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Short-exposure chill injury to investigate intraspecific cold tolerance of the giant salvinia weevil, *Cyrtobagous salviniae* Calder & Sands (Coleoptera: Curculionidae)

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ABSTRACT

Failure of biological control to adequately suppress target weeds is sometimes due to thermal limitations of introduced agents. However, adaptation of biological control agents to local climates after release may provide a resource for improving performance of the agents in regions where control has been poor. To date, *Cyrtobagous salviniae* has not provided adequate control of *Salvinia molesta* in the northern extent of its invasive range in the United States. This has spurred interest in improving control by identifying and leveraging spatial variation in thermal biology of the agent. To compare cold tolerance of *C. salviniae* populations, we used a modification of the upper limit of chill injury zone (ULCIZ) metric that we term short-exposure chill injury temperature (SECIT). SECIT reflects the relationship between temperature, exposure duration, and mortality and provides a tool for extracting substantial information about an organism's cold tolerance while reducing the overall effort associated with a comprehensive ULCIZ. Four populations of *C. salviniae* were sourced from across a latitudinal gradient at field sites in Louisiana and Texas, and SECIT was modeled through a two-factorial assay of chill temperature and exposure durations. SECIT results were then used to predict the estimated mortality of each population based on historical weather data. Differences in SECIT were detected among the four populations (lowest/most cold tolerant: −3.01 C; highest/least cold tolerant: −1.12 C) and when used to estimate mortality based on historical cold events reflects an approximately 183,000 km^2 (36.5%) difference in marginally to highly suitable area of *S. molesta*–infested watersheds in the southeastern United States. These results demonstrate the utility of SECIT to compare cold tolerance between populations of a biological control agent and provide information that can be used to inform management of aquatic weeds using biological control.

Key words: aquatic weeds, biological control, chill injury, cold tolerance, invasive species.

INTRODUCTION

Variable success in the biological control of invasive aquatic weeds can sometimes be attributed to a mismatch between host and agent thermal tolerances (Julien et al. 1995, Mukherjee et al. 2014, Reddy et al. 2019). For example, biological control of the aquatic weed, giant salvinia, *Salvinia molesta* D.S. Mitch., by the giant salvinia weevil, *Cyrtobagous salviniae* Calder and Sands (Coleoptera: Curculionidae), has been widely implemented across the southeastern United States, with particular focus in Texas and Louisiana (Mukherjee et al. 2014; Nachtrieb 2019), but cool winter temperatures have prevented agent establishment and population build-up in higher latitudes (Mukherjee et al. 2014). Cold temperature limitations have led numerous researchers to investigate the utility of sourcing new populations of the agent from its native range (Russell et al. 2017).

For biological control agents with widespread distributions in their introduced range, local adaptation to climate may be detectable through various cold tolerance assays (e.g., Coetzee et al. 2007, Griffith et al. 2019, Knight et al. 2023) and may provide a resource for improving control by redistributing populations with enhanced cold tolerance traits (Harms et al. 2021). Because cold tolerance is a multifaceted trait, a range of metrics have been used for testing different insect species (Lee 2010, Harms et al. 2021). Depending on the aspect of low-temperature biology that is important for a particular agent (e.g., low-temperature survival, fecundity), different metrics may be reported. For *C. salviniae*, supercooling point, pulsed exposures to low temperature, chill coma, chill coma recovery, and temperature-dependent development have been studied to explain the limited distribution of *C. salviniae* in regions where it has been introduced, but only limited attention has been paid to differences among U.S. populations (Mukherjee et al. 2014, Obeysekara et al. 2015, Russell et al. 2017, Cozad et al. 2019). In the United States, surveys to determine whether locally adapted populations of *C. salviniae* exist have been limited to a few introduced or native populations (Mukherjee et al. 2014, Russell et al. 2017, Cozad et al. 2019).

