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1 Insect immunity varies idiosyncratically during overwintering

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ABSTRACT

Overwintering insects face multiple stressors, including pathogen and parasite pressures that shift with seasons. However, we know little of how the insect immune system fluctuates with season, particularly in the overwintering period. To understand how immune activity changes across autumn, winter, and spring, we tracked immune activity three temperate insects that overwinter as larvae: a weevil (*Curculio* sp., Coleoptera), gallfly (*Eurosta solidaginis*, Diptera) and larvae of the lepidopteran *Pyrrharctia isabella*. We measured baseline circulating haemocyte numbers, phenoloxidase activity, and humoral antimicrobial activity, as well as survival of fungal infection and melanisation response at both 12 °C and 25 °C to capture any potential plasticity in thermal performance. In *Curculio* sp. and *E. solidaginis*, haemocyte concentrations remained unchanged across seasons and antimicrobial activity against gram-positive bacteria was lowest in autumn; however, *Curculio* sp. were less likely to survive fungal infection in autumn, whereas *E. solidaginis* were less likely to survive infection during the winter. Further, haemocyte concentrations and antimicrobial activity decreased in *P. isabella* overwintering beneath snow cover. Overall, seasonal changes in activity were largely species-dependent, thus it may be difficult to create generalizable predictions about the effects of a changing climate on seasonal immune activity in insects. However, we suggest that the relationship between the response to multiple stressors (e.g. cold and pathogens) drives changes in immune activity, and that understanding the physiology underlying these relationships will inform our predictions of the effects of environmental change on insect overwintering success.

Key words: acclimatization, trade-offs, multiple stressors, seasonality, pathogens, humoral immunity

33 INTRODUCTION

34 In temperate, polar, and alpine regions, insects can spend more than half of their lives
35 overwintering, during which they face multiple environmental stressors, including cold,
36 desiccation, and starvation (Williams et al., 2015). However, little is known of how biotic
37 stressors, such as pathogens, affect insects during the winter, or of how insects respond to these
38 stressors. Pathogens are present in the overwintering environment and responsible for
39 overwintering mortality (Steinmann et al., 2015; Webberley and Hurst, 2002; Steenberg et al.,
40 1995; Mills, 1981), thus insects may require immune activity during the winter. However, insect
41 immune activity may trade-off with the physiological response to cold (Ferguson et al., 2016;
42 Linderman et al., 2012; Sinclair et al., 2013), so thermally-induced changes in immunity could
43 modulate the strength of the immune response, potentially determining pathogen-related
44 overwintering survival. Despite this, we know little of how insect immune activity changes
45 across seasons, especially during overwintering, which makes it difficult to evaluate the
46 importance of immunity in insect seasonal biology.

47
48 The ways in which insects modify immune activity across seasons are likely to be
49 mediated by interactions between temperature, pathogen prevalence, and energy reserves
50 (Fedorka et al., 2013; Fedorka et al., 2012; Córdoba-Aguilar et al., 2009). Energy conservation is
51 essential for successful overwintering (Sinclair, 2015) and both immune activity and the
52 response to cold are energetically costly (Sinclair, 2015; MacMillan et al., 2012; Ardia et al.,
53 2012). Thus, immunity may trade-off with the requirements for cold tolerance and energy
54 conservation, leading to decreased immune activity during the winter. Indeed, overwintering
55 honeybees downregulate genes encoding antimicrobial peptides (Steinmann et al., 2015), and

damselflies have reduced resistance to bacterial infections during the winter (Córdoba-Aguilar et al., 2009). Conversely, both cold exposure and diapause induction can activate the immune system in some insects (Le Bourg et al., 2009; Marshall and Sinclair, 2011; Ragland et al., 2010), even in the absence of pathogen infection (Xu and James, 2012; Zhang et al., 2011), perhaps to compensate for trade-offs or cold-induced damage (Salehipour-shiraz et al., 2017). Because of the potential trade-offs associated with increased immunity, this implies that increased immune activity (even if compensatory) may be an adaptive response to overwintering pathogen pressures (Sinclair et al., 2013).

The insect immune system is largely innate and depends on a variety of cellular and humoral activities. Cellular activity is mediated by haemocytes, which circulate through the haemolymph and are primarily responsible for phagocytosis and encapsulation of pathogens and parasites (Lavine and Strand, 2002). Humoral activity includes that of enzymes - for example, phenoloxidase, whose activity ultimately culminates in the production of melanin and cytotoxic and reactive intermediate products (González-Santoyo and Córdoba-Aguilar, 2012) – as well as antimicrobial peptides (Gillespie and Kanost, 1997). These independent components of the immune system may be differentially-altered by or -responsive to environmental stress, and the immune system has the capacity to reconfigure itself, such that cellular activity may increase to compensate for impaired humoral activity, or vice-versa (Adamo, 2014). Therefore, because of this capacity for reconfiguration, it is important to measure several components of the immune system, including constitutive or potential activity, as well as inducible defenses and resistance/tolerance to infection [e.g. realised immunity (Fedorka et al., 2007)] to capture shifts in seasonal activity.

79

80 The activity of immune cells and enzymes is largely temperature-sensitive and thus will
81 depend directly on temperature (Ferguson et al., 2016; Murdock et al., 2012; Catalan et al.,
82 2012). However, immune activity is also phenotypically-plastic; for example, cold-acclimation
83 depresses immune activity at low temperatures in the cricket *Gryllus veletis*, likely as a response
84 to, or product of trade-offs between cold-tolerance and immunity (Ferguson et al., 2016).
85 Therefore, seasonal acclimatisation may shift the optimal temperature of immune activity, or
86 change the breadth of temperatures at which activity can occur (Angilletta, 2009), and immune
87 activity should be measured at both high and low temperatures in an effort to capture plasticity in
88 the thermal performance of immunity across seasons.

89

90 To understand how immune activity fluctuates across seasons, and how activity will shift
91 under a changing climate, we can begin by exploring immune activity in different species of
92 insects that will experience different iterations of temperature, pathogen, and energy stress
93 throughout the winter. Depending on the directions of shifts in activity, we can then begin to
94 predict whether changes in immunity are conserved across species, or if we can predict immune
95 activity based on the types of environmental pressures that species will experience across
96 seasons. Our objective in this study was to explore seasonal changes in immune activity during
97 overwintering in a range of insects, in an effort to understand how overwintering success is
98 mediated by immune activity, and whether or not we can generalise changes in activity across
99 species. We measured a variety of immune responses that incorporate humoral and/or cellular-
100 mediated activity in both constitutive and realised responses, to determine whether immunity
101 changes wholesale across seasons, or if the immune components are regulated differentially.

Further, to capture any seasonal plasticity in the thermal sensitivity of immune activity that might increase or decrease immunocompetence at a given temperature, we measured both constitutive immune activity and realised responses to pathogens at both low (12 °C) and high (25 °C) temperatures. These two temperatures represent typical summer and autumn temperatures for London, Ontario (Environment Canada, www.weather.gc.ca; Fig. 1). This breadth of temperature is likely to be wide enough to capture plasticity in thermal performance (e.g. Kingsolver et al., 2015), while also facilitating our ability to measure immune responses in the laboratory.

METHODS AND MATERIALS

Study species

We measured immune activity of three different species of insects to detect if seasonal patterns of immunity are generalizable, or species-specific. We chose three univoltine species, native to Southwestern Ontario, Canada, for which the overwintering biology (i.e. cold-tolerance strategies, microhabitat) is known: 1) The acorn weevil *Curculio* sp. (Coleoptera: Curculionidae) [derived from the same population that was misidentified as *Curculio glandium* by Udaka & Sinclair (2014)], which overwinters in the soil as freeze-avoidant larvae and emerges in late spring or summer to complete adult development (Udaka and Sinclair, 2014). 2) The goldenrod gall fly, *Eurosta solidaginis* Fitch, 1855 (Diptera: Tephritidae), which overwinters as a diapausing freeze-tolerant larva in a goldenrod stem gall. In mid-winter, *E. solidaginis* terminate diapause, remaining quiescent until temperatures rise in the spring (Irwin et al., 2001). 3) The woolly bear caterpillar, *Pyrrharctia isabella* Smith, 1979 (Lepidoptera: Arctiidae), which

overwinters as a diapausing freeze-tolerant larva beneath leaf litter, before higher temperatures in the spring allow completion of development (Goettel and Philogène, 1978; Layne et al., 1999). Further, we manipulated the overwintering microhabitat of *P. isabella* as either above or below snow cover (cf. Marshall and Sinclair, 2012), to determine whether microclimate (and its resultant effects on energy use) can modify immune activity.

