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Effects of microhabitats and seasonal conditions on the supercooling points of *Tetrastichus planipennisi* (Hymenoptera: Eulophidae) in Vermont, USA

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Abstract Arthropod natural enemies such as *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae) face novel environments when introduced to non-native ranges for classical biocontrol. Their ability to adapt to novel thermal regimes may determine their success in establishment. *T. planipennisi* was introduced to the USA in 2007 for classical biological control of the invasive emerald ash borer *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). Previous studies found that *T. planipennisi* is chill-susceptible and its establishment is affected by winter mortality at higher latitudes. We conducted a field experiment during winter of 2021–2022 in Jericho Research Forest, Vermont, USA, to determine if the temperature at which spontaneous freezing occurs, known as the supercooling point (SCP), of *T. planipennisi* larvae changes in response to microhabitat manipulations of ambient temperature, and we measured eclosion. Larvae were placed under control conditions or artificial shelters that reduced temperature extremes. Larvae in control groups had significantly lower SCP values in January, February, and March compared to those in artificial shelters, with mean SCP values in control-group larvae 2.52 °C lower in January, 1.70 °C lower in February, 1.98 °C lower in March, and higher eclosion success (median 18% in controls). SCP values were negatively correlated with monthly cooling rate and higher in shelter treatments, and degree-hours ≤ -2 °C best predicted eclosion. These findings demonstrate significant inter-individual variation in SCP in response to shifts in local habitat temperatures. Microclimatic variation may play a role in determining insect SCP capacity and emergence, particularly in northern regions with extreme winter conditions.

Keywords Biocontrol · Supercooling Point · *Tetrastichus planipennisi* · Overwintering · Microhabitat

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Introduction

The emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is an invasive wood-boring beetle native to Northeast Asia where it is a pest of ash trees (*Fraxinus* spp.), killing susceptible host trees by feeding on their phloem, cambium, and shallow sapwood (Wei et al. 2007). Due to

a lack of natural enemies and an abundance of available host trees, EAB has rapidly spread and caused severe damage to native ash tree species in North America, following its accidental introduction to southern Michigan, USA in the 1990s (Herms and McCullough 2014; Siegert et al. 2014). Researchers identified parasitoid wasps as potential biocontrol agents against EAB, such as *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae), a larval endoparasitoid. This parasitoid, a specialist that targets late-instar EAB larvae in its native China, was approved for US environmental release in 2007 (Federal Register 2007; Bauer et al. 2015).

Field studies have documented the successful establishment of *T. planipennisi* at many release sites in northern areas of the USA and Canada. A key uncertainty for long-term efficacy is whether it can withstand severe winters in the northern regions of the USA and Canada. Releases now span broad climatic gradients, including regions that experience severe cold outbreaks such as Ontario, New Brunswick, and Quebec, Canada (Butler et al. 2022). Duan et al. (2020) compared *T. planipennisi* deployments in Michigan to Connecticut, USA and reported significant increases in parasitoid mortality following extreme temperature events and substantial among-site variation, suggesting that temperature exposures and environmental factors such as forest type and topographic features can influence overwintering mortality rates across sites.

Overwintering ectotherms experience not only low mean temperatures, but also short-term variability driven by precipitation, wind, forest cover, and solar exposure, which can affect the temperature of the local microclimate. Forest cover can act as a windbreak and dampen temperature fluctuations, reducing solar radiation and nocturnal cooling within sheltered "quiet zones" (Klein et al. 2021). Vermunt et al. (2012) found that the under-bark microclimate in Ontario ash trees differs notably from winter air temperatures, likely due to solar loading. These fine-scale thermal variations could influence the overwintering of *T. planipennisi,* which resides beneath the bark and experiences these specific thermal conditions.

Insects use three main cold tolerance strategies: (1) freeze-tolerant species can survive internal ice formation, (2) freeze-avoidant species stay supercooled to prevent internal ice formation and freezing, and (3) chill-susceptible species suffer lethal non-freezing injuries at low temperatures above their supercooling point (Sinclair et al. 2015). The temperature at which spontaneous freezing occurs is known as the supercooling point (SCP), marking the onset of crystallization and the transition from liquid to solid. Non-diapausing insects enhance supercooling by removing internal ice nucleators (e.g., gut evacuation), synthesizing cryoprotectants, and the production of anti-freeze proteins. These adaptations contribute to lowering the SCP and reducing the risk of ice formation (Bale 1987). Seasonal changes in SCP have been noted in some insects, as freeze-avoidant species enhance supercooling capacities in preparation for winter, decreasing the chance for lethal freezing of body fluids. Multiple studies have shown that seasonal and environmental temperature variations leading up to the initial freezing temperatures can affect SCP adjustments (Irwin and Lee Jr. 2000; Denlinger and Lee Jr. 2010; Chandler et al. 2020).

Hanson et al. (2013) documented significant overwintering mortality of *T. planipennisi* larvae subjected to prolonged low-temperature exposure, highlighting their sensitivity to sustained thermal stress and suggesting that *T. planipennisi* is a chill-susceptible species. However, the extent to which small-scale temporal and spatial variations in climate can affect SCP capacity is not well understood, leading to a need for further research into the extent to which SCP variation occurs in this species (Duan et al. 2020). Understanding the effect of microclimatic conditions on cold tolerance of *T. planipennisi* could provide insights into its thermal limits and predictions of its overwintering mortality and help develop protocols to optimize deployment of biocontrol agents in regions with extreme winter weather conditions.