Determination of the upper limit of chill injury zone (ULCIZ), although not commonly used to measure cold

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tolerance (but see Zhao et al. 2015, Wang et al. 2019), may provide the most information about low-temperature survival because it reflects the time-temperature relationship with mortality. The ULCIZ is the lowest temperature that does not cause chill mortality even after a very long exposure, beyond which chill-injury accumulates, eventually leading to death (Nedvěd et al. 1998). Data collected from such a factorial, exposure time × temperature survival experiment facilitate the calculation of related metrics such as LT_{50} (median lethal time) and the sum of injurious temperatures (SIT). SIT is the degree-day relationship between exposure time and chill injury accumulation and, in combination with other metrics, can be used to predict establishment and persistence of biological control agents across large areas (Zhao et al. 2015; Wang et al. 2019). However, ULCIZ experiments are time- and resource-intensive and may not be feasible for long-lived agents such as *C. salviniae*. Given the value of the data provided by ULCIZ, but considering the limitations in certain systems, we propose a modified approach, conducted over a narrower range of temperatures and for shorter durations, that is useful for describing acute effects of low-temperature events on agent survival and valuable for comparing between multiple populations of the same species.

The goal of this research was to 1) use a modification of the ULCIZ metric that we term short-exposure chill injury temperature (SECIT) to compare cold hardiness of multiple *C. salviniae* populations and 2) discuss the utility of this approach for screening agent cold tolerance to improve biological control efforts. In cases where relative cold-tolerant populations can be detected using these tools, they may be used to augment mass-rearing programs and improve survival and establishment of biological control agents in cooler regions.

MATERIALS AND METHODS

C. salviniae source populations

Releases of *C. salviniae* in Louisiana and Texas have been ongoing for more than 15 yr. To the best of our knowledge, all the populations sampled in this study originated from the same source, which was from imported from Australia and first released by USDA APHIS at Toledo Bend Reservoir, TX, in 2001 (Flores and Wendel 2001).

Palm Lake, LA (30.27°N; 89.80°W). Palm Lake, LA, is a ~37 ha freshwater lake on private land near Slidell, LA. Weevils recovered from this location were an adventive population with no record of intentional release. Collections of *C. salviniae* from infested plants were made on 9 June 2021.

Indian Creek, LA (31.10°N; 92.46°W). Indian Creek Reservoir is a ~900 ha freshwater impoundment, near Woodworth, LA. Releases of *C. salviniae* at Indian Creek were first made in 2015 and again in 2017. Collections of *C. salviniae* from infested plants were made on 15 June 2021.

Lewisville Aquatic Ecosystem Research Facility, TX (LAERF; 33.07°N; 96.95°W). The LAERF is an intermediate-scale research facility designed for the study of biology, ecology, and management of aquatic plants (Smart et al. 1995). *Cyrtobagous salviniae* has been in culture at the LAERF since 2003 (Harms et al. 2009). Since then cultures have been supplemented with individuals recovered from field sites in Louisiana. Cultures are maintained in aboveground wooden culture boxes within open-sided greenhouses (Nachtrieb 2013). During winter months, greenhouses are closed to maintain warm temperatures for early spring population growth. Collections of *C. salviniae*–infested plants were made on 12 and 15 October 2021.

Black Lake, LA (31.96 °N; 93.05°W). Black Lake is a ~5,400 ha freshwater cypress lake in north-central Louisiana. Releases of *C. salviniae* occurred at Black Lake in 2017. In 2021, despite no additional releases, substantial *C. salviniae* populations were observed during monthly lakewide monitoring efforts. Collections of *C. salviniae*–infested plants were made on 18 October 2021.

Weevil extraction and preparation

Adult *C. salviniae* were collected from three field locations and one rearing culture during the summer and fall of 2021 (see previous section). Weevil-infested *S. molesta* was collected in bulk and processed at either the Lewisville Aquatic Ecosystem Research Facility (LAERF), Lewisville, TX, or Louisiana State University, Baton Rouge, LA. Berlese funnel extraction was used for plant material collected at LAERF, Black Lake, and Palm Lake. Indian Creek Lake weevils were extracted with wire transfer screens placed over collection basins in an open-air environment at the LAERF. Moist paper towels were added to collecting jars or basins to provide moisture, substrate, and protection from predation. Following extraction, weevils were transferred to hard-sided containers with a small amount of giant salvinia as a food source. Within 4 d of collection, weevils were shipped overnight to the U.S. Army Engineer Research and Development Center (ERDC), Vicksburg, MS, for experimentation. Because insects were field collected at different times during the year, and we used these individuals in experiments, we were unable to standardize their age or reproductive status. Thus, we made all efforts to standardize conditions leading up to experiments by holding weevils in the laboratory prior to their use in experiments.