Insect collection and field housing

We collected red oak (*Quercus rubra*) acorns from deciduous forests on Pelee Island, Ontario, Canada (41°46'N 82°39'W) in October, 2014 and transported the acorn (containing larvae of *Curculio* sp.) to London, Ontario, Canada (42°59'N 81°14'W). We housed acorns in an urban garden over 2 × 2 cm plastic grids in 68 L plastic bins containing moistened terrarium soil (Eco Earth Coconut Fibre Substrate, San Luis Obispo, CA, USA), and collected *Curculio* sp. larvae from the bottom of the bin after they emerged from the acorns and fell through the grid. We grouped five individuals of *Curculio* sp. in 35 mL plastic *Drosophila* vials filled with moistened terrarium soil (n = 70 vials). We then immersed all vials in soil contained in plastic bins (68 L) buried 10 cm below the soil surface and covered the vials with mesh bags of leaves to provide the thermal barrier offered by leaf litter. Larvae were housed in these containers for the duration of the overwintering period.

We collected approximately 200 goldenrod galls containing *Eurosta solidaginis* from fields and urban parks in London, Ontario, Canada in late September and early October of 2014, and

transported them to the same urban garden as *Curculio* sp. We housed all galls grouped in a mesh bag suspended approximately 1 m above the soil.

We collected *P. isabella* caterpillars (n = 71) from the same locations as *E. solidaginis* and housed each individual in a 120 mL plastic container with moistened terrarium soil and burdock (*Arctium* sp.) leaves as food, collected from the same area in which caterpillars were found. We grouped all containers in one of two 68 L plastic bins, covered the containers with mesh bags of fallen leaves and situated one bin approximately 50 cm above soil level (above snow cover) and one below (below snow cover), such that the caterpillars were level with the soil surface (Marshall and Sinclair, 2012).

Individual containers of insects were haphazardly chosen for collection in October (autumn) November (*P. isabella* only, representing the point at which feeding ceased; *E. solidaginis* and *Curculio* sp. ceased feeding before collection), December (early winter), February (mid-winter), and April (spring). Insects were transported to the lab and immediately used for experiments. We recorded mortality of all species at each retrieval by visually assessing movement following physical stimulation with a blunt probe. Animals that were alive all responded to the probe, and those that were dead did not move or showed signs of infection (e.g. hard to the touch and covered in fungal spores). We monitored the temperature of the microhabitats for each species using Hobo® Pro V2 temperature loggers (Onset Computers, Bourne, MA, USA).

Constitutive levels of immune activity

167 We briefly surface-sterilised each insect with 70 % ethanol before haemolymph collection. We
168 pierced the insects with a 32 G needle and collected the haemolymph that welled from the
169 wound. We collected 1 µL of haemolymph from individual insects and mixed it with
170 anticoagulant (98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, 41 mM citric acid, pH 6.8) for
171 immediate haemocyte counts (final dilutions: 1:24 *P. isabella* ; 1:24 *E. solidaginis*; 1:20
172 *Curculio* sp.). We collected 2 µL (*Curculio* sp.) or 4 µL (*P. isabella* and *E. solidaginis*) of
173 haemolymph from individual insects and mixed it with either 50 µL (*Curculio* sp.) or 100 µL (*P.*
174 *isabella* and *E. solidaginis*) of phosphate-buffered saline (PBS; for PO activity) or left it
175 unmixed for humoral antimicrobial activity. We snap-froze samples in liquid nitrogen, and
176 stored them at -80 °C until use.

177

178 To estimate the circulating haemocyte concentration (CHC), we counted haemocytes in freshly
179 diluted haemolymph in a Neubauer improved hemocytometer (Hausser Scientific, Blue Bell, PA,
180 USA) at 400 × magnification. We used five individuals per species, per season, and per
181 microhabitat.

182

183 We measured baseline (e.g. spontaneously-activated) levels of phenoloxidase activity (PO)
184 spectrophotometrically, following Adamo (2004) with some modifications. We thawed
185 haemolymph mixed with PBS and added 20 uL to 900 uL of 4 mg/mL L-DOPA (Sigma Aldrich,
186 Oakville, ON, Canada) in a plastic cuvette (final dilution of 1:1150). We measured absorbance
187 over 30 min at 490 nm at both 12 °C and 25 °C, for each species and time point (Carey 100
188 Spectrophotometer with Peltier-effect Temperature Controller, Agilent, Santa Clara, CA, USA),

and standardised the change in absorbance during the linear portion of the reaction to a control sample that did not contain hemolymph (n = 4-6 per species, per season, per temperature, and per microhabitat).

We quantified haemolymph antimicrobial activity, following Haine et al. (2008) in five individuals per species/ season/ temperature/ microhabitat combination. Briefly, we thawed and diluted 1 μ L (*Curculio* sp.) or 2 μ L (*P. isabella*, *E. solidaginis*) of haemolymph in 24 or 23 μ L of PBS, respectively, and added 1 μ L of a suspension (for a final volume of 25 μ L) of streptomycin-resistant *Escherichia coli* (gram-negative) or *Bacillus subtilis* (gram-positive) at approximately 5×10^6 colony forming units (CFU)/mL in PBS (final dilutions of 1:25). We incubated the haemolymph-bacteria suspensions for 2 h at either 12 °C or 25 °C on a shaking plate at 150 rpm. We diluted and spotted the suspensions on lysogeny broth (LB) agar containing 25 μ g/mL streptomycin and averaged the number of CFU over three replicate spots, following 24 h at 37 °C. We plated a control suspension containing no haemolymph to obtain the average number of CFU added to each suspension, and calculated the proportion of bacteria cleared from each suspension as: (CFU remaining in sample)/(CFU added to sample).

Realised immune responses

In *Curculio* sp. we measured the strength of the melanisation response against an imitation parasite (n = 3-7 per season, per temperature) by inserting a 2 mm length of nylon filament (0.25 mm diameter) behind the head capsule, towards the posterior end. Due to sample size and logistic constraints, we did not measure melanisation in *P. isabella* or *E. solidaginis*. Following

24 h incubation at either 0 °C, 12 °C, or 25 °C, we removed the filament, allowed it to dry, and photographed two sides of the length of the filament (Krams et al., 2011) at 30 × magnification using a Nikon DSFI1 camera (Nikon Instruments Inc. Melville, NY, USA) attached to a stereomicroscope. The strength of the melanisation response was calculated as the average darkness (grey value, GV), of each filament, using ImageJ (Rasband, 1997), and expressed as 255-GV, such that a higher value indicates increased melanisation.

We infected *E. solidaginis* and *Curculio* sp. with a cold-active strain of *Metarhizium brunneum* (provided by Michael Bidochka, Brock University, ON, Canada). We were unable to obtain enough individuals of *P. isabella* to include them in the fungal infections. We diluted *M. brunneum* spores to 5×10^7 spores/mL in 0.01% Tween 80 and briefly dipped each insect in the suspension (De La Rosa et al., 2002) (n = 14-29 per species, per season, per temperature). Control insects were dipped in 0.01% Tween 80 (n = 12-18 per species per temperature). We housed insects on moist filter paper in plastic 6-well plates at either 12 °C or 25 °C and assessed mortality daily. Mortality due to *M. brunneum* infection was confirmed by growth of characteristic green spores on the exterior of the insect.

Statistical Analysis

All analyses were performed in R v3.1.2 (R Development Core Team, 2010) and preliminary data exploration was conducted according to Zuur et al. (2010). We compared CHC, PO activity, melanisation, and humoral antimicrobial activity against each species of bacteria across months using ANOVA and detected differences between months or temperatures using Tukey's HSD.

To satisfy the assumptions of the ANOVA, we square-root- (CHC for all insects and PO activity of *P. isabella*), log- (PO activity of *Curculio* sp.) or arcsine-square-root-transformed (humoral antimicrobial activity of all insects) response variables. We assessed the assumptions of ANOVA by plotting residuals against fitted values to confirm homogeneity of variance, and standardised residuals against theoretical quantiles to assess normality (Crawley, 2007). We used the ‘survival’ package in R (Therneau and Grambsch, 2015) to use a lognormal model (based on log-likelihood values compared among models) to detect differences between survival curves of fungal infections. Due to constraints on survival models when no “event” occurs, when no mortality occurred in a group (e.g. uninfected controls) we did not include these groups in the analysis.

RESULTS

Overwintering mortality and temperatures in the field

Curculio sp. had high winter mortality: although all individuals retrieved in October were alive (n= 20 vials), 51 % \pm 5 % (SEM; n = 29 vials) of *Curculio* sp. retrieved in early winter and 74 %, \pm 5 % (SEM; n = 25 vials) retrieved in mid-winter, were dead. Further, due to unforeseen, localised flooding, the majority of weevils were dead (presumably drowned) in the spring. We did not observe any mortality in *P. isabella* during winter (e.g. all animals collected at this time point were alive); however, 26 % (5/19) of the caterpillars housed above snow cover were dead at the time of the spring collection, compared to 58 % (5/12) of those housed below snow cover. When opening galls throughout the study, we did not observe any dead *E. solidaginis*.

