among all populations of *T. japonicus* and *T. cultratus*, median longevity after exposure to -23 and 25 °C ranged from 0.78 to 1.33, and 4.28 to 6.44 d, respectively (Fig. 2). Maximum likelihood ratio tests revealed that the interaction of population and temperature was

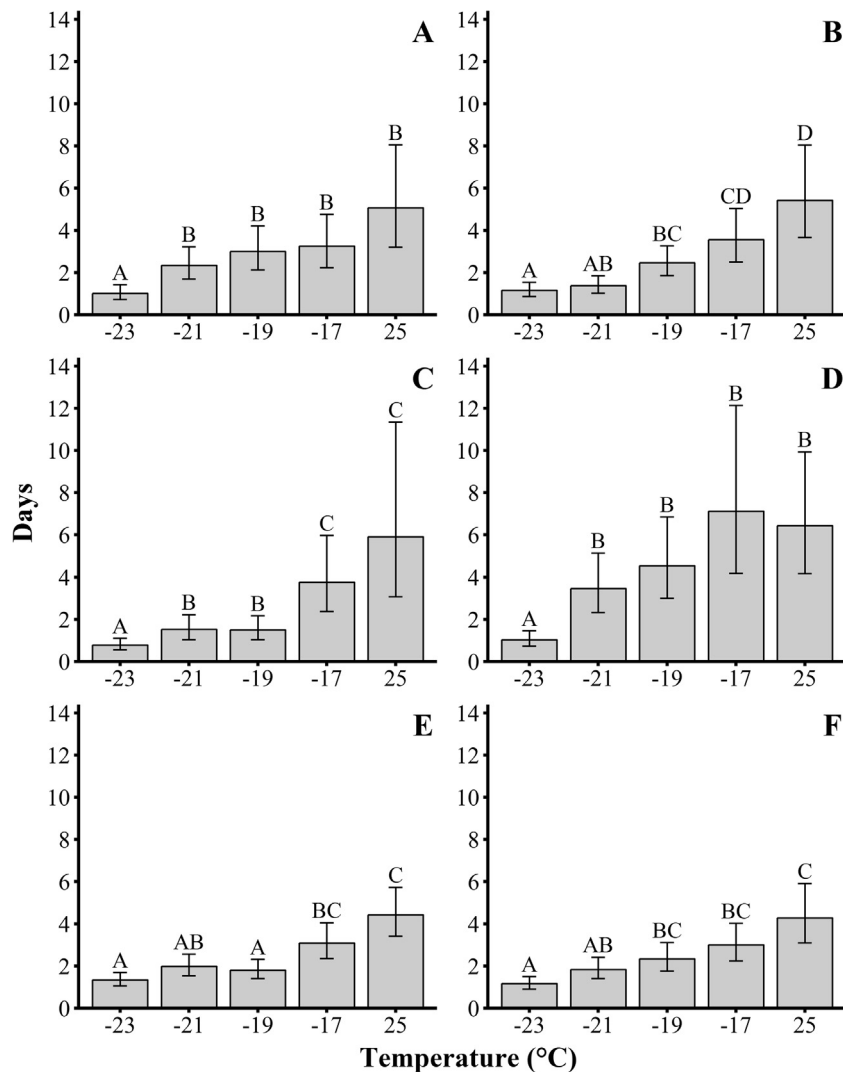


Fig. 2. Median longevity ($\pm 95\%$ CI), following brief exposure to low temperatures for populations of *Trissolcus japonicus* (A: Beijing, China; B: Pucheon, South Korea; C: Tsukuba, Japan; D: Nanjing, China) and *T. cultratus* (E: Tsukuba 2007, Japan; F: Tsukuba 2012, Japan).