We conducted a field experiment measuring the supercooling points of overwintering *T. planipennisi* to determine the effects of microclimate conditions on the cold tolerance of *T. planipennisi* in the New England region, USA. We enclosed *T. planipennisi* larvae in U-Line plastic jars that were attached to ash trees in Jericho Research Forest, Vermont, from November 2021 to March 2022. We hypothesized that shelters would buffer jar microclimates by reducing exposure to wind, precipitation, and light, reducing temperature variance and altering cooling dynamics experienced by *T. planipennisi*. We predicted that sheltered jars would experience fewer extreme minimum temperatures but also reduced daytime warming, potentially

increasing the duration of exposure to sub-zero conditions. Finally, we quantified spring eclosion to evaluate whether microhabitat conditions could affect emergence. We predicted individuals in shelters to exhibit higher SCP values compared with their counterparts exposed to natural temperature fluctuations in the no-shelter control group and that prolonged exposure to low temperatures driven by local microclimate conditions in those shelters would reduce winter survival and eclosion success of *T. planipennisi* in the treatment group relative to controls.

Materials and methods

Parasitoid rearing and experimental design

All *T. planipennisi* larvae used for the study were reared on late instars of EAB larvae infesting green ash bolts (~5 cm in diameter × 15 cm long) according to methods described in Duan et al. (2019). Each ash bolt was infested with 7–10 fertilized EAB eggs, then incubated for approximately four to five weeks in an environmental chamber (AR-66L2, Percival Scientific, Perry, IA, USA) at 25 ± 1.5 °C, 65 ± 10% RH, and a L:D 16:8 photoperiod. EAB larvae inside the infested ash bolts that had reached late (3rd–4th) instars were exposed to gravid females of *T. planipennisi* for one–two weeks. After exposure to adult parasitoids, the green ash bolts were shipped overnight to Burlington, Vermont, USA, and placed in jars to be deployed in the field the following day.

This study was conducted in the Jericho Research Forest in Jericho, Vermont, USA, from November 2021 to March 2022 (Supplementary Figure S1, GPS coordinates in Supplementary Table S1). Jars were dispersed throughout three plots identified as dominant/codominant green or white ash tree stands that had similar environmental conditions including slope, elevation, and tree density, which served as blocking factors for this experiment. To account for variation between blocks, multiple replicates of treatment groups were present in every block, allowing for comparisons between and within blocks. Within each block, six ash trees were identified within 20 m of the center and randomly marked as either a control or treatment. Ash bolts were pooled by treatment group within jars prior to analysis due to destructive sampling constraints. As a result, tree-level variation could not be assessed, and site-level replication (n = 3 per treatment) was used to evaluate treatment effects. This experiment used a nested randomized block design and sampling without replacement over the course of the fall/winter season for both the control group and treatment group.

At each site, one-gallon U-Line jars that held green ash bolts containing *T. planipennisi* larvae were attached to the south-facing side of each tree at 1.5 m above ground level. Each jar housed a minimum stock of six ash bolts at the start of the experiment in November. Each ash bolt had on average five parasitized EAB larvae, each containing five to eight *T. planipennisi* larvae on average. Control group jars were left uncovered, whereas sheltered treatment jars were covered on three sides by vinyl paneling surrounding each jar to provide shelter from precipitation, wind, and solar exposure and daytime warming. All jars were attached to ash trees using steel wire, and each jar had an attached iButton data logger for monitoring the temperature profiles within the jars every 30 min for the entire season. Every month starting in November, a bolt from each jar at each site was collected and dissected for *T. planipennisi* larvae and SCP was measured as described below. Seasonal eclosion success was measured by using ash bolts with parasitized EAB larvae, kept during the field experiment and collected in May. Free-flying *T. planipennisi* were noted, and bolts were dissected to identify non-eclosed individuals and their developmental stages.

Sampling techniques: ash bolt dissection and supercooling point assays

Each month, one ash bolt per jar was collected and held at 4 °C until dissection. *T. planipennisi* larvae were removed and placed into size four gelatin capsules (Capsuline, Dania Beach, Florida, USA). Larvae were visually inspected and only healthy individuals were assayed (Liu et al. 2007). An average of eight healthy *T. planipennisi* larvae were sampled per month from each jar. The total count was 129 larvae from control jars across blocks (49 in block 1, 39 in block 2, 41 in block 3) and 143 individuals from treatment jars (51 in block 1, 41 in block 2, 51 in block 3). For emergence assessments, larvae from eclosion bolts were extracted similarly and scored as eclosed

adults, non-eclosed adults, or non-eclosed individuals at earlier developmental stages.

To measure the supercooling points in *T. planipennisi,* we adapted methodology described in Hanson and Venette (2013). Because larvae are fragile, individuals were assayed in gelatin capsules (placed in open 1.5 ml tubes to stabilize them) and a Type-K thermocouple was attached to each capsule. Body temperature was recorded using a HUATO S220-T8 Datalogger (HUATO ELECTRIC, Shenzhen, China) and recorded every 2 s while being cooled from 4 °C to − 30 °C at a cooling rate of 0.5 °C per minute for each run in a PolyScience circulating water bath in Polycool HC-50 coolant. SCP was defined as the temperature at the onset of the exotherm (a sudden temperature spike) indicating spontaneous freezing (Wu et al. 2007; Hanson et al. 2013).

Data analysis

All statistical analyses were done in R, version 4.4.0 (R Core Team 2024). As ash bolts from each replicate jar are destructively sampled every month and replicate jars for both treatment and control groups are spatially nested within each block, this study has a nested randomized block design that has sampling without replacement. SCP values collected from each sample of ash bolt are treated as correlated with each other due to them coming from the same colony and maintaining the same rearing history prior to deployment for this experiment.