Temperatures below soil (i.e. experienced by *Curculio* sp.) remained buffered throughout the period of snow cover and did not reach the average supercooling point of these insects, thus is it unlikely that they experienced temperatures at which they would freeze (Fig. 1A; Udaka and Sinclair, 2014); although freezing inoculated by the surrounding soil might be responsible for some of the mortality in *Curculio* sp. Temperatures 1 m above soil level were highly variable throughout the winter and *E. solidaginis* were likely to experience both prolonged as well as repeated freezing and thawing (Fig. 1B; Baust and Lee, 1981). *Pyrrharctia isabella* below snow cover remained buffered from extreme temperatures and were potentially unfrozen for the winter, based on their hydrophobicity average supercooling points in previous years (B.J. Sinclair, Pers. Obs.). *P. isabella* above snow cover experienced greater variation in temperature than below snow cover and were likely to freeze (possibly repeatedly) throughout the winter (Fig. 1C; Marshall and Sinclair, 2011).

Baseline immune activity

We did not detect any changes in circulating haemocyte counts or PO activity among seasons (Fig. 2A: $F_{3,17} = 2.32$, $p = 0.11$; Fig. 3A; $F_{3,29} = 0.68$, $p = 0.57$) or temperatures (Fig. 3A; $F_{1,29} = 0.89$, $p = 0.35$) in *Curculio* sp. Humoral antimicrobial activity was higher at 12 °C than 25 °C against the gram-negative bacterium, *E. coli* (Fig. 4A; $F_{1,34} = 4.37$, $p = 0.04$), although activity remained unchanged across months ($F_{3,34} = 2.21$, $p = 0.10$) and there was no significant interaction between month and temperature. Conversely, humoral activity against the gram-positive bacterium, *B. subtilis*, was lowest in autumn compared to all other months (Fig. 4A; $F_{3,35} = 4.36$, $p = 0.01$); however, temperature did not affect clearance ($F_{1,35} = 0.53$, $p = 0.47$). There was no significant interaction between season and temperature ($F_{3,32} = 0.19$, $p = 0.90$).

278

279 We did not detect any seasonal changes in circulating haemocyte counts in *E. solidaginis* (Fig.
280 2B; $F_{3,16} = 1.56$, $p = 0.24$) and were unable to detect any baseline PO activity in haemolymph
281 samples from *E. solidaginis* at any time point; further, we could not detect PO using α -
282 chymotrypsin to activate the zymogen form of PO (proPO) to PO. Humoral antimicrobial
283 activity by *E. solidaginis* against *E. coli* did not change across seasons (Fig. 4B; $F_{3,31} = 2.01$; $p =$
284 0.13); however, humoral activity against *B. subtilis* was highest in mid-winter and early spring
285 (Fig. 4B; $F_{3,32} = 18.97$, $p < 0.001$), and was significantly higher at 12 °C than 25 °C (Fig. 4B;
286 $F_{1,32} = 20.34$, $p < 0.001$), although there was no significant interaction between month and
287 temperature ($F_{1,31} = 1.92$; $p = 0.15$).

288

289 In *P. isabella*, CHC decreased in mid-winter and spring, but only in caterpillars housed beneath
290 snow cover (Fig. 2C; $F_{4,28} = 6.51$, $p < 0.001$). Phenoloxidase activity was higher at 12 °C
291 compared to 25 °C (Fig. 3B; $F_{1,55} = 8.73$, $p < 0.01$) but we did not detect a seasonal change in PO
292 activity (Fig. 3B; $F_{4,55} = 2.27$, $p = 0.05$). Humoral activity against *B. subtilis* remained unchanged
293 with season ($F_{4,55} = 1.87$, $p = 0.10$), and although we detected a significant effect of temperature
294 on activity ($F_{1,55} = 7.10$, $p = 0.01$) we were unable to find specific significant differences
295 between groups using Tukey's HSD (Fig. 4C). However, humoral antimicrobial activity against
296 gram-negative *E. coli* was lower in *P. isabella* housed beneath snow cover than in those housed
297 above snow cover (Fig. 4D; $F_{6,57} = 6.94$, $p < 0.0001$). Antimicrobial activity by *P. isabella*
298 against *E. coli* was unaffected by temperature ($F_{1,57} = 1.68$, $p = 0.20$).

299

Realised immune responses

Melanisation in *Curculio* sp. was lower in autumn compared to early winter (Fig. 5; $F_{3, 39} = 3.78$, $p = 0.018$) but was unaffected by temperature ($F_{2, 39} = 1.66$, $p = 0.20$). The ability of *Curculio* sp. to survive fungal infection was lowest in autumn compared to mid-winter, and trended towards lower survival when compared to early winter, although these differences were only detectable at 12 °C (Fig. 6A,B; Table 1).

At 12 °C, *E. solidaginis* were more likely to survive fungal infection in autumn and spring, and least likely to survive in early and mid-winter (Fig. 6D; Table 1); however, at 25 °C, survival was higher only in the spring (Fig 6C; Table 1). At 25 °C in April, all *E. solidaginis* pupated within 48 h of inoculation (including controls) and survival was measured as survival to eclosion. At 12 °C in April, all *E. solidaginis* pupated, but did not emerge; however, controls emerged when returned to 25 °C, whereas infected flies did not, and fungus was observed growing on the outside of the infected pupae.

DISCUSSION

We found that *Curculio* sp., *E. solidaginis* and *P. isabella* all shift their immune activity across seasons; however, changes in immunity were inconsistent among species (summarised in Fig. 7), and even among measures of immune activity. Overall, this suggests that the ways in which insect immunity responds to changes in season will be species-specific with respect to both the host and the pathogen, and that different measures of immune activity can reflect

different seasonal patterns. Thus, it may be difficult to generalize about seasonal shifts in insect immunity.

The patterns of immune activity among and within species may reflect trade-offs between the demand on different physiological responses to multiple, seasonal pressures. For example, the response to cold may trade-off with immune activity in insects (Linderman et al., 2012; Ferguson et al., 2016), leading to decreased immune activity during times in which insects are physiologically preparing for overwintering. Both *E. solidaginis* and *Curculio* sp. displayed low antimicrobial activity in autumn, the timing of which coincides with increased production of cryoprotectants (Storey and Storey, 1986) and transition from acorn to an overwintering state in the soil, respectively. In the face of responding to multiple stressors and potential trade-offs, the immune system may reconfigure activity to maintain some level of protection (Adamo, 2014), leading to differences in activity among measures of immunity, such as those we observed in this study. For example, in mid-winter when *E. solidaginis* was least likely to survive fungal infection, they simultaneously displayed increased humoral activity, compared to autumn. Overall, it appears that seasonal shifts in immune activity are, at least in part, governed by the relationship between the immune system and the physiological response to concurrent stressors, such as cold.

Eurosta solidaginis displayed increased bacterial clearance and survival of fungal infection in the spring, and *Curculio* sp. increased bacterial clearance after autumn. This increased immune activity in the spring could also represent a (potentially prophylactic) response

to an increase in pathogen stress that may either act to increase immunocompetence overall, or compensate for any damage to, or trade-offs experienced by, the immune system (Salehipour-shirazi et al., in press). As temperatures increase, infection by new pathogens, or growth of pathogens overwintering in the insect, may increase (Altizer et al., 2006; Harvell et al., 2002), thereby initiating increased immune activity in response to, or in preparation for, increased pathogen stress (Sinclair et al., 2013). Conversely, increases in immune activity from autumn to spring may represent selection for individuals with stronger immune responses (Krams et al., 2011); this explanation is supported by high overwintering mortality in *Curculio* sp. as a potential source of selection, but is unlikely in *E. solidaginis* for which we observed no mortality. In either case, increased immune activity in spring, coupled with high overwintering mortality in *Curculio* sp., indicates that overwintering is likely an important period of pathogen exposure and/or selection on immunocompetence.