Upon receipt at the ERDC, weevils were placed on fresh giant salvinia in a mesh-covered dish and held in a plant growth chamber (E-41L2, Percival Scientific, Perry, ID) at 20 C. Prior to the experiment, weevils were sorted individually into 44 ml (1.5 oz) plastic cups with a piece of moist paper towel and a frond of salvinia. Temperatures were reduced from 20 to 13 C over 24 h (−0.46 C/h), then weevils were acclimated at 13 C for 72 h (Hennecke and Postle 2006, Russell 2017). Following acclimation, cups with weevils were transferred to cold chambers (LT-41VL, Percival Scientific) and temperature was ramped from 13 C to the experimental temperature at a rate of 0.5 C/min. Recording of exposure time began when chambers reached their experimental temperature.

Short-exposure chill injury temperature experiment

A full-factorial temperature by exposure time experiment was conducted with four temperatures (−2, −4, −6, −8 C) and five exposure durations (6, 12, 24, 48, 96 h). Ten replicate weevils were assigned to each treatment combination.

Due to mortality in transit, the coldest and longest treatments were omitted for some populations. At each experimental time point, weevils were removed from the cold chamber and placed at room temperature for 24 h, then observed for survival. To assess survival, individuals were moved from their location in the cup, placed on a paper towel, and monitored for movement. If the individual righted itself within 15 min or responded to stimulation, then they were considered alive. For each temperature and time combination, proportion survival was calculated.

Calculation of cold tolerance metrics

The relationship among weevil survival, temperature, and exposure duration was modeled following Nedvěd et al. (1998) as

$$S(t, T) = \frac{e^{[a+bt(T-c)]}}{1 + e^{[a+bt(T-c)]}}$$

where $S(t, T)$ is the proportion survival at time (t; hours) and temperature (T; C). Model parameters a, b, and c were estimated. The parameter c in this case is the short-exposure chill injury temperature and represents the temperature at which the T_{50} is indistinguishable across measured exposure durations. Modeling was done with nonlinear least squares regression (Statistica 64, v12, Stat Soft, Inc., Tulsa, OK).

Additionally, the temperature that resulted in 50% mortality at each exposure duration (LT_{50}) was calculated as

$$T = c - \frac{a}{b} \times \frac{1}{t} \left(\text{Nedvěd et al. 1998}\right).$$

SECIT mortality map

To visualize how SECIT can be used to understand differences in cold tolerance among populations, a map showing mean predicted mortality was calculated following a modified method described in Wang et al. (2019) to predict the resulting mortality of the annual single-most severe cold event for each *C. salviniae* population. Raw 1-min resolution temperature data from 2003–2024 was downloaded from the National Oceanic and Atmospheric Administration (ftp://ftp.ncdc.noaa.gov/pub/data/asos-onemin/), then cleaned following Wang et al. (2019). Data were further aggregated for each site year such that a single year of temperature data spanned September though the following August of each year. Site and year combinations missing more than 25% of data from any month between October and April and any site for which there were fewer than 10 yr of data were omitted from the analysis. The duration of the longest single cold event for which the temperature dropped below a particular temperature was calculated for −2, −4, −6, −8, and −12 C for each site-year combination. A cold event was defined as the duration of time a location was below a threshold temperature. Missing time points that occurred during an event were included in the event duration if the duration of the missing data did not exceed 6 h. Predicted mortality based on each temperature threshold and event duration was calculated for each location and year, and the highest estimated mortality was chosen from this set and averaged across years for each site. Resulting mean predicted mortality was then interpolated using Inverse Distance Weighting across the entire United States for each *C. salviniae* population (ArcMap, ESRI, Redlands, CA). For purposes of discussion, regions with predicted mortality less than 25% in response to the chill event are defined as highly suitable, between 25% and 50% as marginally suitable, between 50% and 75% as marginally unsuitable, and greater than 75% as highly unsuitable.

RESULTS AND DISCUSSION

Survival of *C. salviniae* at low temperatures was variable among populations and did not clearly reflect their source latitude. The Indian Creek population displayed higher survival at lowest temperatures for longer durations than other populations (Figures 1 and 2). Survival for short durations was similar between Indian Creek and Palm Lake populations, but LT_{50} at 96 h (4 days) was 1.5 C colder for Indian Creek than Palm Lake weevils (Figure 2A). The sole mass-culture population (LAERF) was nominally the least cold tolerant but similar to individuals from Black Lake across exposure temperatures and durations. Although not explicitly tested, the relatively consistent and mild overwintering conditions in culture may prevent the development of increased cold tolerance. Additionally, we standardized pre-experiment holding conditions to limit the effect of environmental exposure before field collection, but there is a possibility of seasonal evolution that could influence cold tolerance differences between seasons within a single population. Seasonal variation in thermal tolerances over multiple generations would be a valuable line of future research for biological control programs with multivoltine agents.

