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Physiological Ecology

Cold tolerance of field-collected soybean gall midge (Diptera: Cecidomyiidae) under laboratory overwintering conditions

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Soybean gall midge, *Resseliella maxima* Gagné, was first described in 2019 following widespread outbreaks in soybean across the midwestern United States. Larval feeding inside stems causes lesions, lodging, and yield loss. Third instars overwinter in the soil within silken cocoons, but their cold tolerance, an important factor for survival in the temperate midwestern United States, is unknown. In 2022 and 2023, late-season soybean stems with *R. maxima* were collected from southwestern Minnesota. Third instars were retrieved and transferred to vials of sand, where they successfully burrowed and formed cocoons. Vials with cocoons were stored at 13 and 3 °C, and larval supercooling points and lower lethal temperatures (LTs) were measured after 1 and 2 months. All individuals remained larvae by the time of measurements, suggesting they were in diapause. Supercooling points and lower LTs were primarily between −20 and −25 °C. Alignment between these measurements suggests that *R. maxima* is freeze-intolerant. There were no consistent effects of storage time or temperature, indicating a lack of further acclimation. The temperature causing 50% mortality (LT_{50}) was −19.8 °C in both 2022 (95% CI: −26.4 to −13.2 °C) and 2023 (95% CI: −26.6 to −13.0 °C). Historical soil temperature data reveal the coldest temperature recorded from 2015 to 2024 near the northern portion of *R. maxima*'s known range (−17.7 °C) would cause <50% mortality to *R. maxima* populations, while average coldest temperatures were substantially warmer (−3.1 to −8.5 °C). Short-term exposures to winter soil temperatures may not cause significant mortality to overwintering *R. maxima* in the midwestern United States.

Keywords: cold hardiness, Cecidomyiidae, thermal ecology

Introduction

Soybean gall midge, *Resseliella maxima* Gagné (Diptera: Cecidomyiidae), is a new pest of soybean (*Glycine max* [L.] Merrill) in the midwestern United States. Small-scale outbreaks are suspected to have occurred sporadically throughout the 2010s, but *R. maxima* did not have a widespread economically significant outbreak until 2018 and was described as a new species in 2019 (Gagné et al. 2019, McMechan et al. 2021a). *R. maxima* can significantly impact soybean production, causing up to 92% yield loss within 30 m of the edge of heavily impacted fields (McMechan et al. 2021a). *R. maxima* adults typically lay eggs in naturally occurring fissures near the base of the soybean stem, or opportunistically at other sites of mechanical injury (McMechan et al. 2021a, 2021b). Larvae eclose, move inside the stem, and begin feeding internally, causing lesions in the stem tissue that disrupt water and nutrient flow; occasionally, they penetrate into the pith of the stems (Gagné et al. 2019, McMechan et al. 2021a). Feeding injury often leads to lodging, wilting and death of the plant (Helton et al. 2022).

Although morphological features such as head capsule width and presence of the spatula (ie a sclerotized structure located on the ventral prothorax of most cecidomyiid third instars) can be used to reliably distinguish instars of other Cecidomyiidae (Gagné and Hatchett 1989, Elsayed et al. 2020, Gagné 2024), such features remain largely unknown for *R. maxima* larvae. The description of *R. maxima* only includes some features of the third instar (eg presence of larval spatula) (Gagné et al. 2019). In general, for *R. maxima*, early instars are smaller and less pigmented than later instars, which may appear orange (Castro 2022), similar to a congener *Resseliella theobaldi* (Barnes) (Gordon and Williamson 1991). Third instars ultimately drop

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out of the plants, burrow into the soil, and construct a silken cocoon (McMechan et al. 2021b). The majority of cocoons (~75%) are found in the top 2 cm of soil and are rarely found deeper than 6 cm (da Silva Lima 2023), much like *R. theobaldi* (Pitcher 1952, Gordon et al. 1989). During early to mid-summer, these larvae will pupate in their cocoons, emerge as adults, and reproduce. However, in late summer, cocooned third instars cease development and overwinter, resuming development and pupating the following spring (McMechan et al. 2021b, Castro 2022) which further parallels *R. theobaldi* (Pitcher 1952, Stenseth 1973). After emergence of the overwintering generation, field observations indicate 2 to 3 further summer generations per year (McMechan et al. 2021b, Castro 2022).

As of 2025, *R. maxima* had been detected in Iowa, Kansas, Minnesota, Missouri, Nebraska, North Dakota, and South Dakota, and is being detected in new counties within these states yearly (Soybean Gall Midge Alert Network 2025). Because of its relative novelty, the potential for *R. maxima* range expansion has yet to be thoroughly studied. Cold tolerance can be one of the most important determinants of an insect's geographic range (Andersen et al. 2015, Sinclair et al. 2015, Lawton et al. 2022) and is particularly relevant in the temperate climate of the midwestern United States where *R. maxima* is established. Assessing the cold tolerance of a newly establishing pest is important for understanding what areas may be at risk by forecasting the likelihood of overwintering (McCornack et al. 2005, Lawton et al. 2022).

Overwintering in the soil likely buffers *R. maxima* from extreme air temperatures. In addition, constructing a cocoon may help protect a larva from mechanical damage or inoculative freezing as ice forms in the surrounding soil, depending on the cocoons' morphology and imperviousness to water (Danks 2004). However, insects also have physiological mechanisms to tolerate cold temperatures, including the depression of their freezing point (ie supercooling) or the ability to survive internal ice formation through controlled freezing processes (Lee 2010, Sinclair et al. 2015). These adaptations contribute to different cold tolerance strategies, known as chill-susceptible (ie experiences mortality before freezing), freeze-avoidant (ie suppresses freezing point but experiences mortality upon freezing), and freeze-tolerant (ie does not experience mortality until sometime after freezing) (Andersen et al. 2015, Sinclair et al. 2015). The strategy *R. maxima* uses to contend with low temperatures is not yet known.