Quantile–quantile plots were visually inspected to assess normality of the SCP variable. Bartlett's test was used to determine the homogeneity of variances of the data. To determine whether SCP values differed between control and treatment jars, we used a linear mixed-effects model (LMM) with treatment group and month as fixed effects and block (site) as a random intercept to account for hierarchical structure and spatial variation. Interaction effects between treatment and month were included.

Temperature data from iButton loggers were summarized as minimum, mean, and maximum temperatures at daily, weekly, and monthly intervals (monthly summary statistics in Supplementary Table S9). To assess whether the observed differences in environmental temperature between treatment groups occurred by chance, we employed a randomization test using the pracma package in R (Borchers 2023). Treatment labels were permuted among jars within blocks and the resulting treatment difference over time was quantified by the area under the curve (AUC) of the treatment–control difference (computed using the trapezoidal rule). The p-value was calculated as the proportion of permuted AUC values more extreme than the observed value (10,000 permutations; one-tailed).

We evaluated whether SCP covaried with thermal conditions using LMMs with SCP as the response and candidate predictors derived from logger data. This step was guided by findings from Chandler et al. (2020), which highlighted that SCP values were most closely correlated to ambient temperatures during the two weeks leading up to collection. Month and treatment were also considered as fixed effects, with block as a random intercept. We assessed multicollinearity among all candidate predictors using generalized variance inflation factors (GVIF). Variables with $GVIF^{\frac{1}{2df}} > 4$ were considered collinear and excluded from model selection. The month variable showed high collinearity with temperature measures (minimum, mean, maximum; GVIF > 10), so each was tested in separate models. Interaction effects were considered, and candidate models were fit using maximum likelihood and compared using Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC). For each linear mixed-effects model, β estimates representing the slope of each fixed effect and SE were reported. Fixed effects were evaluated using Type III ANOVA F-tests with denominator degrees of freedom approximated using Satterthwaite's method, and coefficient-level t-values (β/SE) were provided as well. Models were fit using the lme4 package, and inferential tests (t-tests, F-tests, and p-values) were obtained using the lmerTest package (Bates et al. 2015; Kuznetsova et al. 2017).

We then expanded our focus towards quantifying the thermal stress experienced by *T. planipennisi* throughout the field season by calculating the rate of cooling that occurs during each daily thermoperiodic cycle. We calculated this using the difference between the maximum temperature collected from each jar's iButton datalogger on a given afternoon and the minimum temperature collected during the early morning of the next day, divided by the time it took to reach that lowest temperature point. We summarized cooling rate at the monthly scale and evaluated

its relationship with SCP using LMMs that included monthly cooling rate together with treatment, month, and other temperature metrics as fixed effects and block as a random intercept. Model selection followed the same AIC/BIC workflow as above. Likelihood ratio tests were used to compare nested model pairs as a secondary check. We used the performance package to gather performance metrics for model selection and comparison (Lüdecke et al. 2021).

To test for a treatment effect on eclosion success, we used Generalized Linear Mixed Models (GLMMs) with Binomial distributions and a logit link function. Treatment was a fixed effect, and block was included as a random intercept. We compared this to a null (random intercept only) model using AIC and BIC. Treatment effects were assessed using pairwise contrasts of estimated marginal means and reported as odds ratios with 95% Wald confidence intervals and Wald z-tests, obtained by back-transforming logit-scale contrasts using the emmeans package (Lenth et al. 2025). Models were fit using glmmTMB (Brooks et al. 2017). We also conducted a permutation test to evaluate whether the effect of treatment improved model fit beyond chance. Treatment labels for each jar per block were randomly assigned and the likelihood ratio statistic (null deviance – residual deviance) was recalculated 5,000 times. The p-value was calculated as the proportion of permuted statistics greater than or equal to the observed test statistic.

To evaluate whether cumulative cold exposure predicted eclosion success, we calculated degree-hours at or below candidate threshold temperatures as proxies for chill injury. For each threshold, degree-hours were computed as the temperature deficit summed across hours when temperatures were at or below the threshold. We evaluated degree-hour thresholds of $0, -2$, and -5 °C, and additionally tested January degree-hours at or below the median SCP. We fit binomial GLMMs including each degree-hour metric (and treatment where applicable), screened predictors for multicollinearity prior to model selection, and compared candidate models to a null model using AIC and BIC. Coefficients were interpreted on the logit scale. Effects are reported as odds ratios, including degree-hour effects within the reference treatment level and treatment effects evaluated at the mean degree-hour.

Results

Effect of artificial shelters and microclimate on SCP

Supercooling point (SCP) values of *Tetrastichus planipennisi* were collected from November 2021 to March 2022 in three blocks under treatment and control conditions (Fig. 1). In an LMM with block as a random intercept, SCP differed by treatment and month (Treatment: $F_{1,228.69} = 56.47$, $p < 0.001$; Month: $F_{4,229.11} = 25.50$, $p < 0.001$). Sheltered treatment jars had higher SCP values than controls ($\beta = 1.45 \pm 0.19$, Supplementary Table S2) and SCP values became more negative over winter (Supplementary Table S2).