Cyrtobagous salviniae collected from Indian Creek had the lowest short-exposure chill injury temperature over 96 h (SECIT = −3.01 ± 0.47 C) of the tested populations. The population at Indian Creek was established by state and regional water resource agencies during 2015 and 2017 but had not been monitored prior to 2021, when *C. salviniae* was collected for experiments. The 2015 release included 8,100 individuals from cultures maintained by the Red River Waterway Commission, likely originally sourced from established populations in Louisiana. The 2017 release of 1,200 weevils was made by state personnel, using weevils harvested from Lake Iatt, LA; weevils at Lake Iatt were originally established from cultures at the LAERF. Given the relatively poor performance of the LAERF population in this study, these results may highlight the risk of reduced fitness in long-term laboratory cultures (Hoffmann and Ross 2018). Regardless of source, the Indian Creek weevil population survived multiple winters in the field, including harsh temperatures during winter 2021, in which air temperatures were as low as −12 C for 2 d in a row, and average daily temperatures were below freezing for five straight days (NOAA.gov).

The relative cold hardiness of *C. salviniae* populations can be visualized by mapping predicted mortality based on annual winter weather events. For Palm Lake and Indian Creek populations, mortality from the single worst cold event is estimated to result in less than 30% mortality for

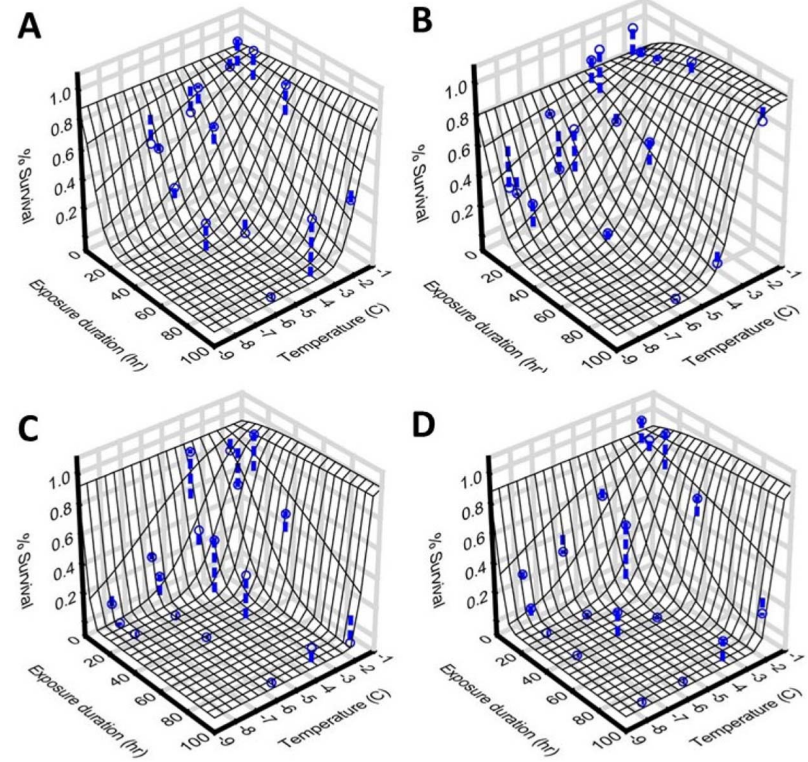

Figure 1. Survival of *C. salviniae* adults modeled for combinations of temperature and exposure period. Populations shown here were sourced from (A) Palm Lake, LA, (B) Indian Creek, LA, (C) LAERF, TX, and (D) Black Lake, LA.

most of southern Louisiana (Figure 3). Palm Lake weevils had the highest area within the invasive range of *S. molesta* in the eastern United States with predicted mortality less than 25%, whereas Indian Creek had the greatest total area with predicted mortality less than 50% (Table 1). In contrast, the LAERF population was predicted to be the least suitable, with 43.66% of the modeled area estimated to be marginally to highly unsuitable.