Species-specific patterns of seasonal immune activity may also be explained by overwintering habitat, wherein the temperatures experienced in these microhabitats determine both the amount of temperature stress experienced by the insect, as well as the probability of pathogen encounter. *Pyrrharctia isabella* that overwintered above the snow cover had increased numbers of circulating haemocytes and humoral antibacterial activity, compared to those that overwintered below snow cover, and experienced more severe and variable low temperatures (Fig. 1C). Warmer conditions beneath snow cover, or during a milder winter, may increase energy consumption (Irwin and Lee, 2003; Marshall and Sinclair, 2012), and consequently decrease energy available for immune activity; insects exposed to higher temperatures through the winter may thus be immunocompromised in the spring and vulnerable to infection. Indeed, a

higher proportion of *P. isabella* died in the spring, with some evidence of fungal infection, when housed beneath snow cover for the winter. Further, repeated freezing and thawing may occur either above snow cover (i.e. in more variable temperatures), or below cover if inoculative freezing occurs; in either case, tissue damage from freezing and thawing may also increase immune activity (Marshall and Sinclair, 2011; Sinclair et al., 2013). Therefore, microhabitat is likely to act as an important driver of protection against pathogens, overwintering success, and fitness in the spring.

In addition to species-specific responses in the context of season, it is interesting to note overall, species-specific differences in immune activity. For example, we were unable to detect PO activity in the haemolymph of *E. solidaginis* – neither spontaneous activity, nor activity when we added the activator α -chymotrypsin. Although another activator [e.g. cetylpridinium chloride (Adamo et al., 2016)] may have allowed us to detect PO, we also observed that the substrate for PO activity, L-DOPA, as well as haemolymph samples from *Curculio sp.* and *P. isabella*, would darken (e.g. auto-oxidation) over time (within two hours at room temperature); however, samples of L-DOPA containing haemolymph, as well as pure haemolymph, from *E. solidaginis* remained clear, potentially from a lack of PO activity due to inhibitors [e.g. serine protease inhibitors (Sugumaran et al., 1985)]. To our knowledge, this is the first investigation of immune activity in *E. solidaginis*. *Eurosta solidaginis* larvae do have genes coding the PO enzyme (H. Udaka, A.B. Dennis & B.J. Sinclair, unpublished data), which suggests that *E. solidaginis* larvae suppress PO activity in winter, possibly to avoid toxic by-products of the melanisation response (González-Santoyo and Córdoba-Aguilar, 2012), or as a trade-off between immunity and other physiological systems. The gall environment likely provides some protection

389 from pathogens, and we did not observe fungus-killed larvae in field collections for this or other
390 studies (L.V. Ferguson & B.J. Sinclair, unpublished observations); thus, these larvae may not
391 require PO activity while in the gall.

392
393 Temperature also governed the response to pathogens across seasons, in part through the
394 thermal performance of the immune system. We detected seasonal differences in the ability to
395 survive fungal infection at 12 °C, which indicates that the interaction between host and parasite
396 changes depending on temperature. This may result from shifts in the thermal performance of the
397 immune system across season, and highlights the importance of considering temperature when
398 predicting the outcome of infection (Thomas and Blanford, 2003). Despite this, most measures
399 of immune activity did not change with measurement temperature, which suggests that these
400 insects have a broad range of thermal immune performance, and that their immune systems may
401 be well-suited to function over the large fluctuations in temperature that insects experience
402 across seasons (Fig. 1). We do note that we did not measure activity at temperatures lower than
403 12 °C and thus may not have captured all temperature-dependent changes in immune activity.

404
405 The interaction between temperature and phenology may override immune activity when
406 development coincides with pathogen challenge. In spring, high survival of *E. solidaginis* against
407 fungal infection coincided with rapid pupation (within 48 h of inoculation) at 25 °C, which
408 indicates that the pre-pupal cuticle and/or puparium may have formed before the fungus was able
409 to penetrate the cuticle and establish in the haemolymph [e.g. *M. anispoliae* reaches the
410 haemolymph in 48 h post-inoculation at 28 °C in *Schistocerca gregaria* (Gillespie et al., 2000)].

Therefore, the effects of temperature on survival of infection across seasons is dependent on both the thermal sensitivity and plasticity of the immune system, as well as other physiological systems governing activity such as growth and reproduction.

CONCLUSIONS

Here we show that immune activity in three species of overwintering insects fluctuates by season, but that these changes in activity largely vary by species (summarised in Fig. 7). These variations in activity may depend on the interaction between the physiological responses to multiple stressors, and are also governed by the thermal performance of the immune system. Further, immune activity is affected by overwintering microhabitat, in which the relationship between multiple, seasonal pressures may shift. Based on the idiosyncratic nature of our results, we cannot generalise the effects of season on insect immunity; however, we suggest that further understanding of the mechanisms underlying these species-specific shifts in immune activity (e.g. trade-offs between cold tolerance and immunity) will allow us to make broader predictions of the effects of season and climate change on overwintering success.

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FIGURE LEGENDS

Fig. 1. Microhabitat temperatures. We measured temperature using Hobo® Pro V2 temperature loggers (Onset Computers, Bourne, MA, USA) with probes situated in each microhabitat. **A.** Temperatures experienced by *Curculio* sp., situated approximately 10 cm beneath soil surface. **B.** Temperatures experienced by *Eurosta solidaginis*, situated approximately 1 m above the soil surface. **C.** Temperatures experienced by *Pyrrharctia isabella* either below (black line) or above (grey line) snow cover.

Fig. 2. Circulating haemocyte counts across season. Haemocytes were counted in standardised volumes of haemolymph extracted from three different insects in months throughout autumn, winter, and spring. **A.** *Curculio* sp. (n = 5 per month). **B.** *Eurosta solidaginis* (n = 5 per season). **C.** *Pyrrharctia isabella* above and below snow cover (n = 5 per month, per season). Error bars represent SEM.

Fig. 3. Baseline phenoloxidase activity. Circulating activity of phenoloxidase activity was measured spectrophotometrically in standardised volumes of haemolymph extracted from three different insects in months throughout autumn, winter, and spring. Neither baseline nor activated phenoloxidase activity was detected in *Eurosta solidaginis*. Activity was measured at either 12 °C or 25 °C **A.** *Curculio* sp. (n = 5 per season, per temperature, except n = 4 for October, February, and April at 25 °C) **B.** *Pyrrharctia isabella* (n = 5 per season, per temperature, except n = 6 December, 12 °C; n = 4 October 12 °C & 25 °C, April 12 °C & 25 °C). Error bars represent SEM.

Fig. 4. Humoral antimicrobial activity across seasons. Baseline levels of humoral antimicrobial activity were measured *in vitro* with standardised volumes of haemolymph extracted from three species of insects in months throughout autumn, winter, and spring. Activity was measured at either 12 °C or 25 °C against a gram-positive (*Bacillus subtilis*) or gram-negative (*Escherichia coli*) bacteria. Negative activity indicates samples in which bacteria grew, suggesting that any antimicrobial activity present was not sufficient to overcome infection. **A.** *Curculio* sp. (n = 5 per month, per temperature, per bacteria). **B.** *Eurosta solidaginis* (n = 5 per month, per temperature, per bacteria). **C.** Activity of *Pyrrharctia isabella* housed above and below snow cover against *Bacillus subtilis* (n = 5 per season, per temperature, per microhabitat). **D.** Activity of *Pyrrharctia isabella* housed above and below snow cover against *Escherichia coli* (n = 5 per season, per temperature, per microhabitat). Error bars represent SEM.

Fig. 5. Melanisation response in *Curculio* sp. across seasons. Melanisation was measured as the darkness (grey value) of melanin deposited on a nylon filament (2 mm) introduced into the body cavity of the insect for 24 h at either 0 °C (except for in April), 12 °C, or 25 °C (n = 5 per month, per temperature except: n = 7 October 12 °C; n = 6 October 25 °C; n = 4 December, all temperatures; n = 3 February and April, all temperatures). Error bars represent SEM.