Well-developed methods exist for studying and describing insect cold tolerance. One method is measuring the supercooling point (SCP) of the insect. Several mechanisms enable supercooling, including inherent properties of a small body size/low internal water volume, voiding of the gut, dehydration, accumulation of cryoprotectants, and production of antifreeze proteins (Lee 2010). When the insect eventually begins to freeze, the latent heat of crystallization is released. This release of heat is called the "exotherm," and the temperature at which freezing begins is the supercooling point (Andersen et al. 2015, Sinclair et al. 2015). Supercooling points are relatively easy to measure with contact thermocouple thermometry, providing a natural starting point for understanding cold tolerance, but without additional data their ecological relevance has been questioned (Renault et al. 2002, Sinclair et al. 2015). Other measurements can provide additional insights into insect cold tolerance under real-world conditions. The lower lethal temperature (LT) is a measure of the extent of mortality after momentary exposure to low temperatures (the LT is equivalent to the SCP in freeze-intolerant insects), and the lethal time (sometimes referred to as "lethal time at a low temperature") describes the extent of mortality after prolonged exposure to constant cold temperatures (Andersen et al. 2015, Sinclair et al. 2015). Together, these measurements give us valuable information on the potential response of an insect to cold conditions in the short and long term.

The objectives of this study are to (i) measure the supercooling point and lower LT of the overwintering, cocooned, third instars of *R. maxima*, (ii) to test for acclimation potential by storing cocooned larvae at different temperatures and testing their cold hardiness over time, and (iii) to classify their cold tolerance strategy. These measures of cold hardiness are placed into context by comparing them to historical soil temperatures in a north–south gradient ranging from southwest Minnesota to northeast North Dakota, covering and extending beyond the higher latitudes of *R. maxima's* known range. Overall, this study will help us to better understand the areas likely not at immediate risk to *R. maxima* range expansion, better predict population trends following winter conditions, and inform potential future expansion risks under climate change.

Materials and Methods

Field Collections and Laboratory Acclimation

Soybean plants showing symptoms of infestation by *R. maxima* (ie dark lesions at base of plant stems; Gagné et al. 2019) were collected from an 80-m transect along the edge of a commercial field on 6 September 2022 near Luverne, Minnesota, United States and on 29 August 2023 near Dell Rapids, South Dakota, United States. At the time of collection, the plants were generally senescing (R6 to R7 growth stage; Fehr et al. 1971). After collection, the stems were cut above the lesions created by *R. maxima* and placed into plastic bags which were placed into coolers and brought to the laboratory (Melotto et al. 2023). The following day, the stems were dissected using scalpels to retrieve the *R. maxima* larvae (Melotto et al. 2023). Large, orange-colored larvae, presumed third instars, were chosen for use in these experiments (Melotto et al. 2023). Presence of the larval spatula, a feature of third instars described by Gagné et al. (2019), was confirmed on a subset of the larvae examined. Larvae of *R. maxima* extracted from stems in this manner successfully completed development to adulthood in other studies (Stillwell Jardine et al. 2026).

To produce the overwintering stage (ie cocooned third instars) of *R. maxima*, we adapted methods developed for swede midge, *Contarinia nasturtii* Kieffer, and *Aphidoletes aphidimyza* (Rondani) (Diptera: Cecidomyiidae) (Košťál and Havelka 2000, Des Marteaux et al. 2012). Seven-dram polystyrene vials were filled with 3 cm of sand (QUIKRETE Premium Play Sand, Quikrete, Elburn, Illinois, United States) which had been sifted through a size 60 soil sieve. The sand in each vial was moistened with 3.4 ml of deionized water and mixed with the cylindrical handle of a fine-tipped paintbrush. Ten larvae were placed on the surface of the sand in each vial and the vials were capped. All vials were held in growth chambers at 25 °C for 10 days, to allow time for larvae to dig into the sand and construct cocoons.

Vials containing *R. maxima* larvae were transferred to environmental growth chambers (Percival Scientific, Perry, Iowa,

United States) set to artificial acclimation conditions. In 2022, 2 storage temperatures were used: 13 and 3 °C. In 2023, only 3 °C was used. In all cases, larvae were held at a 10L:14D light cycle, a photoperiod shown to induce diapause in *A. aphidimyza* and *C. nasturtii* (Košťál and Havelka 2000, Des Marteaux et al. 2012). After 1 and 2 months at these conditions, subsets of vials were removed from the chambers. Larvae within intact cocoons were retrieved from the vials by emptying the sand into a dish and rinsing it using deionized water, applied using a laboratory wash bottle. Cocooned larvae were transferred to Petri dishes lined with filter paper to dry for 30 to 60 min prior to use for cold hardiness measurements to prevent possible inoculative freezing from external ice crystal formation. In 2022, further subsets of vials were examined after the initial 10 days of acclimation at 25 °C and after 1 and 2 months at the storage temperatures to record rates of successful cocooning for validation of this method for producing cocooned third instars of *R. maxima*.

Supercooling Points

Supercooling points of cocooned larvae were measured following methods modified from Wittman et al. (2021). Cocooned larvae extracted from sand were attached individually to copper-constantan thermocouples with a small amount of high vacuum grease (Dow Corning, Midland, Michigan, United States). A microcentrifuge tube was fitted over each probe, and the probes were placed into individual glass test tubes within a silicon-oil chiller bath (A40, Thermo Fisher Scientific, Waltham, Massachusetts, United States) programmed to cool at a rate of ~1 °C per minute. Thermocouples transferred temperature data to TRACERDAQ PRO software (Measurement Computing, Bourne, Massachusetts, United States) once per second, and larval temperatures were observed for exotherms in real time. Approximately 16 larvae were placed into the chiller bath for each given "run", divided about evenly between the storage temperatures and durations, and randomly placed within the chiller bath.

When an exotherm was observed (indicated by distinct, rapid increases in an otherwise descending temperature over time), the lowest temperature the larva reached before the exotherm was recorded as its supercooling point. Occasionally, a cocoon would fall off the thermocouple, or an individual thermocouple would malfunction. A run was concluded either when a supercooling point or a thermocouple malfunction was observed for each larva. Runs were repeated until sample sizes of 14 to 30 individuals were reached for each treatment of storage time and temperature, with sample sizes depending on availability of cocooned larvae (Supplementary Table 1).