not significant in either model for *T. japonicus* ($\chi^2_3 = 3.32$, $P = 0.34$) nor *T. cultratus* ($\chi^2_1 < 0.002$, $P = 0.97$). Therefore, among populations within a species, the relative differences in longevity were constant across all exposure temperatures. In the reduced model for *T. japonicus* population was significant ($\chi^2_3 = 23.5$, $P < 0.001$), and the median longevity ($\pm 95\%$ CI) for Nanjing was 1.50 (1.12, 2.00) days longer than Beijing ($Z = 2.75$, $P = 0.03$), 1.73 (1.32, 2.27) days longer than Pucheon ($Z = 3.92$, $P < 0.001$), and 1.98 (1.46, 2.69) days longer than Tsukuba ($Z = 4.37$, $P < 0.001$). Population was not significant in the reduced model for *T. cultratus* ($\chi^2_1 < 0.001$, $P = 0.97$), indicating there were no differences in longevity between the two populations. In the final model for each species, temperature was significant for both *T. japonicus* ($Z = 6.98$, $P < 0.001$) and *T. cultratus* ($Z = 6.48$, $P < 0.001$), indicating that longevity within a population increased with increasing (i.e., warmer) exposure temperatures (Fig. 2).

3.3. Cold-tolerance strategy of *T. japonicus*

From the comparisons of curves that described cumulative freezing and cumulative mortality within each population of *T. japonicus*, mortality appeared to precede freezing as temperature

declined (Fig. 1A–D). For each population, the slope and intercept of the logistic regression model characterized the cumulative frequency of supercooling points. In the logistic regression models, supercooling points intercepts and slopes were all significantly lower than the lower lethal temperature intercepts and slopes (Table 2).

3.4. Cold acclimation of *T. japonicus*

From the logistic regressions of cumulative percent of individuals dying as a function of temperature, there were no significant differences among the intercepts and slopes of generations within rearing conditions or rearing conditions within a generation (Table 3, Supplemental Table 2). The 50th percentile mortality temperature ($\pm$ SE) for the summer and fall conditions were similar, spanning from -17.95 ± 0.50 to -15.09 ± 0.76 °C (Table 3). As temperature decreased, mortality increased at the same rate among all generations within a rearing condition, as well as between rearing conditions for a given generation (Fig. 3A–D). Therefore, exposure over three generations to conditions known to induce reproductive diapause in *H. halys* did not affect the likelihood of survival of *T. japonicus*.

Table 3

Coefficients ($\pm$ SE) for logistic regression models to describe the extent of mortality after brief cold exposure among *Trissolcus japonicus* adults that had been reared under summer or fall conditions for up to three generations.

Generation	df	Intercept (b_0)				Slope (b_1)				50th percentile ($^{\circ}$ C)	
		Summer	Fall	Z	P	Summer	Fall	Z	P	Summer	Fall
P*	76	$-12.60 \pm 2.89_a$	–	–	–	$-0.70 \pm 0.16_a$	–	–	–	-17.95 ± 0.50	–
F ₁	130	$-8.19 \pm 2.29_a$	$-7.68 \pm 2.79_a$	0.06	0.95	$-0.50 \pm 0.13_a$	$-0.50 \pm 0.16_a$	0.14	0.89	-16.24 ± 0.71	-15.44 ± 0.91
F ₂	126	$-7.65 \pm 2.25_a$	$-8.52 \pm 2.51_a$	0.26	0.80	$-0.45 \pm 0.12_a$	$-0.51 \pm 0.14_a$	0.33	0.74	-16.92 ± 0.79	-16.56 ± 0.74
F ₃	139	$-8.73 \pm 2.84_a$	$-14.05 \pm 4.31_a$	1.03	0.30	$-0.58 \pm 0.17_a$	$-0.88 \pm 0.26_a$	0.96	0.33	-15.09 ± 0.76	-15.98 ± 0.52

Models followed the general equation: $P(\text{Insect dies}) = 1/[1 + e^{-(b_0 + b_1 \cdot \text{temperature})}]$.

Within rows (i.e. within generations), intercepts and slopes from the summer conditions were not significantly different from those of the fall conditions at $\alpha = 0.05$.

Within columns, different letters indicate significant differences for intercepts or slopes among generations for summer ($df = 291$) or fall ($df = 256$) conditions at an adjusted $\alpha = 0.008$. The summer P generation was also included in comparison among those reared under fall conditions.

* The P generation was only reared under summer conditions, hence no comparison for this generation to a fall counterpart, nor a predicted fall 50th percentile mortality temperature.