Randomization tests indicate that control jars experienced higher monthly maxima and lower minima than sheltered jars (Minimum Temp AUC $= -32.92$, $p < 0.001$, Maximum Temp AUC $= 466.96$, $p < 0.001$; Supplementary Table S10). In LMMs using two-week pre-collection temperatures, SCP increased with two-week minimum temperature ($\beta = 0.13 \pm 0.02$) and decreased with monthly temperature range ($\beta = -0.12 \pm 0.02$), with a significant treatment and minimum temperature interaction indicating a reduced minimum-temperature effect in sheltered jars (interaction $\beta = -0.11 \pm 0.03$; Supplementary Tables S3–S4). Type III ANOVA F-tests indicated significant effects of two-week minimum temperatures ($F_{1,233} = 27.95$, $p < 0.001$), monthly range ($F_{1,235.5} = 59.81$, $p < 0.001$), and the interaction between treatment and two-week minimum temperature ($F_{1,233.52} = 15.57$, $p < 0.001$).

Effect of cooling rate on SCP

Mean monthly cooling rate was included as an additional predictor of SCP (Fig. 2). In the best-supported LMM (Table 1; Supplementary Table S5), SCP decreased with increasing cooling rate ($\beta = -3.95 \pm 0.97$, $F_{1,229} = 16.61$, $p < 0.001$). It also had a significant positive effect of two-week minimum temperatures ($F_{1,228} = 35.82$, $p < 0.001$, Table 1), as well as the interaction effect of treatment and two-week minimum temperatures ($F_{1,228} = 20.57$, $p < 0.001$). Nested model comparisons supported retaining the treatment and minimum temperature interaction ($\chi^2 = 15.08$, $df = 1$, $p < 0.001$; Supplementary Table S7).

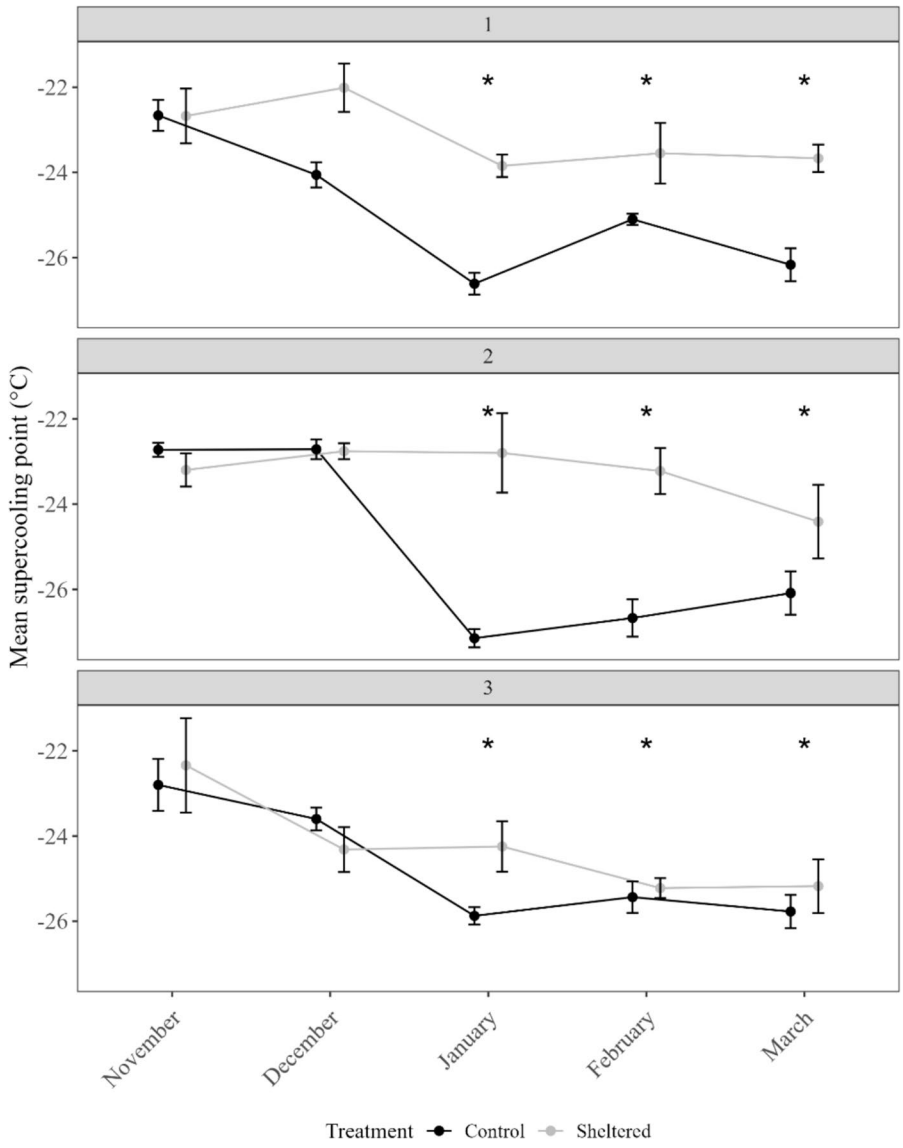

Fig. 1 Mean Supercooling Point values for control *vs*. treatment groups for three blocks (1–3) from November to March 2021–2022. Mean SCP values for each treatment group are represented as points with SE) bars. Linear mixed model results indicated a significant fixed effect of Treatment on SCP ($\beta = 1.45$, $t = 7.52$, $df = 237$, $p < 0.05$). Asterisks indicate coefficient-level significance for month indicators relative to December. Significant fixed effects of January ($\beta = -1.92$, $t = -6.65$, $df = 237$, $p < 0.05$), February ($\beta = -1.76$, $t = -5.79$, $df = 237$, $p < 0.001$), and March ($\beta = -1.77$, $t = -5.91$, $df = 237$, $p < 0.05$) are indicated on SCP. Further details are listed in Supplementary Table S2

Effect of treatments and chilling injury on eclosion success

The proportion of eclosion success of *T. planipennisi* from experimental jars varied across treatments (permutation test (Δ deviance) = 445.51, $p < 0.001$). Across each block, control group jars had higher proportions of eclosion success relative to treatment jars, with median eclosion success between 12–21% for control groups, and approximately 2% for treatment groups. Holm-adjusted pairwise contrasts of estimated marginal means of a binomial GLMM with treatment as a fixed effect indicate a significant effect of treatment (Fig. 3, Odds Ratio (OR) = 0.16 [95% CI: 0.12–0.21], $z = -13.49$, $p < 0.001$).