Although useful for short-exposure estimations of survival, SECIT does not provide an estimate of the temperature at which actual chill injury begins to accumulate; that is the basis of ULCIZ. Thus, for longer exposures at warmer temperatures than used here, chill injury can occur and, depending on the length of exposure, may lead to chill-induced mortality. Traditional ULCIZ has applications for predicting the distribution of biocontrol agents in introduced areas, assuming distribution is largely determined by winter temperatures. Zhao et al. (2015) demonstrated this utility for *Agasicles hygrophila* Selman & Vogt by calculating the ULCIZ and plotting the predicted distribution of *A. hygrophila* in China against mean winter temperature isotherms. In their model, supercooling point (SCP) was used to delineate the northern distribution of *A. hygrophila* and then describe the intermediate zone between the ULCIZ and the SCP as unstable habitat where *A. hygrophila* would be expected to occur during some years. Except for freeze-tolerant species, SCP functionally represents the absolute lowest temperature where mortality is 100% as exposure time approaches zero. Supercooling point was not estimated during the current study; however, SCP has previously been

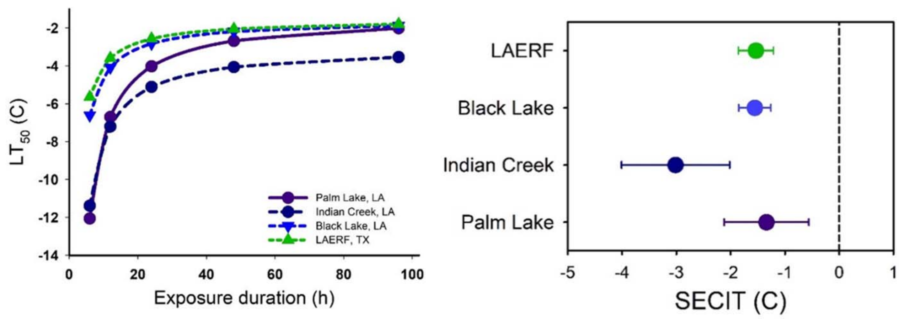

Figure 2. (A) Temperatures that caused 50% mortality at each of five exposure periods for four populations of *C. salviniae*. (B) Short exposure chill injury temperature (± 95% CI) for four populations of *C. salviniae* over a 96 h exposure.

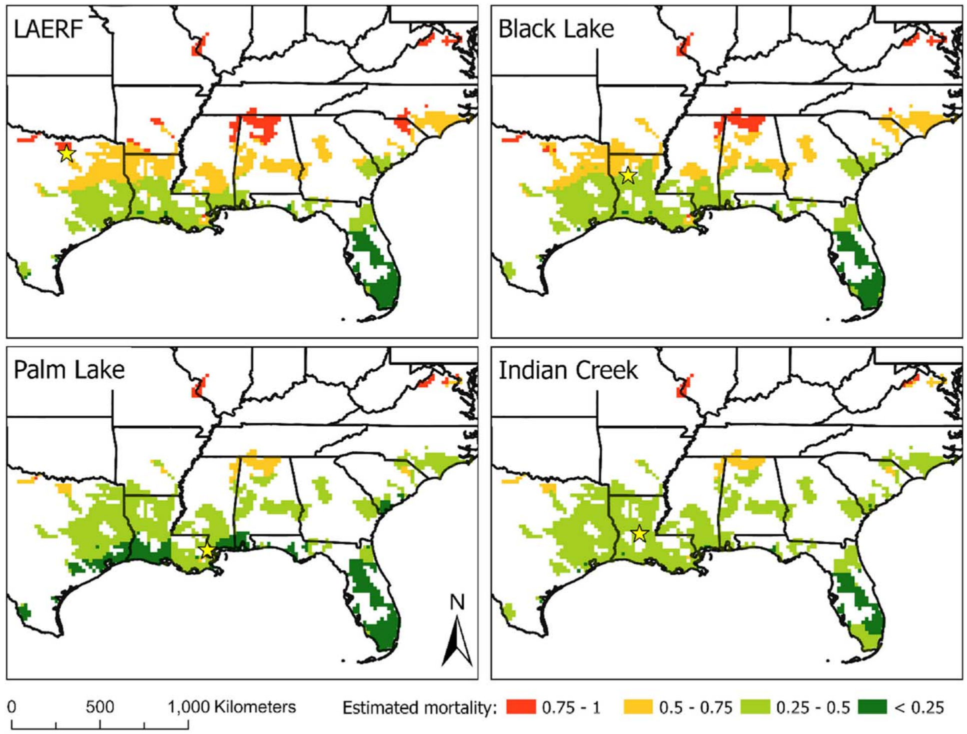

Figure 3. Estimated mortality from the average deadliest chill event. Colored range represents USGS Hydrologic Units (Steeves and Nebert 1994) where *S. molesta* is known to occur (U.S. Geological Survey 2024). The area outside the colored range are regions where giant salvinia has not been reported. Stars indicate the coordinates where each population was collected.