Lower LT

Lower LTs were measured following methods modified from Stephens et al. (2015) and Wittman et al. (2021). Cocooned larvae were placed in gelatin capsules, which were then placed within microcentrifuge tubes in immediate proximity to thermocouple wires. The thermocouples were lowered into glass test tubes in the same silicon chiller bath described above. The larvae were randomly assigned to be cooled to 1 of 5 exposure temperatures: −10, −15, −20, −25, or −30 °C. These temperatures were chosen to encompass ranges observed in initial measurements of supercooling points. The chiller bath was set to cool at ~1 °C per minute, and temperatures were observed over time as previously described.

Upon reaching their assigned temperature, the cocooned larvae were immediately removed from the chiller bath and returned to room temperature. After 1 h the cocoons were carefully dissected open using insect mounting pins and larvae inside the cocoons were inspected for survival. Larvae which survived moved when prodded, while those that died did not. Live larvae were orange, and dead larvae had a distinctive discoloration and change in internal appearance (Fig. 1). Additional preliminary observations on a subset of these larvae after 24 h showed no recovery of larvae classified as dead.

In 2022, each thermocouple had 2 cocooned larvae in its associated microcentrifuge tube, 1 from each of the storage temperatures (13 and 3 °C). The exposure temperature was randomly assigned at the thermocouple level, so both cocooned larvae would be removed upon reaching the same target exposure temperature. In 2023, larvae were maintained at only 1 storage temperature (ie 3 °C), so only 1 cocooned larva was used per thermocouple. In either case, the 32 (in 2022) and 16 (in 2023) individuals for each run were approximately evenly distributed among the exposure temperatures, for ~3

Fig. 1. Healthy, living third-instar *R. maxima* (left) and a dead third instar (right) displaying a distinctive postmortem change in internal appearance after freezing, including cloudiness and a seeming breakdown of internal tissues. Larvae shown were extracted from cocoons prior to photographing.

replications of each exposure temperature and storage temperature combination per run. Runs were repeated until ~10 replications of each exposure temperature were completed for each set of storage conditions.

Soil Temperature Data

Hourly soil temperature data from a depth of 5 cm were obtained from the University of Minnesota Research and Outreach Centers located at Morris and Lamberton, Minnesota, as well as from the North Dakota Agricultural Weather Network (NDAWN) for Grand Forks, North Dakota (NDAWN 2024) (Fig. 2). The Grand Forks data were from 2015 to 2022, the Morris data from 2015 to 2021, and Lamberton data from 2015 to 2024. These data were processed using R version 4.4.1 to retrieve minimum monthly temperatures for each year at each location (R Core Team 2024).

Statistical Analyses

All statistical analyses were performed with R version 4.4.1 (R Core Team 2024). For the supercooling and lower LT data, Kaplan–Meier analyses were used with the absolute value of temperature instead of time as the predictor to initially summarize the data and account for censoring within the lower LT data as per Cira et al. (2016) and Moore et al. (2025). The supercooling data were considered uncensored, because the temperature at which individuals began to freeze was known. The lower LT data were considered censored. In the case of dead individuals, mortality could have occurred at any temperature between the start of a trial and the removal temperature. Live individuals would likely have died had they been exposed to a lower temperature. The resulting survival curves were then analyzed with the "betareg" package using a beta-regression with a logit link to account for the proportional data (constrained between 0 and 1) resulting from Kaplan–Meier analysis and the "xbetax" distribution to account for the existence of values equal to exactly 1 (Zeileis et al. 2024, Kosmidis and Zeileis 2026).

Beta regressions for both the supercooling and lower LT data used the cumulative proportion of either frozen or dead individuals as the response. Temperature was a continuous predictor, representing the supercooling point data and exposure temperatures from the lower LT trials. Both storage time and, in 2022, storage temperature were used as categorical predictors. Effects of the storage conditions on both the intercept (fixed effect) and slope (interaction with temperature) were tested using pairwise comparisons with the Tukey HSD test. Final models were then created which pooled data on nonsignificant predictors found in the earlier pairwise analyses.

Predicted proportions frozen (for the supercooling study) and dead (for the lower LT study) as well as 0.025 and 0.975 quantiles were generated from these models for every 0.1 °C from 0 to –40 °C using the base-R "'predict" function and used to plot final survival curves. The predicted temperatures to cause 50%, 90%, and 95% of the population to begin to freeze ($SCP_{50, 90, \text{and } 95}$) and the predicted temperatures to cause 50%, 90%, and 95% mortality ($LT_{50, 90, \text{and } 95}$) were estimated by finding the temperature with a predicted proportion freezing or proportion mortality that was closest to the value in question (eg 0.5, 0.9, and 0.95), and 95% confidence intervals were estimated using the 0.025 and 0.975 quantiles.

After analyzing these experiments separately, cold tolerance strategy was assessed by combining Kaplan–Meier curves from the supercooling point and lower LT experiments into 1 dataset. Beta regressions were calculated, with cumulative probability of an event (freezing or death) as the response, temperature