Fig. 6. Survival of infection with *Metarhizium brunneum* across seasons. Insects were infected topically with spores of *M. brunneum* suspended in 0.01% Tween80, and infection progressed at either 12 °C or 25 °C. Controls were inoculated with sterile 0.01% Tween80 and are shown as grey lines **A.** *Curculio* sp. at 25 °C; **B.** *Curculio* sp. at 12 °C; (n = 18 October 25

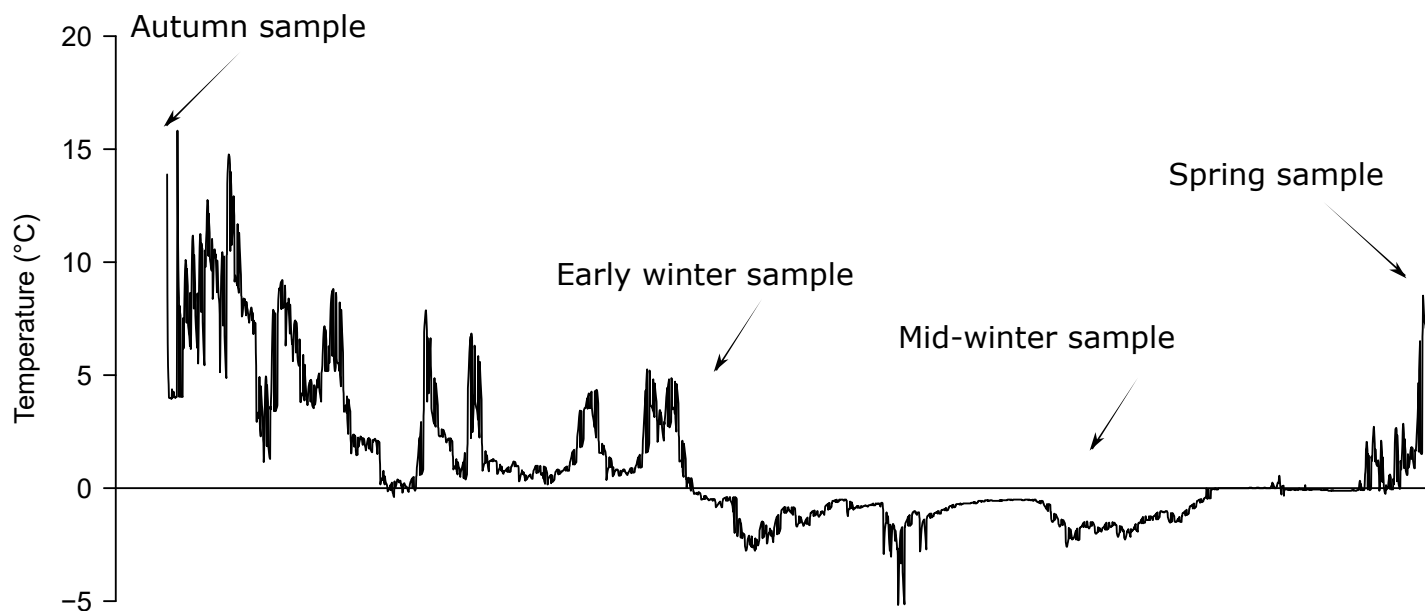
°C, December 12 °C; n = 25 October 12 °C; n = 17 December 25 °C; n = 14 February 25 °C; n = 15 February 12 °C; controls: n = 12-15). **C.** *Eurosta solidaginis* at 25 °C; **D.** *Eurosta solidaginis* at 12 °C; (n = 29 October 12 °C, n = 24 October 25 °C; n = 15 December; n = 18 February & April; controls: n = 15-18). In April, pupal cases formed rapidly at 25 °C and the majority of flies survived to eclosion as adults. At 12 °C, mortality was difficult to detect after the pupal case was formed; however, these flies did not eclose, even after transfer to 25 °C, and were thus determined dead at the end of the study.

Fig. 7. Summary of humoral immunity across season to highlight idiosyncrasy of activity among species. Arrows depict the change in season from autumn (October) through spring (April). Autumn is shown with a circle at the start of the arrow. Circles represent *Curculio* sp., squares represent *Eurosta solidaginis*, and triangles represent *Pyrrharctia isabella*. Each panel represents humoral activity against bacteria, plotted against circulating haemocyte concentrations. Each species demonstrates a different direction of change across season. Dashed lines represent the direction of change in *P. isabella* beneath snow cover.

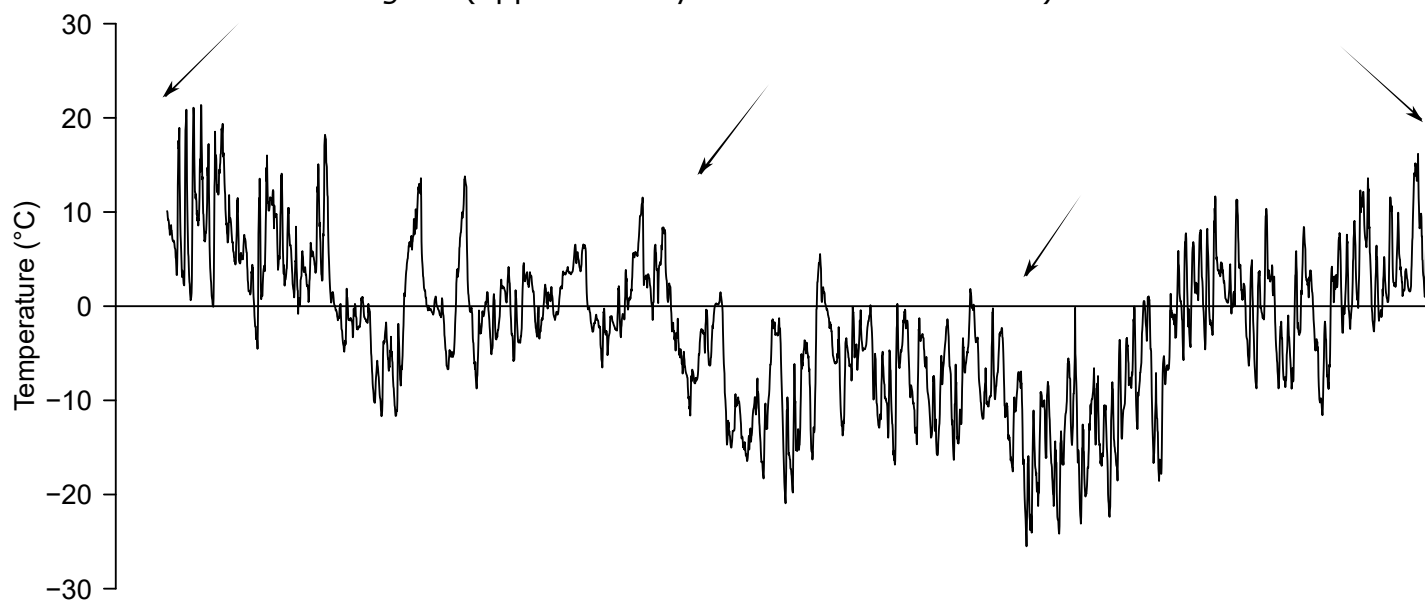
Table 1. Statistical results from lognormal models comparing survival curves. Survival was monitored at 12 °C and 25 °C for *Eurosta solidaginis* and *Curculio* sp. infected with the entomopathogenic fungus *Metarhizium brunneum*. Bolded p-values indicate significant differences.

Species	Temperature (°C)	Comparison	Z	P
<i>E. solidaginis</i>	12	Autumn vs Early winter	2.19	0.03
		Autumn vs Mid-winter	6.73	<0.001
		Autumn vs Spring	1.30	0.02
		Early winter vs Mid-winter	3.79	<0.001
		Early winter vs Spring	3.12	<0.01
		Mid-winter vs Spring	7.25	<0.001
	25	Autumn vs Early winter	0.29	0.77
		Autumn vs Mid-winter	1.91	0.06
		Autumn vs Spring	7.12	<0.001
		Early winter vs Mid-winter	1.44	0.15
		Early winter vs Spring	6.79	<0.001
		Mid-winter vs Spring	8.43	<0.001
<i>Curculio</i> sp.	12	Autumn vs Early winter	1.88	0.06
		Autumn vs Mid-winter	1.01	0.03
		Early winter vs Mid-winter	0.96	0.3
	25	Autumn vs Early winter	0.04	0.97
		Autumn vs Mid-winter	0.14	0.88
		Early winter vs Mid-winter	0.11	0.91