measured as −14 C for a Louisiana population of *C. salviniae* (Russell et al. 2017). As demonstrated here, for all populations assayed, mortality was high long before supercooling point was reached for all but the shortest exposure durations (< 6 h). Compared to ULCIZ, determination of SECIT requires less effort and time because the number of treatment combinations is significantly reduced. It does however effectively describe mortality across a range of ecologically relevant temperatures and exposure durations.

Variation in cold tolerance among introduced biological control agent populations is a topic of great interest, given that climate limitations on control success are common globally (Harms et al. 2020), and effects of extreme temperatures on insects are expected to increase with climate change (Weaving et al. 2022). Although rare in practice, comparisons of thermal tolerances between populations of a biological control agent provide insights into evolution and adaptive capacity and assist the identification of source populations for selective breeding programs (Harms et al. 2021). All populations used in the current study except the LAERF cultures were putatively overwintered *in situ* and had not been supplemented with new weevil releases during the current year. We expected that if differences in cold tolerance were observed, they might be evident as a latitudinal pattern, in which individuals from the northern-most population would be more cold-tolerant than individuals from southern populations, a pattern that we did not observe. Myriad reasons could explain the lack of a clear pattern (e.g., microhabitat variation and protection from cold temperatures or seasonal differences in basal cold tolerance), but it is evident that the three field populations collected in Louisiana were not enough to provide a full picture of the true variation in *C. salviniae* cold tolerance within the southeastern United States. This study demonstrated the utility of SECIT for comparing low-temperature survival of multiple populations across short-exposure times. These metrics do not negate the value of a complete ULCIZ experiment that is conducted over much longer durations, but instead provides a relatively swift and ecologically relevant assessment of lethal temperature and exposure limits due to relatively short chill events experienced during a cold front, for example. Additionally, SECIT and ULCIZ exist on a spectrum of timescales from relatively short (SECIT) to long (ULCIZ) but can be tailored to the organism under investigation, taking into account life history and likelihood of cold exposure in the environment. Without additional populations for testing, we cannot draw larger conclusions about local adaptation of *C. salviniae* to temperature extremes.

Table 1. Estimated *C. salviniae* mortality from the average deadliest chill events and the area (km^2) of *S. molesta* invasive range (U.S. Geological Survey 2024) in each tier of estimated mortality.

Site	Area of suitable habitat (Estimated Mortality%)			
	Highly suitable (<25%)	Marginally suitable (25–50%)	Marginally unsuitable (50–75%)	Highly unsuitable (>75%)
LAERF	48,038.98	190,524.37	165,219.49	49,139.10
Black Lake	46,114.25	234,869.78	143,634.93	30,429.05
Palm Lake	121,496.53	292,660.40	29,222.45	8,015.90
Indian Creek	32,869.49	388,600.79	27,389.02	5,803.13

A number of research directions have been identified through this work. First, a larger survey of cold tolerances across the introduced range, spanning climate zones, is needed. We used only four populations in our experiments and did not have access to native range (Argentinian/Uruguayan) populations for comparison. However, we did find evidence that insects from Indian Creek had the greatest cold tolerance values of those tested. Further work is needed to determine whether low-temperature survival will correlate with other important cold tolerance traits, such as fecundity and development (Reddy et al. 2019). Second, it will be important to understand the temperatures that are experienced at field sites throughout the year. The structure of a salvinia mat acts to insulate the water surface from large temperature fluctuations, but the level of insulating capacity is largely unknown. Knowing the range of temperatures experienced in a salvinia mat during winter months would improve our ability to predict when and where mass-mortality events are likely to occur during each winter. And, if there are fitness tradeoffs with cold tolerance (e.g., in longevity or fecundity), then introductions of cold-tolerant populations should be strategic, only to areas where they are critical for establishment, rather than broadly across the invaded range where overall control may be reduced.

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