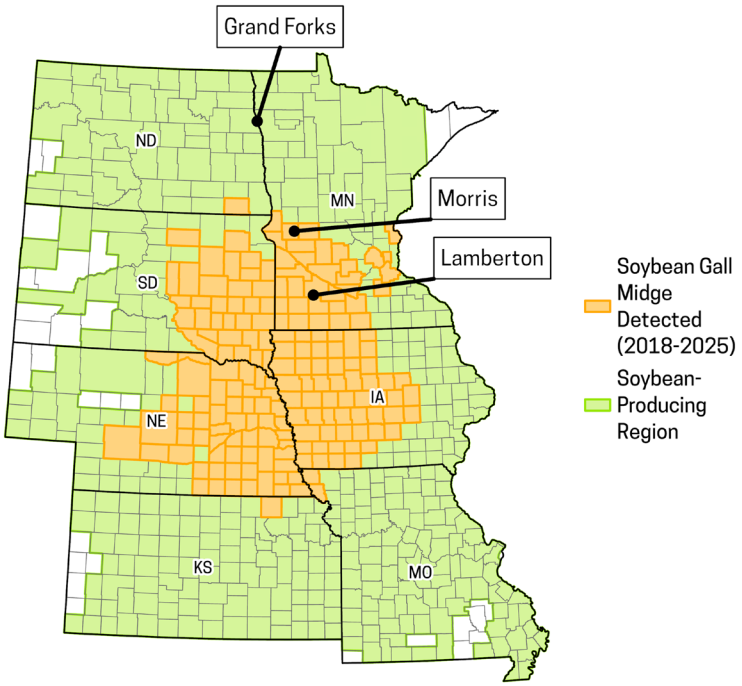

Fig. 2. Map of the 7 states in which *Resseliella maxima* is currently known to occur. Counties highlighted in orange had positive *R. maxima* detections at some point from 2018 to 2025. Counties highlighted in green were recorded as producing soybean in the 2022 USDA-NASS Census of Agriculture (USDA-NASS 2025). Labeled black points indicate field stations where historic 5-cm depth soil temperatures were recorded, with a latitude range from ~44°N (Lamberton, Minnesota) to 48°N (Grand Forks, North Dakota).

as a continuous predictor, and experiment (supercooling or lower LT) as a categorical predictor. Pairwise comparisons were then used to test for any effect of experiment on the intercept or slope of the models. A significant difference would indicate that freezing and dying tend to occur at different temperatures and would provide evidence toward cold tolerance strategies like freeze-tolerant or chill-susceptible.

Results

Cocooning Rates

To determine the success of laboratory methods to produce cocooned larvae, we recorded rates of successful cocooning of *R. maxima* larvae in laboratory sand vials after storage for 10 days at 25 °C, then after 1 and 2 months of storage at 13 or 3 °C. Across the acclimation conditions, most larvae (75% to 83%) placed into sand vials formed cocoons, though small fractions were found free-living and alive (0.8% to 17%) or dead (6% to 25%) (Fig. 3). For free-living larvae, survival was slightly lower as the storage time increased, ranging from a maximum of 70% of larvae being alive at 0 months down to 3% alive after 2 months at 13 °C (Fig. 3). Missing larvae were assumed to have decayed and were included with dead larvae for these calculations.

Supercooling Points

To assess the capacity of overwintering *R. maxima* to suppress their freezing points via supercooling, we recorded the supercooling points of cocooned *R. maxima* larvae in 2 years and tested for acclimation by storing cocoons at 2 temperatures (13 and 3 °C) for 2 different periods of time (1 and 2 months). Across years and storage treatments, 87% of cocooned *R. maxima* larvae began to freeze at temperatures colder than −20 °C, and 58% began to freeze between −20 and −25 °C. Mean supercooling points ranged from −24.3 to −21.9 °C across years and treatments, with an overall mean of −22.9 °C (Supplementary Table 1). The warmest individual supercooling point was −7.5 °C, and the coldest was −27.3 °C (Fig. 4). As exposure temperature decreased, a greater proportion of *R. maxima* began to freeze (Fig. 4). This relationship was significant in both 2022 and 2023 ($|Z| = 4.46$ to 5.77, $P \leq 0.00001$). There was no significant effect of storage time (1 vs 2 months) on the model intercept or slope in either 2022 or 2023 ($|Z| = 2.11$ to 2.28, $P \geq 0.102$), indicating no differences in the accumulation of freezing onset events. In 2022, the populations stored at 3 °C for both durations (1 and 2 months) had significantly lower intercepts ($|Z| = 4.50$ to 7.03, $P \leq 0.00004$) and higher slopes ($|Z| = 4.45$ to 6.92, $P \leq 0.00005$) than those stored at 13 °C at both time points, indicating that larvae began freezing at a lower temperature and freezing onset events accumulated more rapidly. For 2022, storage durations (1 and 2 months) were pooled to create separate models for the storage temperatures (ie 13 and 3 °C). For 2023, due to having no measurable effects of storage time at 3 °C one fully pooled model was created. Model parameters and temperatures needed to cause 50%, 90%, or 95% of the population to begin to freeze (SCP_{50}, SCP_{90}, and SCP_{95}) resulting from these models are reported in Table 1.

Lower LT

To assess the mortality of overwintering *R. maxima* when exposed to cold temperatures, we recorded the lower LT of cocooned *R. maxima* larvae in 2 years and tested for acclimation by storing cocoons at 2 temperatures (13 and 3 °C) for 2 different periods of time (1 and 2 months). Overall, cocooned *R. maxima* larvae had low mortality (~4.9%) with exposure to −10 to −20 °C, followed by a rapid increase in mortality between −20 and −25 °C (Fig. 5). No individual across any set of storage conditions or year survived exposure to −30 °C (Fig. 5). As the exposure temperature decreased, a greater proportion of larvae died. This effect of exposure temperature was significant in both 2022 and 2023 ($|Z| = 2.05$ to 3.04, $P \leq 0.04$). There was no significant effect of storage time or storage temperature treatments on the intercepts or slopes of the model in either year ($|Z| = 0.01$ to 1.83, $P \geq 0.256$), indicating no differences in the distribution of mortality events by temperature. Due to a lack of significant differences among storage conditions, a fully pooled final model was created for each year, and the parameters for the models, as well as predicted lower LTs to kill 50%, 90%, or 95% of exposed individuals (LT_{50}, LT_{90}, and LT_{95} values), are reported in Table 2.

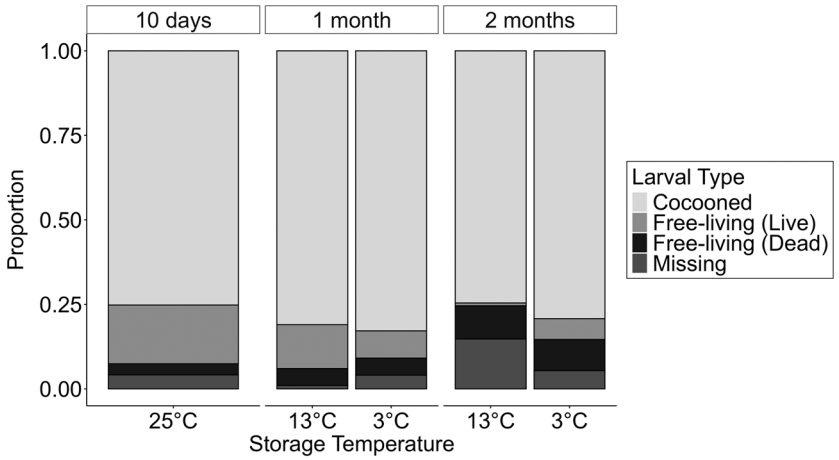

Fig. 3. Proportions of larvae in different states either immediately, 1 month, or 2 months following the introduction and 10-day acclimation of presumed third-instar *Resseliella maxima* larvae to sand vials across time points. n=100 larvae for each storage temperature/storage time combination. Cocooned larvae were always alive.

