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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

INTRODUCTION

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

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

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

To understand how immune activity fluctuates across seasons, and how activity will shift under a changing climate, we can begin by exploring immune activity in different species of insects that will experience different iterations of temperature, pathogen, and energy stress throughout the winter. Depending on the directions of shifts in activity, we can then begin to predict whether changes in immunity are conserved across species, or if we can predict immune activity based on the types of environmental pressures that species will experience across seasons. Our objective in this study was to explore seasonal changes in immune activity during overwintering in a range of insects, in an effort to understand how overwintering success is mediated by immune activity, and whether or not we can generalise changes in activity across species. We measured a variety of immune responses that incorporate humoral and/or cellular-mediated activity in both constitutive and realised responses, to determine whether immunity 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 (*Querucus 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 (42º59'N 81º14'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

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

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

We measured baseline (e.g. spontaneously-activated) levels of phenoloxidase activity (PO) spectrophotometrically, following Adamo (2004) with some modifications. We thawed haemolymph mixed with PBS and added 20 uL to 900 uL of 4 mg/mL L-DOPA (Sigma Aldrich, Oakville, ON, Canada) in a plastic cuvette (final dilution of 1:1150). We measured absorbance over 30 min at 490 nm at both 12 °C and 25 °C, for each species and time point (Carey 100 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 µL *(Curculio* sp.*)* or 2 µL (*P. isabella*, *E. solidaginis*) of haemolymph in 24 or 23 µL of PBS, respectively, and added 1 µL of a suspension (for a final volume of 25 µL) 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 µg/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 % ± 5 % (SEM; n = 29 vials) of *Curculio sp.* retrieved in early winter and 74 %, ± 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 ($F_{3, 32} = 0.19$, $p = 0.90$).

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

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

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

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

373

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

Adamo SA. 2004. Estimating disease resistance in insects: Phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*. J Insect Physiol 50:209-216.

Adamo SA. 2014. The effects of stress hormones on immune function may be vital for the adaptive reconfiguration of the immune system during fight-or-flight behavior. Int Comp Biol 54:419-426.

Adamo SA, Davies G, Easy R, Kovalko I, Turnbull KF. 2016. Reconfiguration of the immune system network during food limitation in the caterpillar *Manduca sexta*. J Exp Biol 219:706-718.

Altizer S, Dobson A, Hosseini P, Hudson P, Pascual M, Rohani P. 2006. Seasonality and the dynamics of infectious diseases. Ecol Lett 9:467-484.

Angilletta MJ, Jr. 2009. Thermal adaptation: A theoretical and empirical synthesis. New York, United States of America: Oxford University Press.

Ardia DR, Gantz JE, Schneider BC, Strebel S. 2012. Costs of immunity in insects: An induced immune response increases metabolic rate and decreases antimicrobial activity. Func Ecol 26:732-739.

Baust JG, Lee RE Jr. 1981. Divergent mechanisms of frost-hardiness in two populations of the gall fly *Eurosta solidaginis*. J Insect Physiol 27: 485-490.

Catalan TP, Wozniak A, Niemeyer HM, Kalergis AM, Bozinovic F. 2012. Interplay between thermal and immune ecology: Effect of environmental temperature on insect immune response and energetic costs after an immune challenge. J Insect Physiol 58:310-317.

Córdoba-Aguilar A, Jiméez-Cortés JG, Lanz-Mendoza H. 2009. Seasonal variation in ornament expression, body size, energetic reserves, immune response, and survival in males of a territorial insect. Ecol Entomol 34:228-239.

Crawley MJ. 2007. The R book. New York John Wiley & Sons, Ltd.

De La Rosa W, Lopez FL, Liedo P. 2002. *Beauveria bassiana* as a pathogen of the Mexican fruit fly (Diptera: Tephritidae) under laboratory conditions. J Econ Entomol 95:36-43.

Fedorka KM, Copeland EK, Winterhalter WE. 2013. Seasonality influences cuticle melanization and immune defense in a cricket: Support for a temperature-dependent immune investment hypothesis in insects. J Exp Biol 216:4005-4010.

Fedorka KM, Lee V, Winterhalter WE. 2012. Thermal environment shapes cuticle melanism and melanin-based immunity in the ground cricket *Allonemobius socius*. Evol Ecol 27:521-531.

Fedorka KM, Linder JE, Winterhalter W, Promislow D. 2007. Post-mating disparity between potential and realized immune response in *Drosophila melanogaster*. Proc R Soc B 274:1211-1217.

Ferguson LV, Heinrichs DE, Sinclair BJ. 2016. Paradoxical acclimation responses in the thermal performance of insect immunity. Oecologia 181:77-85.

Gillespie JP, Burnett C, Charnley AK. 2000. The immune response of the desert locust *schistocerca gregaria* during mycosis of the entomopathogenic fungus, *Metarhizium anisopliae var acridum*. J Insect Physiol 46:429-437.

Goettel MS, Philogène BJR. 1978. Effects of photoperiod and temperature on the development of a univoltine population of the banded woollybear, *Pyrrharctia (isia) isabella*. J Insect Physiol 24:6-7.

González-Santoyo I, Córdoba-Aguilar A. 2012. Phenoloxidase: A key component of the insect immune system. Entomol Exp Appl 142:1-16.

Haine ER, Moret Y, Siva-Jothy MT, Rolff J. 2008. Antimicrobial defense and persistent infection in insects. Science 322:1257-1259.

Harvell DH, Mitchell CE, Ward JR, Altizer S, Dobson AP, Ostfeld RS, Samuel MD. 2002. Climate warming and disease risks for terrestrial and marine biota. Science 296:2158-2162.