Due to the significant difference in eclosion success between treatments and *T. planipennisi's* status as a chill-susceptible species, we suspected that chilling injury and exposure to non-freezing low temperatures could be a driver of this effect. Model selection metrics found the best-fitting GLMM model to have degree-hours ≤ -2 °C as a significant fixed effect (Table 2, AIC = 303.35, ΔAIC = 2.482 to the next best model). The inclusion of degree-hours at or below median SCP in January did not improve model fit. In control jars, degree-hours ≤ -2 °C had a

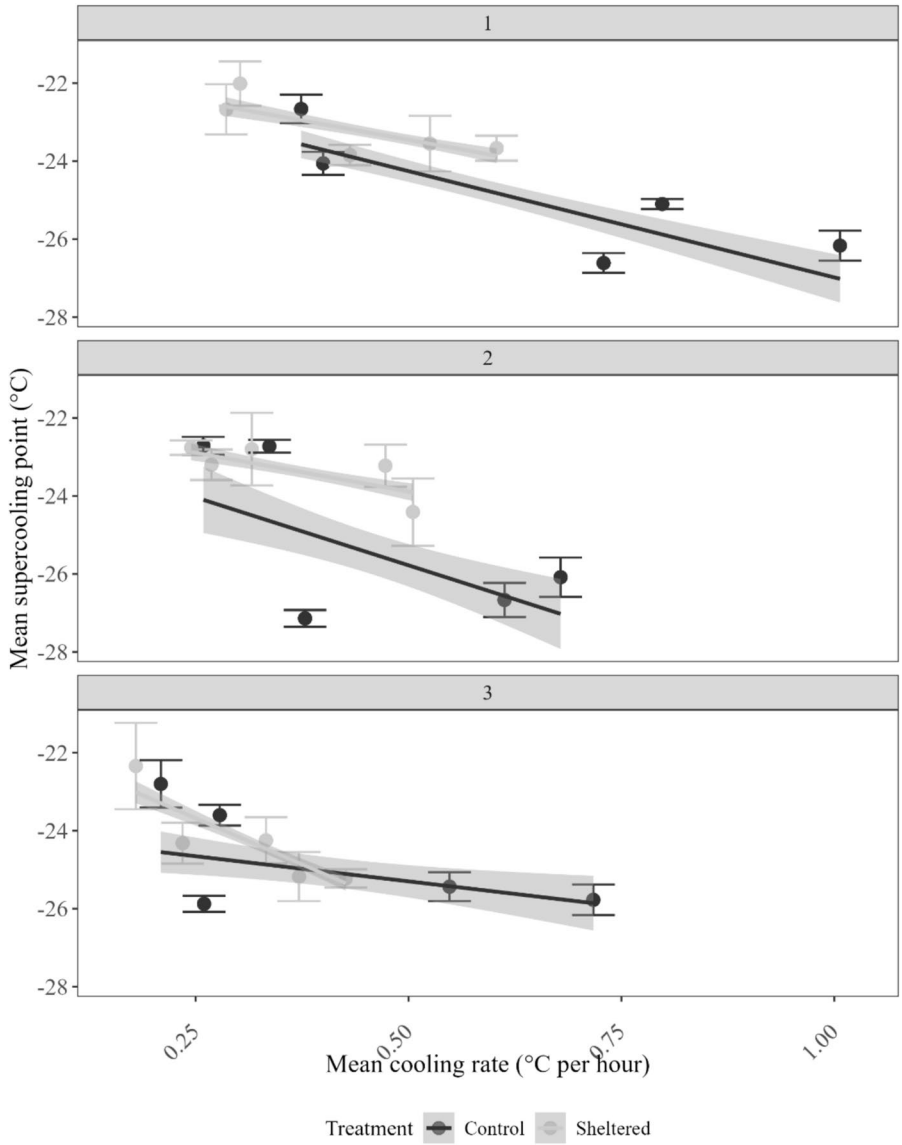

Fig. 2 Scatterplot of Mean Supercooling Point versus Mean Cooling Rate for treatment and control groups across blocks (1–3) from November to March 2021 – 2022. Error bars depict SE, and shadowed areas represent 95% confidence intervals of regression lines using linear mixed models. The best fitting linear mixed model indicated a significant fixed effect of monthly cooling rate (β = -3.95, t = -4.08, *df* = 235, $p < 0.001$), two-week minimum temperature (β = 0.14, t = 7.15, *df* = 233, $p < 0.001$), and an interaction effect between treatment groups and two-week minimum temperature on SCP (β = -0.12, t = -4.58, *df* = 233, $p < 0.001$). Further details are listed in Supplementary Table S6

Table 1 Linear mixed model estimates for final best-fit model of Supercooling Point *vs*. Monthly Cooling Rate and other environmental predictors. Degrees of freedom were estimated using the Satterthwaite approximation