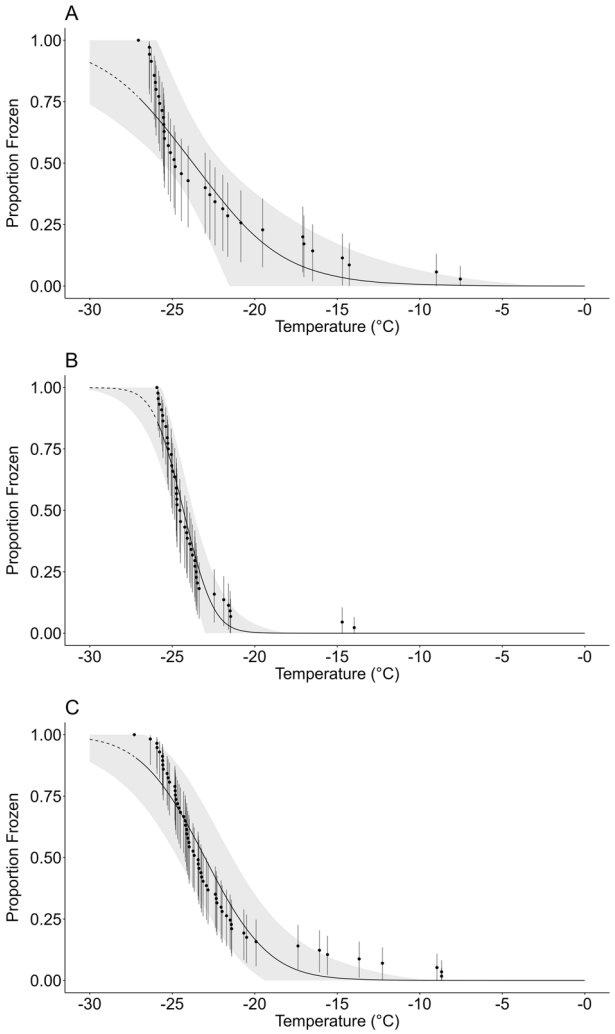

Fig. 4. Effect of subzero exposure temperatures on supercooling points (measured as the cumulative proportion of individuals that had started to freeze) of *Resseliella maxima* cocooned larvae after storage at 13 °C in 2022 (A), after storage at 3 °C in 2022 (B) and after storage at 3 °C in 2023 (C). Dots and bars represent observed cumulative proportions and confidence intervals from Kaplan–Meier analysis. Predicted lines and confidence bands were generated via beta regression. The solid line section represents temperature ranges in which we observed freezing events, while the dashed line section represents modeled projections exceeding our observed data range.

Comparison of Supercooling Points and Lower LTs

To assess the cold tolerance strategy of overwintering *R. maxima*, we compared the distributions of previously recorded supercooling points and lower LTs to each other. Three models were created to test the cold tolerance strategy of *R. maxima*, based on significant effects of storage temperature described in previous sections. For 2022, separate models were prepared for the 13 and 3 °C storage temperatures (pooled across storage times). For 2023, 1 fully pooled model was created across the storage times at 3 °C. In 2022, after storage at 13 °C, there was no significant effect of experiment (SCP or LT) on the intercept ($|Z| = 0.18$, $P = 0.857$) or slope ($|Z| = 0.92$, $P = 0.356$) of the model, indicating that freezing onset and mortality events started occurring at roughly the same temperature and had similar accumulation rates. In 2023, there was also not a significant effect of experiment on the intercept ($|Z| = 1.34$, $P = 0.181$) or slope ($|Z| = 0.82$, $P = 0.410$). However, in the 3 °C storage temperature treatment in 2022, the SCP data had a significantly lower intercept ($|Z| = 4.57$, $P < 0.00001$) and higher slope ($|Z| = 3.65$, $P = 0.0003$) than the LT data, indicating that under these conditions, freezing onset events happened at a colder temperature and accumulated more rapidly than mortality events.

Soil Temperature Data

To place our laboratory-collected data on supercooling and lower LTs in context, we collected and summarized historical monthly minimum soil temperatures at a 5-cm depth from 3 field stations (Fig. 2). Averaged across years, the coldest monthly temperature at the 5-cm depth was in February for all 3 sites. In Grand Forks, the average coldest monthly temperature was −7.5 °C, in Morris it was −8.5 °C, and in Lamberton it was −3.1 °C (Fig. 6). The coldest individual monthly temperatures within any year were −17.7 °C in Grand Forks (24 December 2020), −16.7 °C in Morris (13 January 2018), and −13.9 °C in Lamberton (24 February 2015).