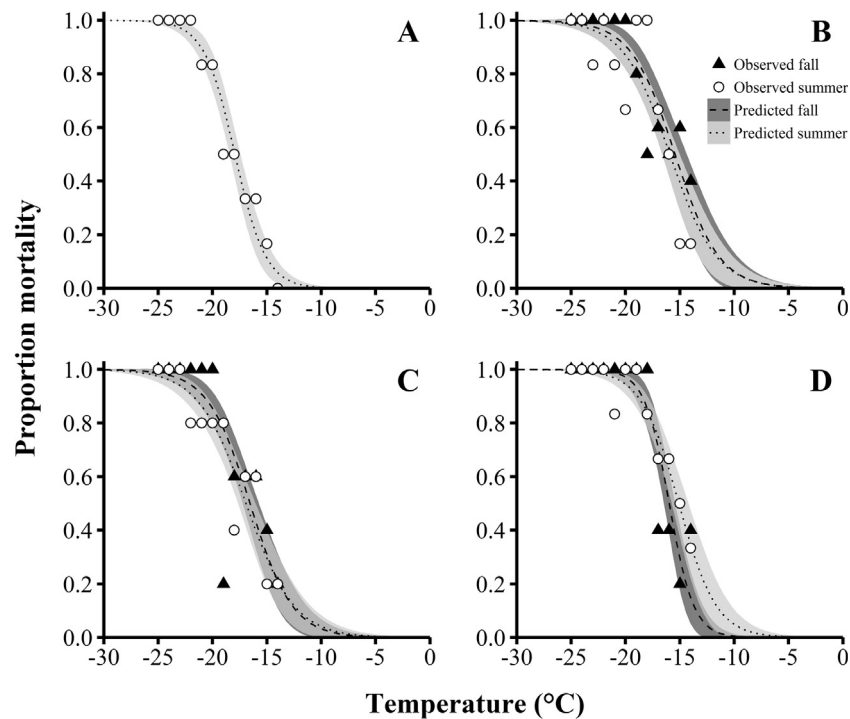


Fig. 3. Observed and predicted mortality ($\pm$ SE band) of *Trissolcus japonicus* reared over multiple generations (A: parental (P) generation; B: first (F₁) generation; C: second (F₂) generation; D: third (F₃) generation) under summer and fall rearing conditions.

4. Discussion

Classical biological control by Asian *Trissolcus* spp. may provide an additional tool for managing *H. halys* beyond North American natural enemies (Herlihy et al., 2016; Ogburn et al., 2016) and the current use of pesticides (Leskey et al., 2012). Recently, populations of *T. japonicus* were detected in both the eastern and western U.S. (Milnes et al., 2016; Talamas et al., 2015). However, these areas currently represent a fraction of the range of *H. halys* in North America (Northeastern IPM Center, 2016), and do not reflect the northern extent of the invaded range of this pest, which seasonally experiences cold winter temperatures (USDA, 2012). The present research is the first assessment of cold tolerance for *T. japonicus* and *T. cultratus*, and will facilitate evaluation of classical biological control for *H. halys*.

Many insects (Crosthwaite et al., 2011; Kim and Song, 2000; Régnière and Bentz, 2007), including parasitic Hymenoptera (Hanson et al., 2013), are known to seasonally acclimatize to cold conditions by increasing cold tolerance as a response to environmental conditions such as photoperiod or temperature

(Denlinger, 1991). Such seasonal acclimatization could affect ecological relevance of cold tolerance estimates attained from unacclimated, laboratory populations. Whether *T. japonicus* seasonally acclimatizes to colder temperatures or shorter photoperiod was unknown to date. Therefore, we began our investigation with unacclimated individuals that were reared under standard conditions (23 ± 1.5 °C, 16:8 h L: D, 65% RH) to determine a reference point for comparing populations reared under cooler temperature or shorter photoperiod conditions.