Term	*Estimate*	*SE*	*t-value*	*df*	*p-value*
(Intercept)	− 20.51	0.62	–33.17	26.04	< 0.001
TreatmentSheltered	–0.34	0.32	–1.08	233.21	0.28
TwoWeekMinTemp	0.14	0.02	7.15	233.01	< 0.001
MonthlyRange	–0.04	0.02	–1.65	233.47	0.11
MonthlyCoolingRate	–3.95	0.97	–4.08	235.31	< 0.001
TreatmentSheltered × TwoWeekMinTemp	− 0.12	0.03	–4.58	233.30	< 0.001

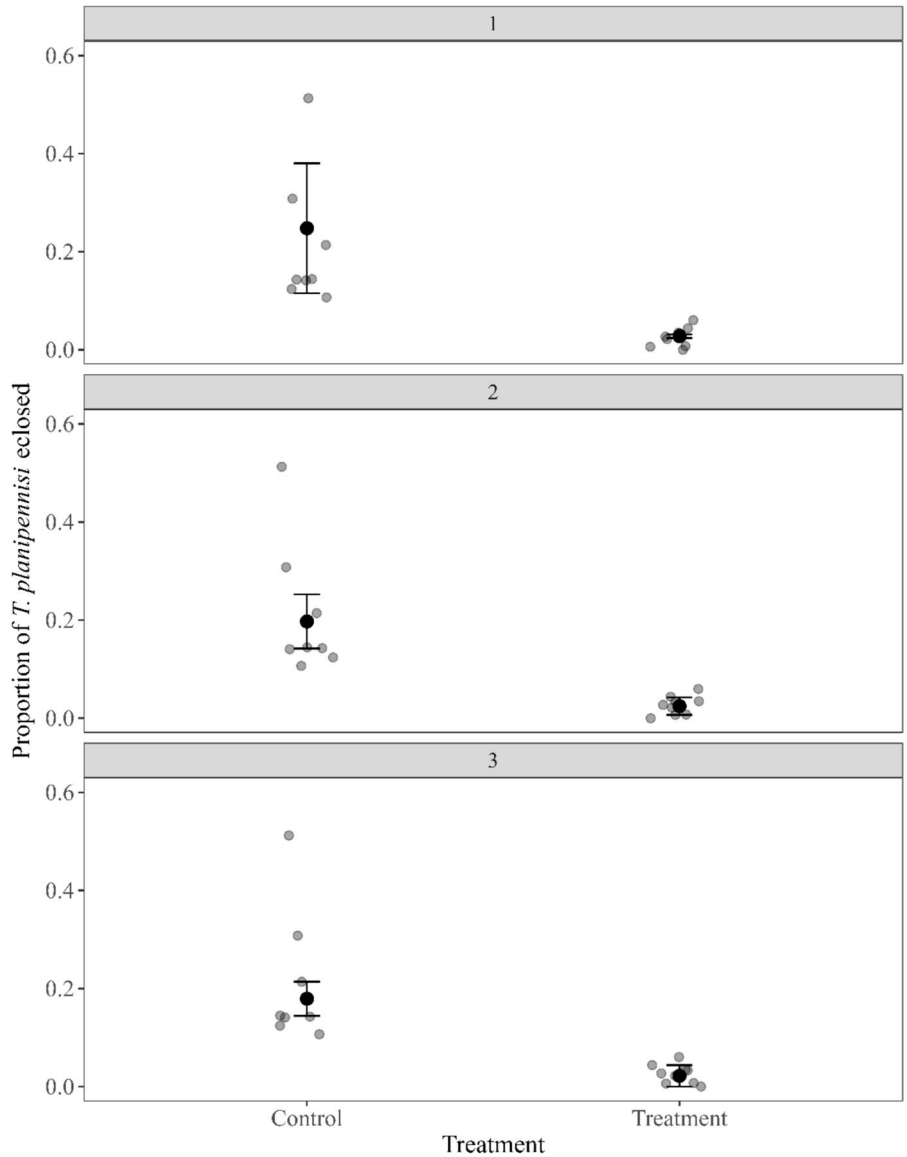

Fig. 3 Observed proportion of eclosed *T. planipennisi* for treatment and control groups across blocks (1–3) from November to March 2021–2022. Light-grey points represent individual jar-level proportions (jittered for visibility), and large points indicate mean proportion for each treatment with accompanying SE bars. Pairwise contrasts of an additive binomial GLMM with treatment as a fixed effect indicate significant differences between treatment groups (Odds Ratio (OR) = 0.16 [95% CI: 0.12–0.21], z = − 13.49, $p < 0.001$) Significance ($p < 0.05$) is based on Holm-adjusted pairwise contrasts of estimated marginal means

Table 2 Model selection metrics comparing the inclusion of January cold stress and treatment as predictors on eclosion using AIC, BIC, and Log-likelihood. Model parameters and details provided in Supplementary Table S9

Model	*Predictors*	*df*	*Log-likelihood*	*AIC*	*BIC*	*ΔAIC*
1	Degree-hours ≤ − 2 °C × treatment	10	–146.67	303.35	306.89	0.00
2	Degree-hours ≤ − 5 °C × treatment + Jan cold stress	9	–146.91	305.83	310.08	2.48
3	Degree-hours ≤ 0 °C × treatment	10	–148.01	306.03	309.57	2.68
4	Degree-hours ≤ − 5 °C + treatment	11	–149.40	306.80	309.64	3.46
5	Treatment	12	–150.48	306.96	309.08	3.61