Discussion

In this study, we produced cocooned third instars (ie the known overwintering stage; McMechan et al. 2021b) by collecting soybean stems infested with *R. maxima* at the end-of-season, dissecting out mature-looking orange larvae, and placing them in vials of moistened sand to study cold tolerance of the

Table 1. Parameters from final beta regression models describing the relationship between cumulative proportion of frozen *R. maxima* cocooned larvae and exposure temperature, as well as temperatures estimated to cause 50%, 90%, and 95% of the population to begin to freeze ($SCP_{50',\ 90',\ and\ 95}$) with 95% confidence intervals

Year/Treatment	Intercept (b_0) ± SE	Slope (b_1) ± SE	Φ-coefficient ± SE	SCP_{50} (°C) (95% CI)	SCP_{90} (°C) (95% CI)	SCP_{95} (°C) (95% CI)	Sample size
2022, 13 °C storage	−4.62 ± 0.46	0.19 ± 0.02	122.80 ± 70.53	−23.7 (−25.6 to −21.9)	−29.7 (−35.4 to −25.3)	−31.7 (−38.5 to −25.6)	35
2022, 3 °C storage	−22.42 ± 2.78	0.92 ± 0.12	53.34 ± 21.97	−24.3 (−24.9 to −23.7)	−26.2 (−27.4 to −25.2)	−26.7 (−28.3 to −25.4)	44
2023	−11.31 ± 1.56	0.49 ± 0.07	24.20 ± 6.54	−23.0 (−24.7 to −21.4)	−27.1 (−30.4 to −24.9)	−28.3 (−32.5 to −25.7)	57

Models for each year/treatment follow the general form: $\ln(\mu/1-\mu) = b_0 + b_1 \times |\text{temperature}|$ and $\text{var}(\mu) = \mu(1-\mu)/1+\varphi$.

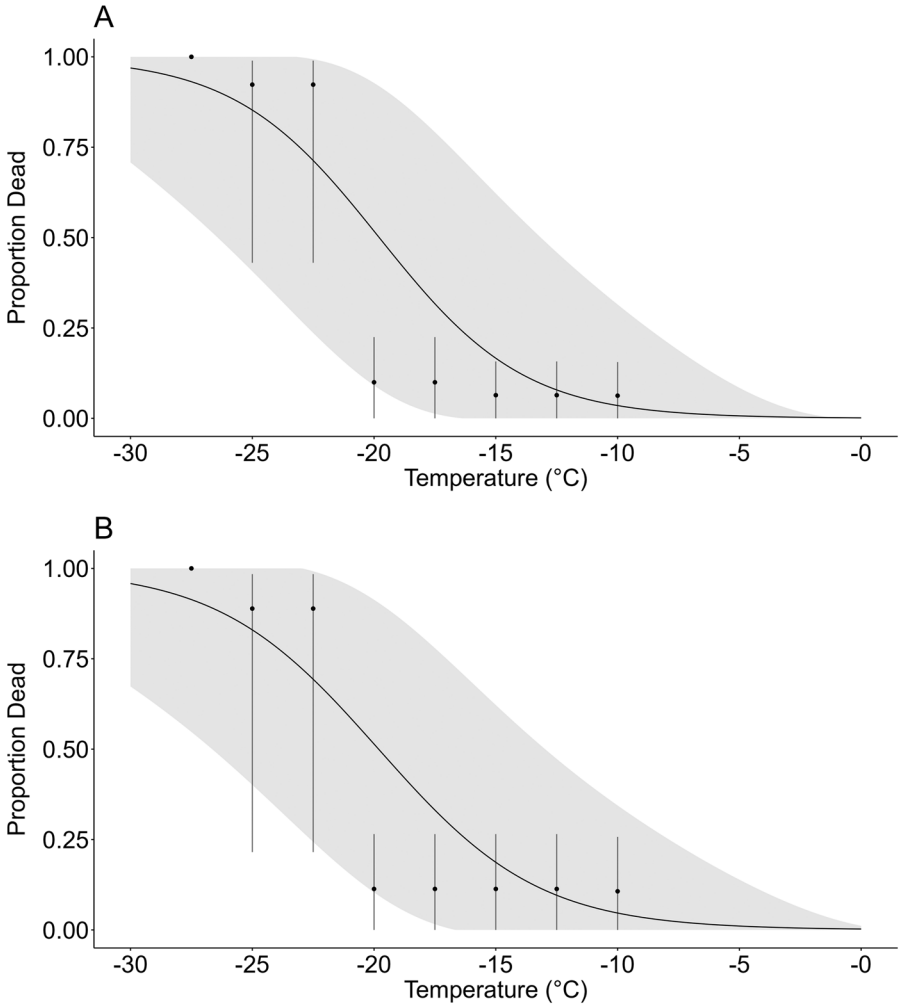

Fig. 5. Effect of brief (ie <3 s) subzero exposure temperatures on the cumulative proportion of dead *Resseliella maxima* cocooned larvae in 2022 (A) and 2023 (B). Dots and bars represent estimated cumulative proportions and confidence intervals from Kaplan–Meier analysis. Predicted lines and confidence bands were generated via beta regression.

Table 2. Parameters from final beta regression models describing the relationship between cumulative proportion of dead *R. maxima* cocooned larvae and exposure temperature, as well as estimated temperatures predicted to cause 50%, 90%, and 95% mortality (LT_{50}, LT_{90} and LT_{95}) with confidence intervals

Year	Intercept ± SE	Slope ± SE	Φ-coefficient ± SE	LT_{50} (°C) (95% CI)	LT_{90} (°C) (95% CI)	LT_{95} (°C) (95% CI)	Sample size
2022	−6.55 ± 2.15	0.33 ± 0.10	3.77 ± 2.49	−19.8 (−26.4 to −13.2)	−26.3 (−34.1 to −19.4)	−28.5 (−35.7 to −20.6)	198
2023	−5.78 ± 1.89	0.29 ± 0.09	4.56 ± 3.12	−19.8 (−26.6 to −13.0)	−27.0 (−35.8 to −19.7)	−29.4 (−37.6 to −20.9)	90

Models for each year/treatment follow the general form: $\ln(\mu/1-\mu) = b_0 + b_1 \times |\text{temperature}|$ and $\text{var}(\mu) = \mu(1-\mu)/1+\varphi$.

overwintering life stage. The success of this method mirrors successful methods used for other soil-overwintering cecidomyiids (Košťál and Havelka 2000, Des Marteaux et al. 2012) and shows promise for use in other experiments with *R. maxima* (eg Stillwell Jardine et al. 2026) and for designing laboratory rearing methods.