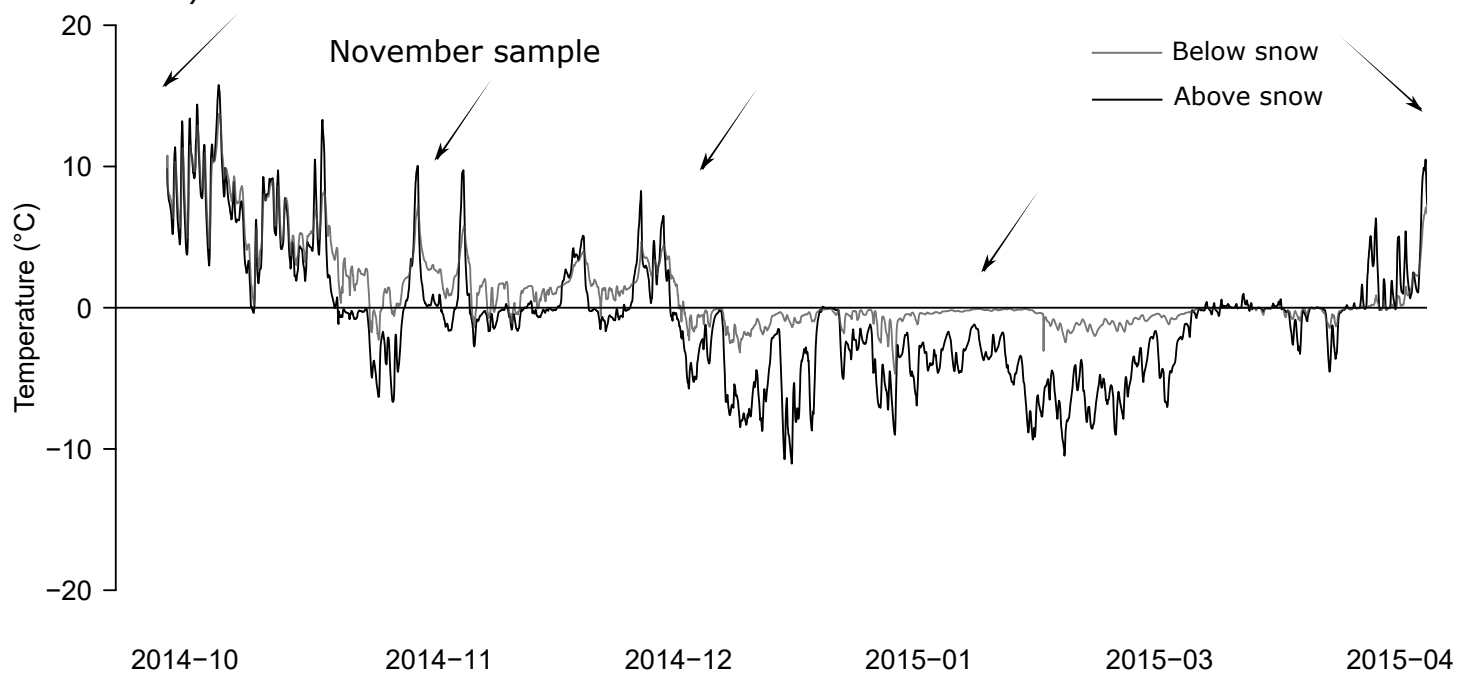
A: *Curculio* sp. (approximately 10 cm beneath soil surface)



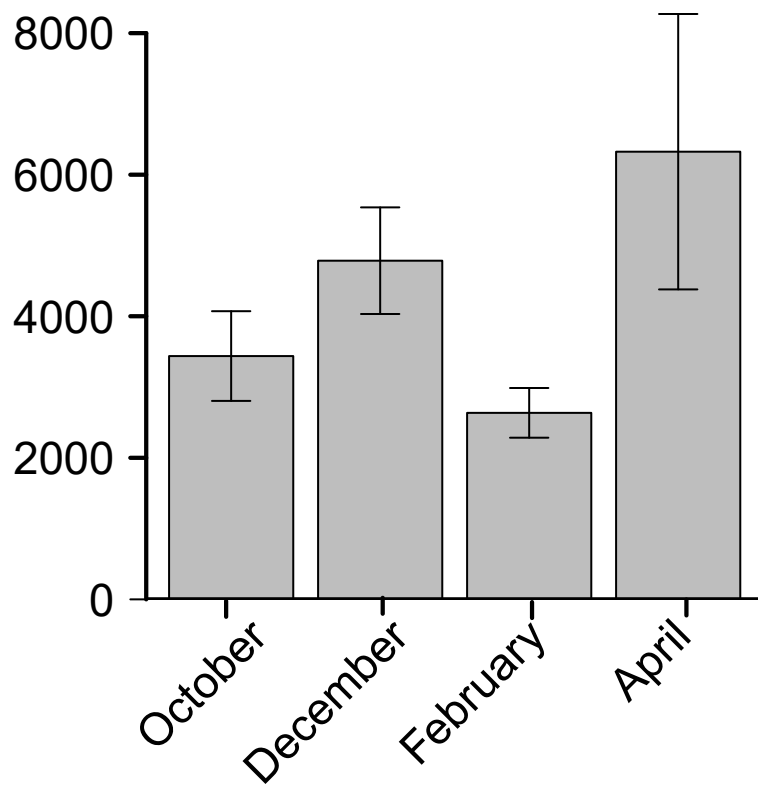
B: *Eurosta solidaginis* (approximately 1 m above soil surface)



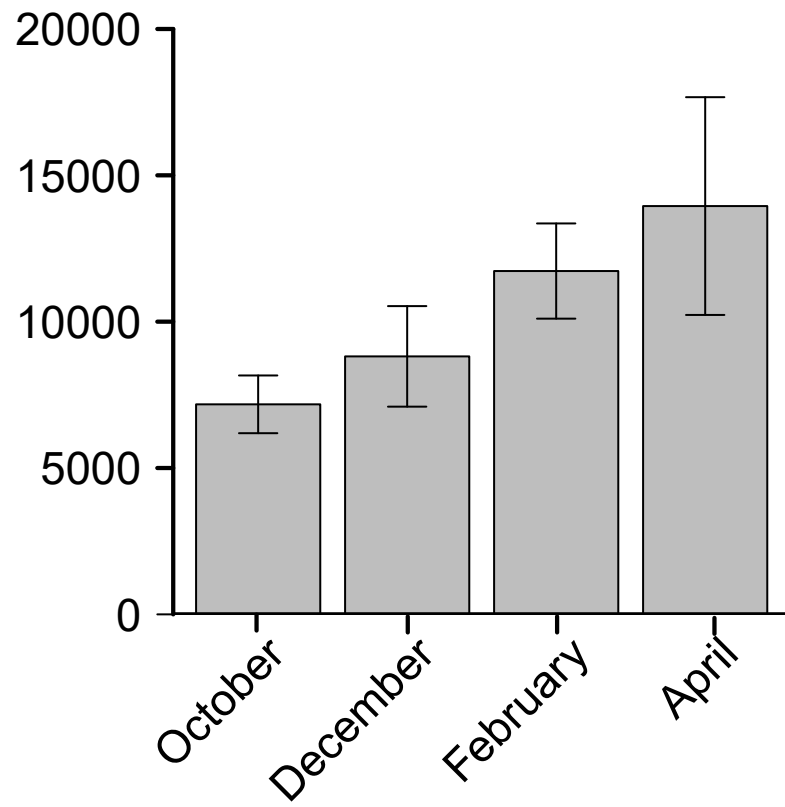
C: *Pyrrharctia isabella* below and above snow cover



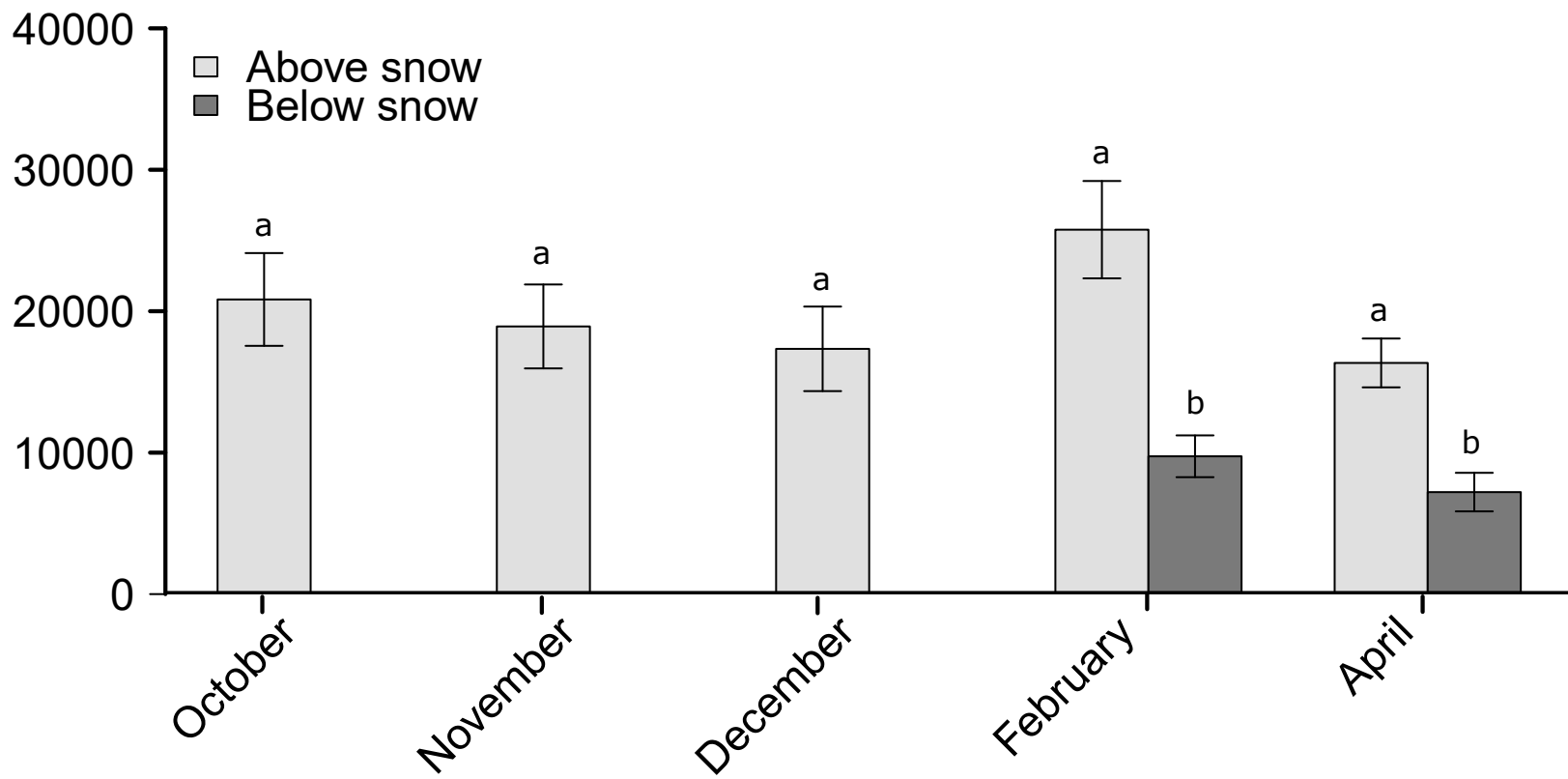
A: *Curculio* sp.