Interestingly, median supercooling point and lower lethal temperature results for unacclimated populations of *T. japonicus* and *T. cultratus* were all below the winter mean supercooling point (-17.06 °C) of *H. halys* from Minnesota, an area that experiences severe winter temperatures (Cira et al., 2016). In addition, prolonged exposure to laboratory conditions for *T. cultratus* (e.g., Tsukuba 2007) did not appear to affect the baseline cold tolerance when compared to a population collected five years later (e.g., Tsukuba 2012). This suggests that both parasitoid species, when unacclimated, are capable of surviving colder temperatures than acclimated *H. halys*, and that this unacclimated cold tolerance is

retained during laboratory rearing. Such results are promising for potential long-term laboratory populations reared for classical biological control. Nevertheless, we evaluated lower lethal temperature of *T. japonicus* under more relevant conditions by performing an experiment that investigated the ability of *T. japonicus* to modify its cold tolerance in response to conditions known to increase cold tolerance in *H. halys*. Our results suggest that unlike *H. halys*, exposure to conditions cooler than 20 °C and a photoperiod of 12:12 h does not increase the cold tolerance *T. japonicus*. Parasitic wasps are dependent on their hosts and for most species, synchrony in behavior to that of their host is crucial to their survivorship (Hance et al., 2007). *Trissolcus japonicus* has been recorded from several other Asian pentatomid species (Matsuo et al., 2016; Yang et al., 2009), and it overwinters as an adult (versus inside of the host) (Yang et al., 2009). This lack of dependency on a single host species and behavior of overwintering outside of the host may in part explain why *T. japonicus* did not respond to the same photoperiod cues as *H. halys*. However, our study does not rule out the possibility that *T. japonicus* can further adjust its cold tolerance if exposed to other conditions. Further work should explore how *T. japonicus* responds to temperature or photoperiod regimes beyond those used in this study.

Supercooling points of *T. japonicus* were not the same for all source populations. Populations originating from the southernmost population (i.e., Nanjing) began to freeze at significantly higher temperatures than the northernmost populations (i.e., Beijing and Pucheon). This pattern aligns with the latitude and 30-year average lowest temperature from January for the source population areas in Asia (Table 1). Similar correlations have also been found with other studies that demonstrate that the mean supercooling points of geographically-distinct populations of a single insect species increase in temperature as latitude decreases (Jing and Kang, 2003; Zhou et al., 2011). However, the small magnitude of difference in median freezing values among the populations tested here is not likely to confer an ecologically-relevant advantage for the populations that supercooled at statistically lower temperatures. Therefore, from the standpoint of using supercooling point as an index of cold tolerance, these four populations of *T. japonicus* should have similar likelihoods for freezing at cold winter temperatures.

We did not find significant differences in mortality as a function of exposure temperature among any of the populations of *T. japonicus* or *T. cultratus*. This suggests that all populations should have similar likelihoods for surviving brief exposure to cold and that all of the populations are equally suitable for introduction with respect to winter survival. Because freezing and mortality can occur at significantly different temperatures (Sømme, 1982), it is important to assess the relationship between these two phenomena in order to determine whether supercooling point and lower lethal temperature are interchangeable indices for mortality (Renault et al., 2002). Our results demonstrate that each of the populations of *T. japonicus* appeared to be dying before freezing. Thus, lower lethal temperature is a more ecologically-relevant source of information than the supercooling point for assessing the cold tolerance of *T. japonicus* (e.g., Baust and Rojas 1985), and should be used for more accurate comparisons of *T. japonicus* cold tolerance to that of *H. halys*. Although informative, our study is limited in that we investigated survivorship after brief exposure to low temperatures (e.g., lower lethal temperature). This deliberate measurement was used to decouple low temperature and exposure time, as duration of exposure to a low temperature can differentially affect survivorship (Renault et al., 2002). Further cold tolerance studies with *T. japonicus* should expand on these initial measures of lower lethal temperature by incorporating the element of time (e.g., lower lethal time). This could help assess how

survivorship of this species is affected under conditions that more closely simulate what the insects may encounter in nature.

Comparison of the temperatures at which insects begin to freeze or die also can be used to infer cold tolerance strategy (e.g., Hanson and Venette 2013), and those that die from cold temperatures before body fluids begin to freeze are considered to be chill-intolerant (Lee, 2010). Our results demonstrate that *T. japonicus* is chill-intolerant, which is a cold-tolerance strategy shared by other parasitoids (Carrillo et al., 2005; Hanson et al., 2013; Rivers et al., 2000), as well as by *H. halys* (Cira et al., 2016). Therefore, similar to *H. halys*, populations of *T. japonicus* are freezing at temperatures significantly lower than what they could survive.