Several lines of evidence lead us to conclude that larvae of *R. maxima* in our trials were likely in diapause. Larvae were collected early in climatological autumn and would have experienced shortened photoperiods in the field. Other cecidomyiids with similar lifestyles are known to enter diapause, including *C. nasturtii* (Readshaw 1966, Des Marteaux et al. 2012), *A. aphidimyza* (Košťál and Havelka 2000), *Sitodiplosis mosellana* (Géhin) (Dufton et al. 2022), and congener *R. theobaldi* (Pitcher 1952, Stenseth 1973). No *R. maxima* in our study developed beyond the third instar, either following the initial 10 days of storage at 25 °C (conditions that would typically produce pupae in early to mid-summer collections; Koch Laboratory unpublished]) or following 2 months of storage at 13 °C. While developmental thresholds for *R. maxima* have not been studied, *C. nasturtii* (Liu et al. 2019), *S. mosellana* (Dufton et al. 2022), and *R. theobaldi* (Gordon et al. 1989) all have developmental thresholds below 13 °C. Cessation of development when conditions are otherwise suitable is a characteristic indicator of diapause

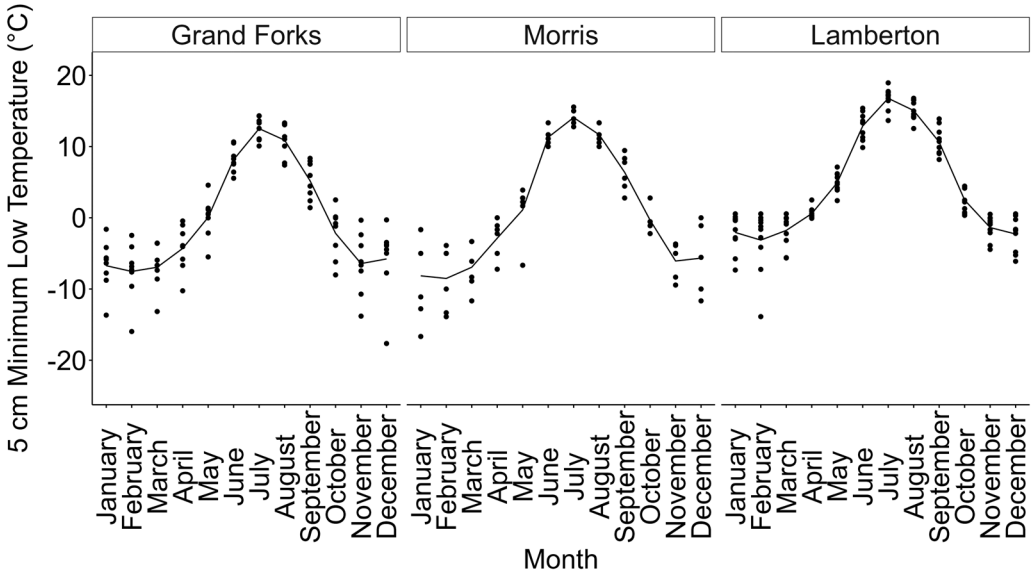

Fig. 6. Soil temperature data sourced from (north to south): Grand Forks, South Dakota; Morris, Minnesota; and Lamberton, Minnesota. The Grand Forks data ranges from 2015 to 2022, the Morris data from 2015 to 2021, and the Lamberton data from 2015 to 2024. Each dot represents the coldest temperature observed at a 5-cm depth from a different year for the given month on the *x*-axis. The solid black line represents the overall average coldest temperature across years by month. See Fig. 2 for location details.

(Koštál 2006) and remaining in the third instar for periods exceeding typical development times has been used to identify diapausing individuals in other cecidomyiids (Readshaw 1966, Des Marteaux et al. 2012, Cheng et al. 2017). Our study is the first to provide evidence indicative of diapause in *R. maxima*, and future studies to identify factors that induce or break the arrested state of development will clarify the existence of diapause and whether it is facultative or obligatory.

Our study is also the first that we are aware of to characterize the cold tolerance of a *Resseliella* species and it further contributes to the broader knowledge on cecidomyiid cold tolerance. There is currently scarce literature comprehensively documenting and comparing cecidomyiid cold tolerance, in particular SCP and LT values, with no examples within *Resseliella*. However, *Resseliella citrifrugis* Jiang (Diptera: Cecidomyiidae), a pest of citrus fruits in China, has been studied in the context of fruit sanitation for trade purposes (Xia et al. 2021). It was found that exposure to 2 °C for 12 days was fatal to mature larvae within the fruits, but the overwintering stage was not used, and this was ultimately a measure of lethal time rather than the SCP or LT (Chen 2010, Xia et al. 2021). Our results are important because of the pest status of *R. maxima* and its potential for continued spread in northern climates. The LT_{50} in particular has been shown in an analysis of several *Drosophila* spp. to be one of the best cold tolerance measurements for predicting geographic distribution (Andersen et al. 2015).

We observed a transition to high rates of both freezing onset and mortality around −20 °C, and by −30 °C, all individuals had started to freeze or had died. This trend and our measurements of cold tolerance in general did not meaningfully vary based on storage time or temperature. It further suggests that our treatments did not prompt further acclimation beyond the general diapause response, or at least that exposure to 13 °C was sufficient. One exception lies in the statistical differences found in the SCP data in 2022, between the populations stored at 13 °C and those stored at 3 °C. These results typically indicate an overall colder distribution of SCPs in the 3 °C population. However, this seems to be driven by a comparative lack of freezing onset events at warmer temperatures (the −5 to −20 °C range) in the population held at 3 °C, and we did not observe notably colder SCPs at the coldest temperatures (−25 °C onward) compared to other datasets. There may be capacity for adaptation to cold conditions beyond this diapause response, perhaps with storage at colder temperatures, for longer periods of time, or when looking at different metrics of cold tolerance, but no such adaptation was seen in our study.

Some more distantly related cecidomyiids such as the willow-gall-forming *Rhabdophaga strobiloides* Osten Sacken and *Mayetiola rigidae* Osten Sacken had far lower SCPs than we observed (−56.1 to −57.8 °C) (Miller and Werner 1987). However, these seem to be exceptional cases, given they are both from an extremely cold climate (ie interior Alaska) and overwinter within their galls, where they would be more directly exposed to air temperatures without insulation provided by the soil (Miller and Werner 1987). Therefore, it is best to compare *R. maxima* to other species which utilize a similar overwintering microhabitat (ie overwintering in the soil in a cocoon) like *A. aphidimyza* (Košťál and Havelka 2000). Mean SCPs of diapausing *A. aphidimyza* ranged from −22.2 to −23.2 °C, without any differences following storage for 0, 20, or 60 days at 3 °C (Košťál and Havelka 2000). These mean SCPs and the lack of acclimation due to storage time align closely to our observations for *R. maxima*.