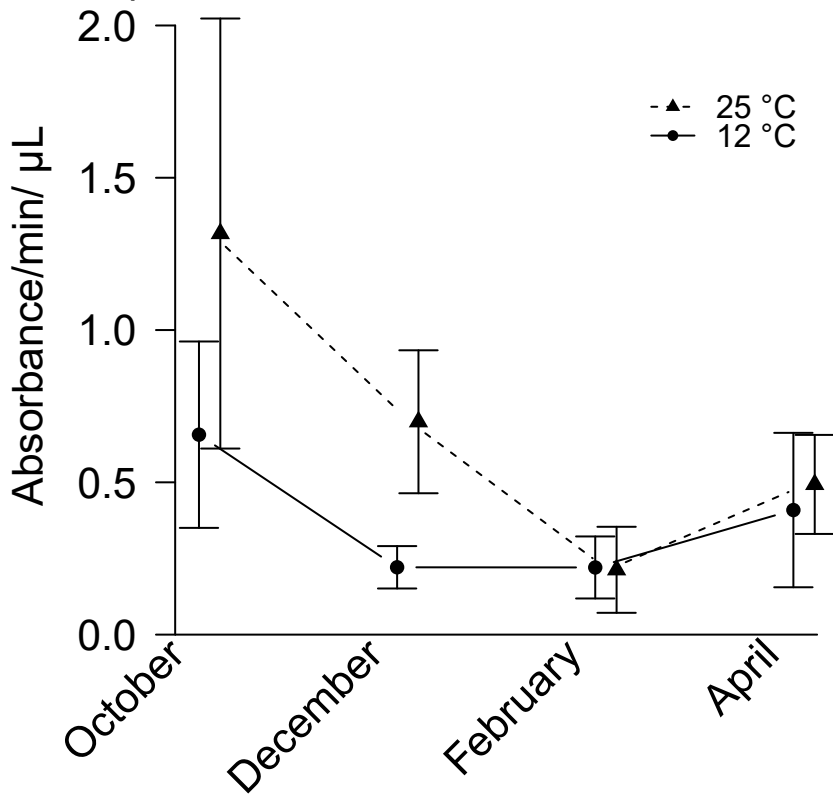
B: *Eurosta solidaginis*



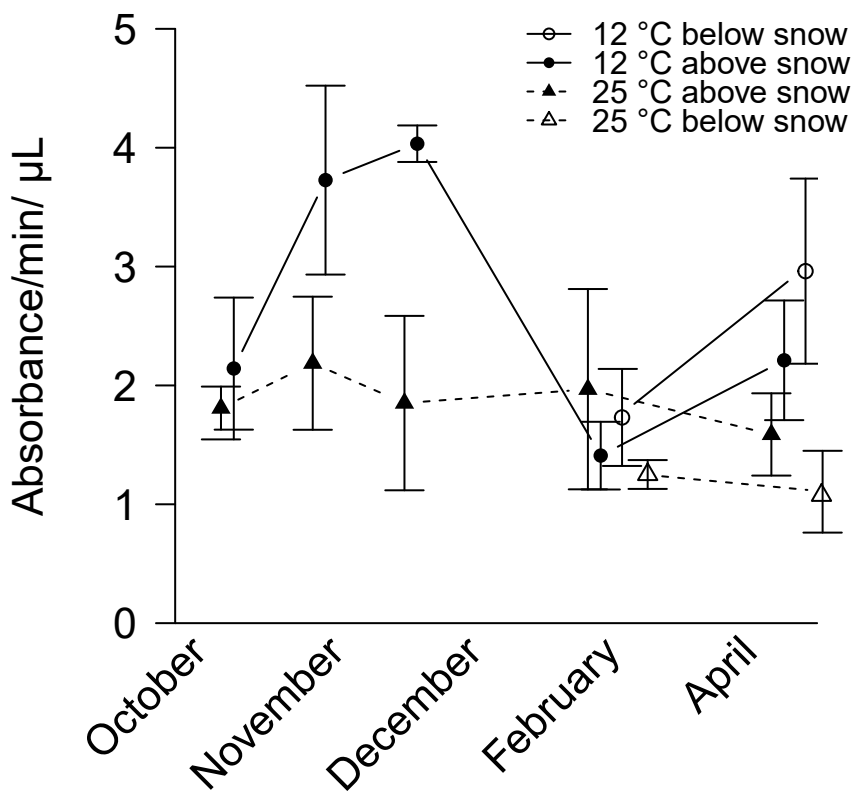
C: *Pyrrharctia isabella*

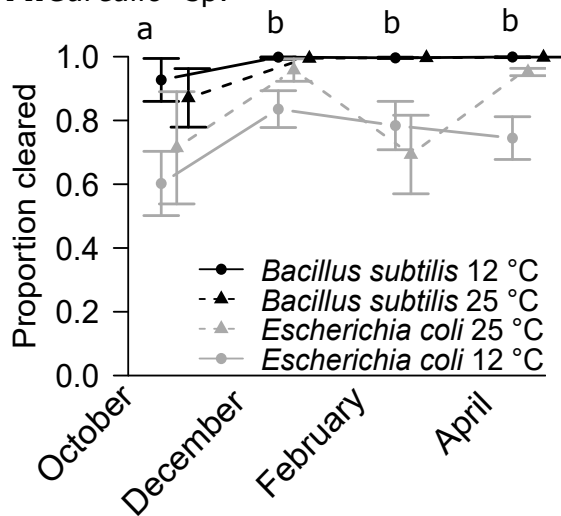
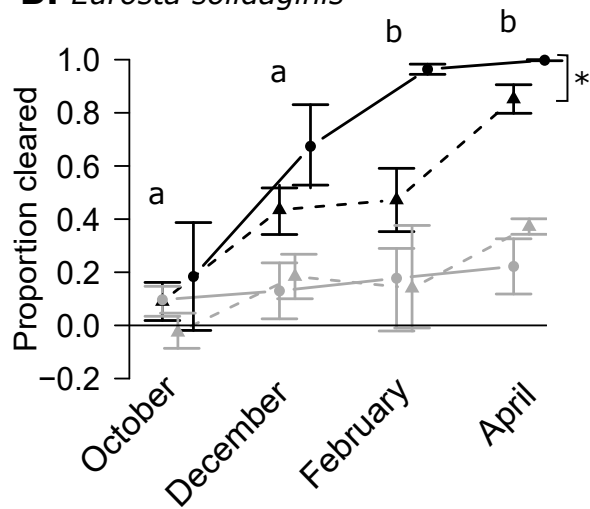
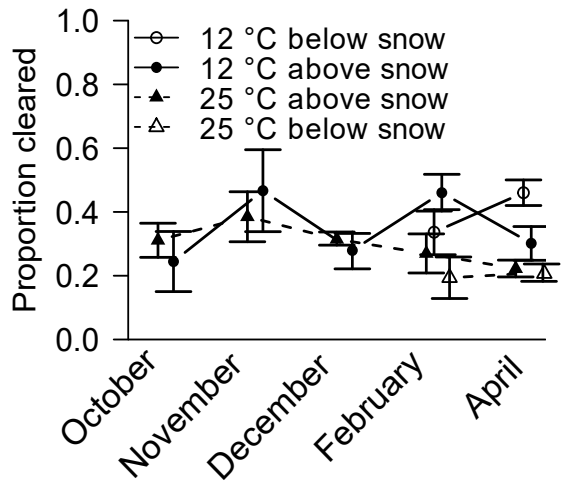
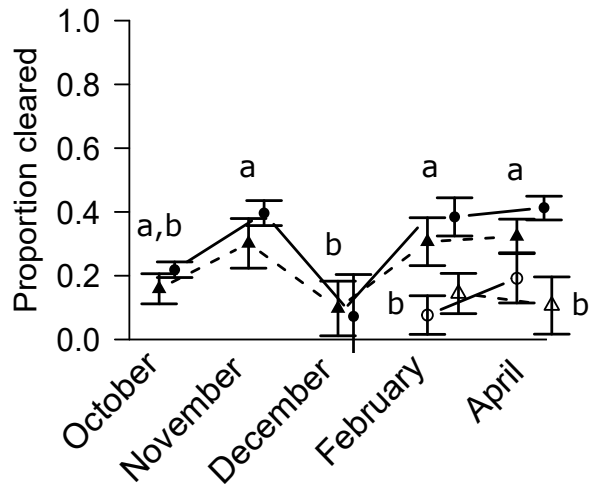