Interestingly, while we did not find differences among populations of either parasitoid in lower lethal temperature, we did find that the southernmost population, Nanjing, lived longer without food after brief exposure to all low temperatures in comparison with the other *T. japonicus* populations. This increase of approximately two days in longevity was relatively small, but it could provide the individuals from this population with additional time to seek nutrition during the spring, when food is not yet available or scarce. Little is known about the natural feeding habits of *T. japonicus* except that it does not host-feed; however, if it is similar to that of other scelionids, this species likely also feeds on flower nectar and insect honeydew (Orr, 1988; Safavi, 1968). Other studies have documented that longevity decreases as a result of prolonged exposure to decreasing temperatures (Colinet et al., 2006; Langer and Hance, 2000; Lysyk, 2004). Our results provide evidence of an effect on longevity after brief cold exposure, where individuals of *T. japonicus* and *T. cultratus* that are able to survive brief exposure to subzero temperatures do so at the expense of their longevity. Such sublethal trade-offs could have negative implications for establishment success of *T. japonicus* or *T. cultratus* in areas that experience even brief subzero temperatures (e.g., Bale 2002).

Localized detections of *T. japonicus* in the U.S. over consecutive summers provide evidence that the parasitoid is able to successfully overwinter or re-colonize. In Winchester, Virginia, the coldest area in the U.S. where *T. japonicus* was found for multiple summers (2015 and 2016, Hoelmer, unpublished data), the average annual extreme minimum temperature ranges from −20.6 to −17.8 °C (USDA, 2012). Based on our laboratory experiments, in which the insects were in direct contact with low temperature, this range of temperatures caused mortality in approximately 25–91% of the laboratory populations of *T. japonicus*. However, some insects seek overwintering habitats that buffer extreme cold as measured by ambient temperature (Inkley, 2012; Koch et al., 2004; Vermunt et al., 2012). Therefore, it is possible that our predictions underestimate survivorship in nature because our methodology does not account for potential thermal buffering provided by an overwintering habitat. *Trissolcus japonicus* may overwinter as an adult below *Zelkova* spp. tree bark (Hirose et al., personal communication), which is a similar behavior to that of its host, *H. halys* (Lee et al., 2014), and other *Trissolcus* spp. (Fathi et al., 2011; Safavi, 1968). It is likely that during this overwintering period *T. japonicus* adults experience some degree of thermal buffering to ambient air temperatures, which could improve their ability to survive in these areas (e.g., Turnock and Fields 2005). Field studies targeting the adventive populations of *T. japonicus* in the U.S. could help investigate the overwintering behavior of the wasps and the degree of thermal buffering provided by bark. In addition, the adventive populations provide a unique opportunity to compare cold tolerance measurements between laboratory and field populations.

As *H. halys* has established in areas that experience cold temperatures, effective biological control agents must be able to withstand similar conditions. Based on our comparison of cold

tolerance indices for geographically-distinct populations of two *Trissolcus* spp., it appears that the populations are equally suited for survival in these areas. In regards to assessing the potential for classical biological control of *H. halys*, these *Trissolcus* spp. appear more cold tolerant than their host and therefore may not be limited by cold in areas that *H. halys* has invaded. However, if microclimate of specific overwintering habitat is not accounted for, resulting estimates of potential geographic range will be conservative. Therefore, to refine estimates of the geographic range of these species, further work is needed to determine the overwintering habitats of these *Trissolcus* spp. and to quantify cold exposure in such habitats.

Finally, arthropod overwintering success is contingent on a number of factors beyond temperature stress. Arthropods employ a number of mechanisms to survive cold exposure including (but not limited to), cryoprotectant and antifreeze protein synthesis, membrane restructuring, adjustments to ion transports, upregulation of heat shock proteins, etc. (Teets and Denlinger, 2013). Further studies with *T. japonicus* or *T. cultratus* should examine additional factors affecting overwintering potential (e.g., Colinet et al., 2007), or the mechanisms that drive that success (e.g., Foray et al., 2013).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biocontrol.2017.01.004>.

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