We also sought to analyze the cold tolerance strategy of *R. maxima* using the approach of comparing the SCP and LT distributions. Our data showed similar distributions of freezing onset and mortality events by temperature and broad overlap between important measures like SCP_{50} and LT_{50}. One exception to these results (statistical differences between LT and SCP data for larvae stored at 3 °C in 2022) appears to result from the same lack of warmer-temperature freezing events in the latter dataset as previously mentioned when discussing acclimation. In both cases, practical differences were small. Due to the otherwise consistent overlap between these measures (ie

the SCP is equal to the LT), our data suggest that overwintering *R. maxima* larvae are likely to be chill-tolerant/freeze-intolerant (freeze avoidant), a cold tolerance strategy in which insects delay the onset of freezing through the suppression of supercooling points and die upon their eventual freezing (reviewed in Sinclair et al. 2015). Comparing the SCP and LT distributions has been previously used to determine the cold tolerance strategy of other insects (eg Slabber and Chown 2004, Bemani et al. 2012, Hanson et al. 2013, Stephens et al. 2015, Cira et al. 2016, Hefty et al. 2017, Nystrom Santacruz et al. 2017, Rosenberger et al. 2017) but this approach has been criticized (Sinclair et al. 2015).

More complex cold tolerance strategies are possible. Košťál and Havelka (2000) evaluated the cold tolerance strategy for *A. aphidimyza* and found it to be freeze intolerant. However, they found that if ice formation was externally inoculated, around one-third of *A. aphidimyza* cocoons could be penetrated by ice crystals and freeze at temperatures above their SCP, and that these individuals survived being held in a frozen state for up to 10 days (Košťál and Havelka 2000). *A. aphidimyza* therefore appears to conditionally tolerate freezing that occurs at warmer temperatures than its SCP. A similar response has been found in several other insect species and is generally considered to be common in soil-overwintering insects which may be in close contact with moisture (Košťál and Havelka 2000, Lee 2010, Sinclair et al. 2015).

Our 3 soil temperature data sites range from southwest Minnesota to northeast North Dakota (from approximately latitude 44°N to 48°N) (Fig. 2). This range covers and extends beyond the northern edge of *R. maxima*'s known range, which as of 2025 was ~46°N (Soybean Gall Midge Alert Network 2025) (Fig. 2). Monthly coldest temperatures at the 5-cm depth across this range of latitudes rarely approach temperatures we have shown to significantly impact *R. maxima* (−20 °C and colder). We would expect few *R. maxima* to be deeper than 5 cm, though many will be at shallower depths where they may be somewhat less buffered against extreme air temperatures (Gordon et al. 1989, da Silva Lima 2023). Soybean is also grown farther north where *R. maxima* has not yet been detected, extending into central Manitoba, Canada to ~51.7°N (USDA-FAS 2025). Nonetheless, even the absolute coldest temperature recorded in any year (−17.7 °C in Grand Forks on 24 December 2020) does not reach either our SCP_{50} (−23.0 to −24.7 °C) or LT_{50} (−19.8 °C in both years) estimates, with 88% of *R. maxima* tested having colder SCPs. On average, we would not expect a 50% mortality event to occur due to brief, extreme cold exposure in even the coldest winter month at any site.

Research comparing cold tolerance to soil temperatures for another soil-overwintering pest in the midwestern United States, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) showed similar results to ours (Morey et al. 2012). SCP and LT values were notably colder than the coldest soil temperatures, even in northern areas where field studies have established *H. zea* cannot overwinter (Morey et al. 2012). The authors concluded that longer term exposure to temperatures warmer than the typical SCP or LT drives mortality, meaning the lethal time is a better predictor of geographic distribution for that species.

Our measures of the cold tolerance of overwintering *R. maxima* larvae suggest that this insect has the capacity to survive brief (ie <3 s) exposure to common winter soil temperatures in the midwestern United States. However, our metrics do not account for the potential of inoculative freezing or prolonged cold exposure to cause mortality. The possibility of different cold tolerance responses based on inoculative freezing could be investigated using the manipulation of moisture or addition of external ice inoculators, as per the example of *A. aphidoletes* (Košťál and Havelka 2000). Exploring relatively warmer temperatures more thoroughly may also shed light on the variability in warmer temperature freezing events exemplified by the SCPs of our 2022 population stored at 3 °C and reflect a more realistic exposure pattern based on soil temperature data. The cooling rate used in our study (1 °C/min), while commonly used in insect cold tolerance research, is also relatively rapid compared to cooling rates that would be experienced in the field, and may affect the ability to relate our measurements of cold tolerance to field conditions. Future research could explore these nuances and better understand the cold tolerance strategy of *R. maxima* by assessing slower, more ecologically relevant cooling rates and by recording mortality following longer durations spent at relatively warmer cold temperatures (ie more chronic exposure at >−10 °C).

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Author Contributions

Pheylan A. Anderson (Conceptualization [equal], Data curation [equal], Formal analysis [equal], Investigation [equal], Methodology [equal], Software [equal], Visualization [equal] Writing—original draft [equal], Writing—review & editing [equal]), Robert C. Venette (Conceptualization [equal], Formal analysis [supporting], Project administration [supporting], Resources [equal], Supervision [supporting], Validation [equal], Writing—review & editing [equal]), Bruce D. Potter (Investigation [supporting], Resources [supporting], Writing—review & editing [equal]), and Robert L. Koch (Conceptualization [equal], Funding acquisition [equal], Methodology [equal], Project administration [equal], Resources [equal], Supervision [equal], Validation [equal], Writing—review & editing [equal])

Supplementary Material

Supplementary material is available at *Environmental Entomology* online.

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Conflicts of Interest

None declared.

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