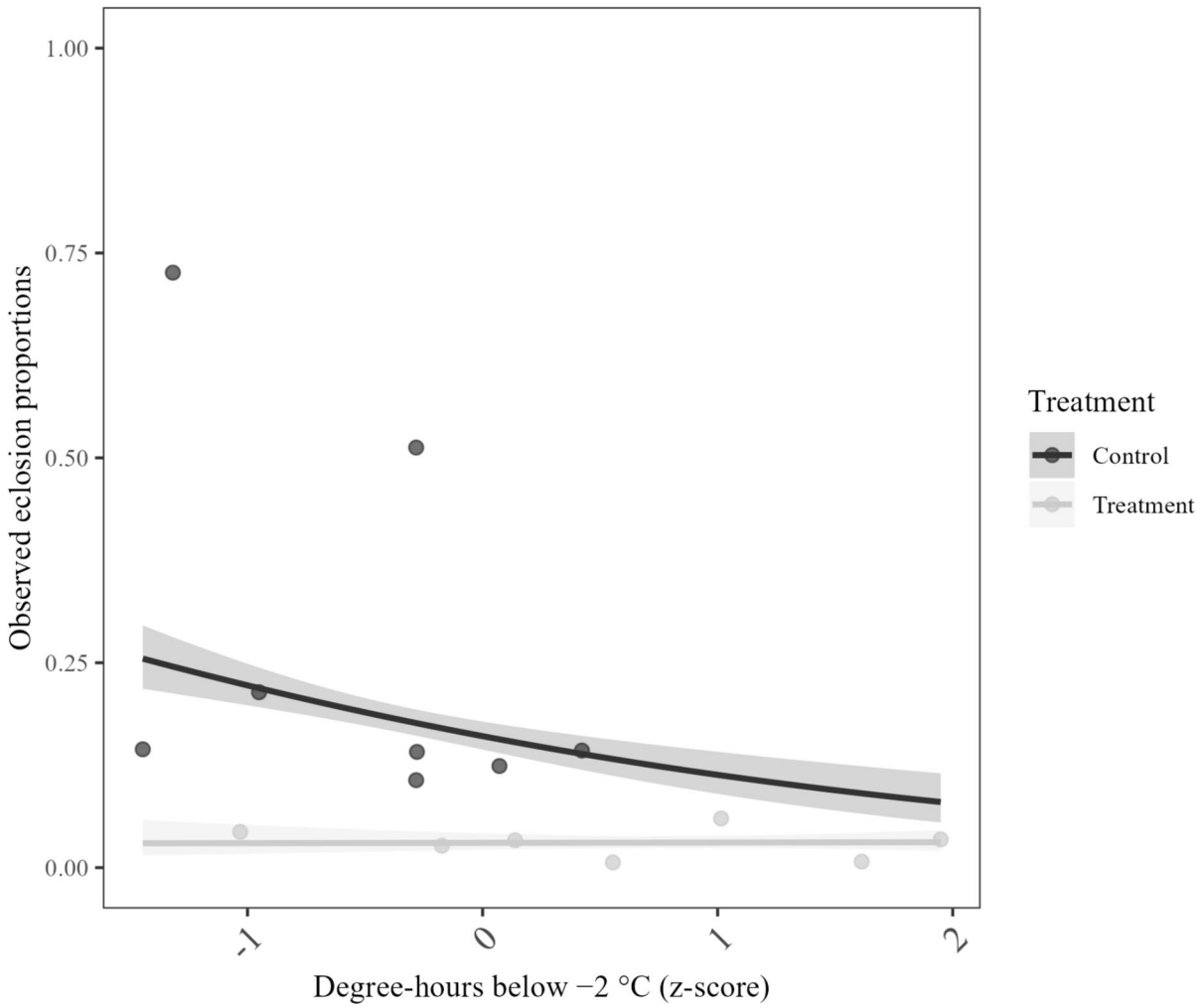

Fig. 4 Observed proportions of eclosed *T. planipennisi* across all three blocks separated by treatment group as a function of z-scored degree-hours ≤ −2 ^{0}C. Lines and shaded ribbons represent predicted eclosion probabilities and 95% CI for the mean predicted probability from the best-fitting binomial GLMM including treatment, degree-hours ≤ −2 C, and their interaction as fixed effects, with block as a random intercept, with further details in Supplementary Table S8

negative effect on eclosion (Fig. 4, $\beta = -0.40 \pm 0.08$, $z = -4.81$, $p < 0.001$), corresponding to an odds ratio of 0.67 [95% CI: 0.57–0.79] per 1-SD increase in degree-hours ≤ −2 °C. At mean degree-hours ≤ −2 °C, treatment jars had lower odds of eclosion than control jars ($\beta = -1.81 \pm 0.18$, $z = -9.91$, OR = 0.16 [95% CI: 0.11–0.23], $p < 0.001$), indicating reduced eclosion success for sheltered jars even at average cold exposure. The interaction between degree-hours ≤ −2 °C and treatment was also significant ($\beta = 0.42 \pm 0.17$, OR = 1.52 [95% CI: 1.09–2.12], $p < 0.0146$; details in Supplementary Table S8), indicating that the negative effect of degree-hours ≤ −2 °C was weaker in treatment jars than in controls.

Discussion

This study evaluated the effects of microclimate conditions on SCP and eclosion success in *Tetrastichus planipennisi* in the New England region of North America (USA). Findings supported our hypothesis that the SCP of *T. planipennisi* is a variable trait that

responds to changes in temperature and facilitates its survival and establishment in variable climate conditions. Sheltered jars significantly buffered temperature extremes relative to controls, providing a microhabitat contrast for assessing temperature effects on SCP. SCP was significantly associated with two-week minimum temperature, treatment, and monthly range, and adding monthly cooling rate improved model fit and had the strongest effect. SCP also declined seasonally, with lowest values in January–March.