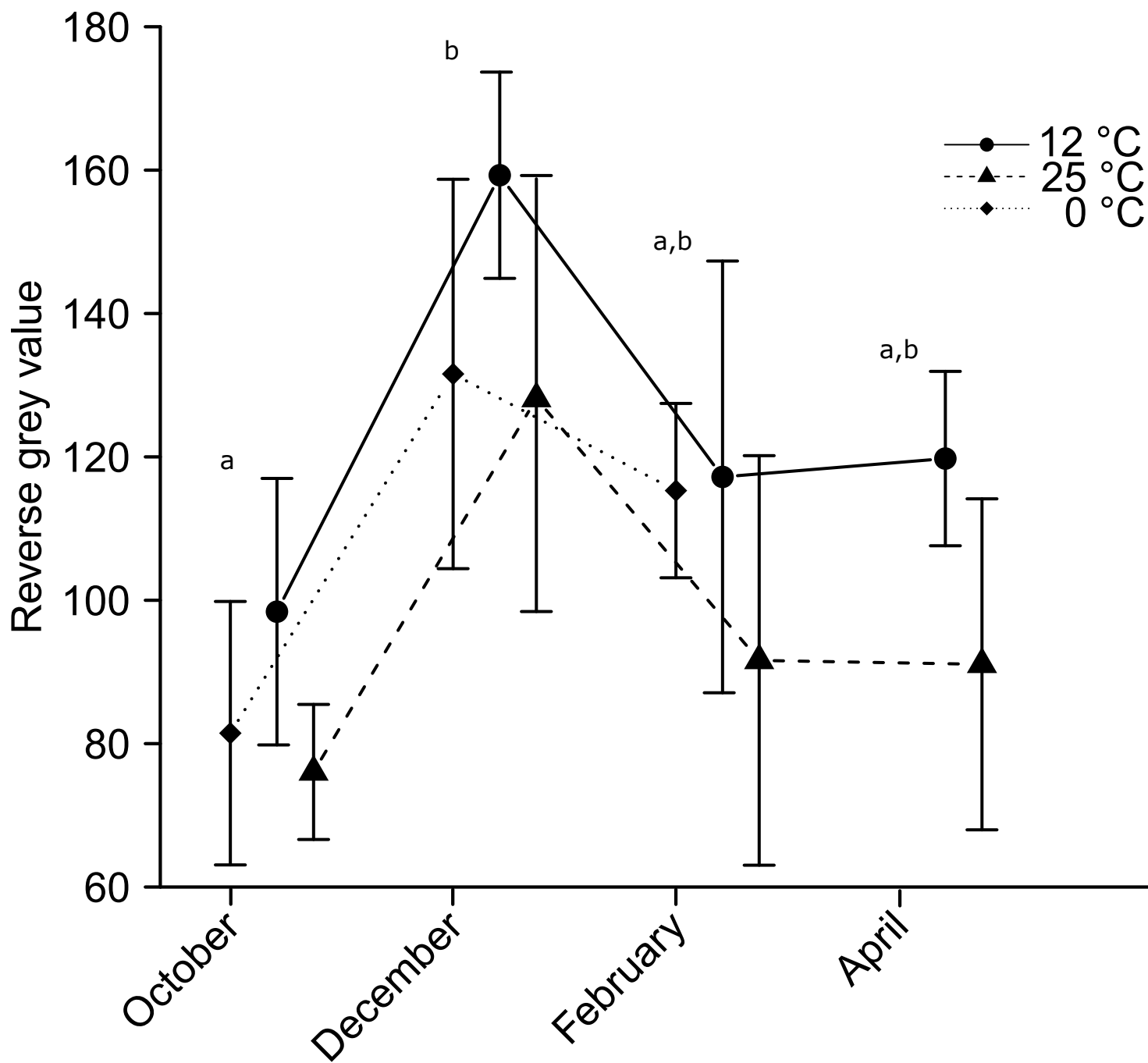
A: *Curculio* sp.

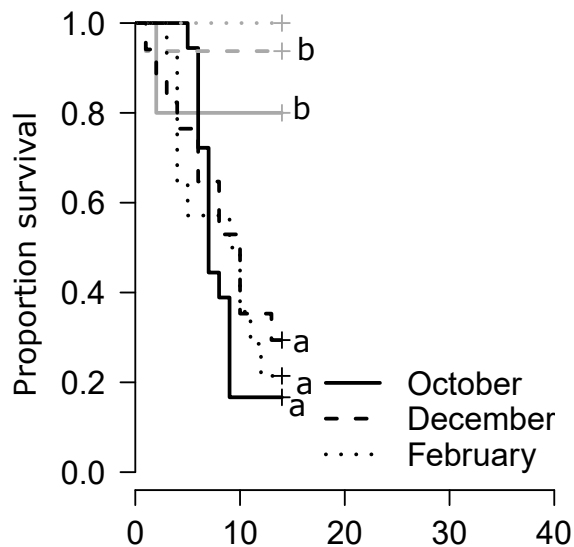
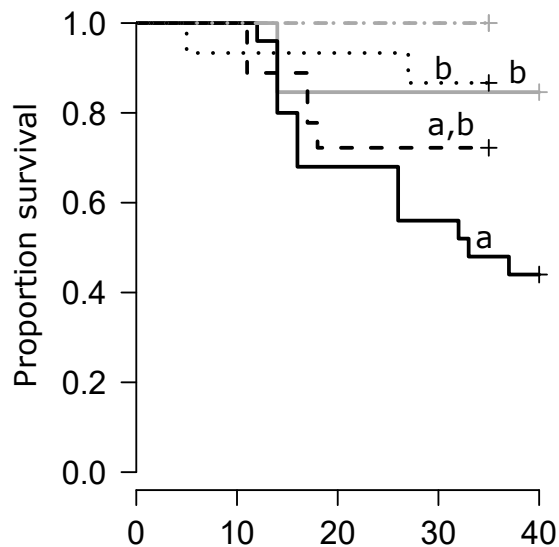
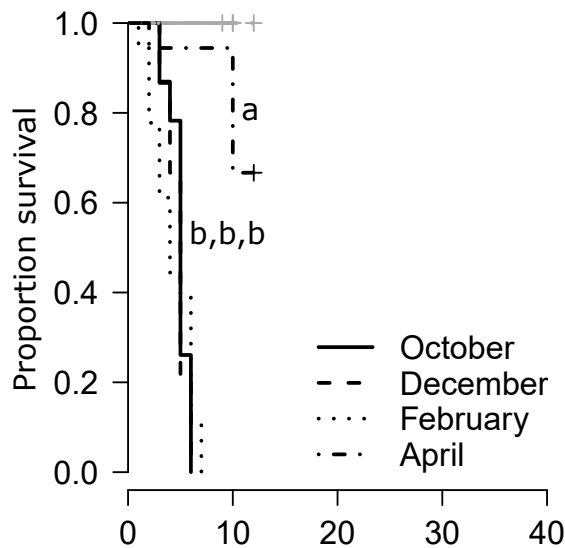


B: *Pyrrharctia isabella*



A: *Curculio* sp.**B:** *Eurosta solidaginis***C:** *Pyrrharctia isabella* vs *Bacillus subtilis***D:** *Pyrrharctia isabella* vs *Escherichia coli*



A: *Curculio* sp. infected at 25 °C**B:** *Curculio* sp. infected at 12 °C**C:** *Eurosta solidaginis* infected at 25 °C**D:** *Eurosta solidaginis* infected at 12 °C