Higher mean monthly cooling rates were associated with lower SCP, and controls cooled faster than treatment groups across winter, consistent with cooling rate contributing to both seasonal declines in SCP and differences between treatment. SCP differences were greatest in January–March, and the control groups that had higher cooling rates also exhibited lower SCP values, consistent with studies conducted on variegated ladybugs *Hippodamia variegata* Goeze (Coleoptera: Coccinellidae) and other ectotherms showing the lowest SCP values in January and February and suggesting that gradual cold acclimatization shifts SCP and cold tolerance (Salt 1966; Khabir et al. 2023). These patterns suggest that, alongside minimum temperature exposure, cooling rate may capture ecologically relevant thermal stress shaping SCP in *T. planipennisi*, potentially through acclimation responses to diurnal fluctuations (e.g., cryoprotectants and membrane stabilization) (Denlinger and Lee Jr. 2010). Cold acclimation can further shift SCP via cryoprotectant accumulation (Bale 1987). Although our SCP values are higher than those reported in Hanson et al. (2013), they remain within the published range for *T. planipennisi* (Wu et al. 2007), and prewinter conditions (e.g., fall acclimation or body size) may contribute to variation (Irwin and Lee Jr. 2000). Future studies pairing field deployments with biochemical assays and increased replication would help link microclimate to mechanisms and quantify block-level variability.

Although SCP differed between treatment groups, previous studies have found evidence of *T. planipennisi* being a chill-susceptible species which limits the relevance of SCP as a single metric for cold tolerance (Hanson et al. 2013). In chill-susceptible insects, mortality can occur at subzero temperatures above SCP through non-freezing chill injury, so SCP is better interpreted as a marker of physiological variation between microhabitats rather than a direct mortality threshold. We therefore quantified jar-level eclosion proportions as a proxy for overwintering survival and tested multiple predictor variables of cumulative cold temperature exposure to quantify chill injury that might have occurred prior to freezing. Consistent with chill susceptibility, our results suggest that reduced eclosion occurred above SCP rather than via freezing.

Eclosion success of *T. planipennisi* was significantly higher in larvae from control jar bolts than sheltered jars. The best-fitting GLMM indicated that greater exposure to temperatures ≤ -2 °C reduced eclosion and that the degree-hour effect was stronger in controls than in sheltered jars. Unbuffered control jars may have experienced greater daytime warming that facilitated partial recovery from chill injury, while sheltered jars likely experienced longer subthreshold exposure (≤ -2 °C), increasing chill-injury risk and reducing eclosion. Reduced diurnal warming may therefore have limited spring eclosion, consistent with studies showing that fluctuating thermal regimes can offset the effects of chill injury and extend survival by providing periodic warming (Renault et al. 2004; Marshall and Sinclair 2012). Hanson et al. (2013) reported similar effects, observing increased eclosion failure after long-term cold exposure in reach-in freezers and suggested pre-freeze mortality occurring above their SCP. Wittman et al. (2021) reported increased mortality and eclosion failure in *Spathius galinae* Belokobylskij & Strazanac (Hymenoptera: Braconidae) after chronic exposure near or below SCP, suggesting that cold stress can prevent spring eclosion without direct freezing and may do so via sublethal effects (e.g., altered polyol production and depleted fat reserves). Overwintering potential is determined by a combination of physiological and ecological factors, including chill injury thresholds, behavioral thermoregulation, insulation from host tissues, and snow cover buffering (Marshall and Sinclair 2012; Sinclair et al. 2015). Although SCP is informative physiologically, it is not a direct predictor of overwintering survival in this chill-susceptible species. Consistent with pre-freeze mortality, degree-hours ≤ -2 °C better explained eclosion success than SCP, indicating that non-freezing cold exposure above SCP drove emergence outcomes (Hanson et al. 2013).

This experiment was conducted over a single winter season and its sites were restricted to

Vermont, USA. These results reflect limited thermal conditions and parasitoid responses, so they may not account for inter-annual variability or the diverse climates within *T. planipennisi's* introduced range and cannot be generalized to regions with different winter severity or across broad latitudinal gradients. Block-level variation in SCP may reflect local buffering (e.g., snow insulation) that may have altered cooling dynamics. Future research should include more locations or longer sampling periods to better assess how temperature and microhabitat affect cold tolerance and eclosion success in *T. planipennisi*.

Our field results document flexible supercooling capacity of *T. planipennisi* in Vermont, USA and indicate that cooling rate, together with minimum temperature exposure, significantly influences its winter cold tolerance. Eclosion patterns indicated cumulative cold stress (degree-hours ≤ -2 °C) predicted spring emergence. Control jars experienced fewer hours below this threshold and had higher eclosion, while sheltered jars experienced longer subthreshold exposure and reduced daytime warming, with lower eclosion. This pattern is consistent with chill-susceptible, non-diapausing larvae and prior cold-exposure assays (Hanson et al. 2013; Duan et al. 2020). Fine-scale monitoring of cooling rates and microclimate (e.g., snowpack insulation, canopy shelter) could help forest managers target releases to naturally buffered sites and identify locations where cold snaps may reduce emergence. Future experiments that explicitly manipulate cooling rates while integrated with cryoprotectant chemistry and larval survival would provide stronger empirical support and refine climate-based forecast models of overwintering survival of *T. planipennisi.*

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Declarations

Conflict of interest The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work on this paper. *Tetrastichus planipennisi* samples were provided by the USDA-ARS Beneficial Insects Introduction Research Unit who had no role in the analysis or interpretation of the research.

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