Huey RB, Berrigan D, Gilchrist GW, Herron JC. 1999. Testing the adaptive significance of acclimation: A strong inference approach. Amer Zool 39:323-336.

Irwin JT, Bennett VA, Lee RE, Jr. 2001. Diapause development in frozen larvae of the goldenrod gall fly, *Eurosta solidaginis* fitch (diptera: Tephritidae). J Comp Physiol B.

Irwin JT, Lee RE, Jr. 2003. Cold winter microenvironments conserve energy and improve overwintering survival and potential fecundity of the goldenrod gall fly, *Eurosta solidaginis*. Oikos 100:71-78.

Krams I, Daukšte J, Kivleniece I, Krama T, Rantala MJ. 2011. Overwinter survival depends on immune defence and body length in male *Aquarius najas* water striders. Entomolo Exp Appl 140:45-51.

Layne JR, Edgar CL, Medwith RE. 1999. Cold hardiness of the woolly bear caterpillar (*Pyrrharctia isabella* Lepidoptera: Arctiidae). Amer Midl Nat 141:293-304.

Lavine MD, Strand MR. 2002. Insect hemocytes and their role in immunity. Insect Biochem Molec Biol 32:1295-1309.

Le Bourg E, Massou I, Gobert V. 2009. Cold stress increases resistance to fungal infection throughout life in *Drosophila melanogaster*. Biogerontol 10:613-625.

Linderman JA, Chambers MC, Gupta AS, Schneider DS. 2012. Infection-related declines in chill coma recovery and negative geotaxis in *Drosophila melanogaster*. PloS One 7:e41907.

MacMillan HA, Williams CM, Staples JF, Sinclair BJ. 2012. Reestablishment of ion homeostasis during chill-coma recovery in the cricket *Gryllus pennsylvanicus*. PNAS USA 109:20750–20755.

Marshall KE, Sinclair BJ. 2011. The sub-lethal effects of repeated freezing in the woolly bear caterpillar *Pyrrharctia isabella*. J Exp Biol 214:1205-1212.

Marshall KE, Sinclair BJ. 2012. Threshold temperatures mediate the impact of reduced snow cover on overwintering freeze-tolerant caterpillars. Naturwissenschaften 99:33-41.

Mills NJ. 1981. The mortality and fat content of *Adalia bipunctata* during hibernation. Entomol Exp Appl 30:265-268.

Murdock CC, Paaijmans KP, Bell AS, King JG, Hillyer JF, Read AF, Thomas MB. 2012. Complex effects of temperature on mosquito immune function. Proc R Soc B 279:3357-3366.

R Development Core Team. 2010. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Ragland GJ, Denlinger DL, Hahn DA. 2010. Mechanisms of suspended animation are revealed by transcript profiling of diapause in the flesh fly. PNAS USA 107:14909-14914.

Rasband WS. 1997-2014. Image j. Version 1.45S. Bethesda, Maryland, USA: National Institutes of Health.

Salehipour-shirazi G, Ferguson LV, Sinclair BJ. 2017. Does cold activate the *Drosophila melanogaster* immune system? J Insect Physiol. 96: 29-34.

Sinclair BJ. 2015. Linking energetics and overwintering in temperate insects. J Therm Biol. 54: 5-11.

Sinclair BJ, Ferguson LV, Salehipour-shirazi G, MacMillan HA. 2013. Cross-tolerance and cross-talk in the cold: Relating low temperatures to desiccation and immune stress in insects. Int Comp Biol 53:545-556.

Steenberg T, Langer V, Esbjerg P. 1995. Entomopathogenic fungi in predatory beetles (col.: *Carabidae* and *Staphylinidae*) from agricultural fields. Entomophaga 40:77-85.

Steinmann N, Corona M, Neumann P, Dainat B. 2015. Overwintering is associated with reduced expression of immune genes and higher susceptibility to virus infection in honey bees. PloS One 10:e0129956.

Storey JM, Storey KB. 1986. Winter survival of the gall fly larva, *Eurosta solidaginis*: Profiles of fuel reserves and cryoprotectants in a natural population. J Insect Physiol 32.

Sugumaran M, Saul SJ, Ramesh N. 1985. Endogenous protease inhibitors prevent undesired activation of prophenolase in insect hemolymph. Biochem Biophys Res Comm 132:1124-1129.

Therneau TM, Grambsch PM. 2015. A package for survival analysis in R. Version 2.38.

Thomas MB, Blanford S. 2003. Thermal biology in insect-parasite interactions. Trends Ecol Evol 18:344-350.

Udaka H, Sinclair BJ. 2014. The overwintering biology of the acorn weevil, *Curculio glandium* in Southwestern Ontario. J Therm Biol 44:103-109.

Webberley KM, Hurst GDD. 2002. The effect of aggregative overwintering on an insect sexually transmitted parasite system. J Parasitol 88:707-712.

Williams CM, Henry HA, Sinclair BJ. 2015. Cold truths: How winter drives responses of terrestrial organisms to climate change. Biol Rev 90:214-235.

Xu J, James RR. 2012. Temperature stress affects the expression of immune response genes in the alfalfa leafcutting bee, *Megachile rotundata*. Insect Molec Biol 21:269-280.

Zhang J, Marshall KE, Westwood JT, Clark MS, Sinclair BJ. 2011. Divergent transcriptomic responses to repeated and single cold exposures in *Drosophila melanogaster*. J Exp Biol 214:4021-4029.

Zuur AF, Ieno EN, Elphick CS. 2010. A protocol for data exploration to avoid common statistical problems. Methods Ecol Evol 1:3-14.

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.

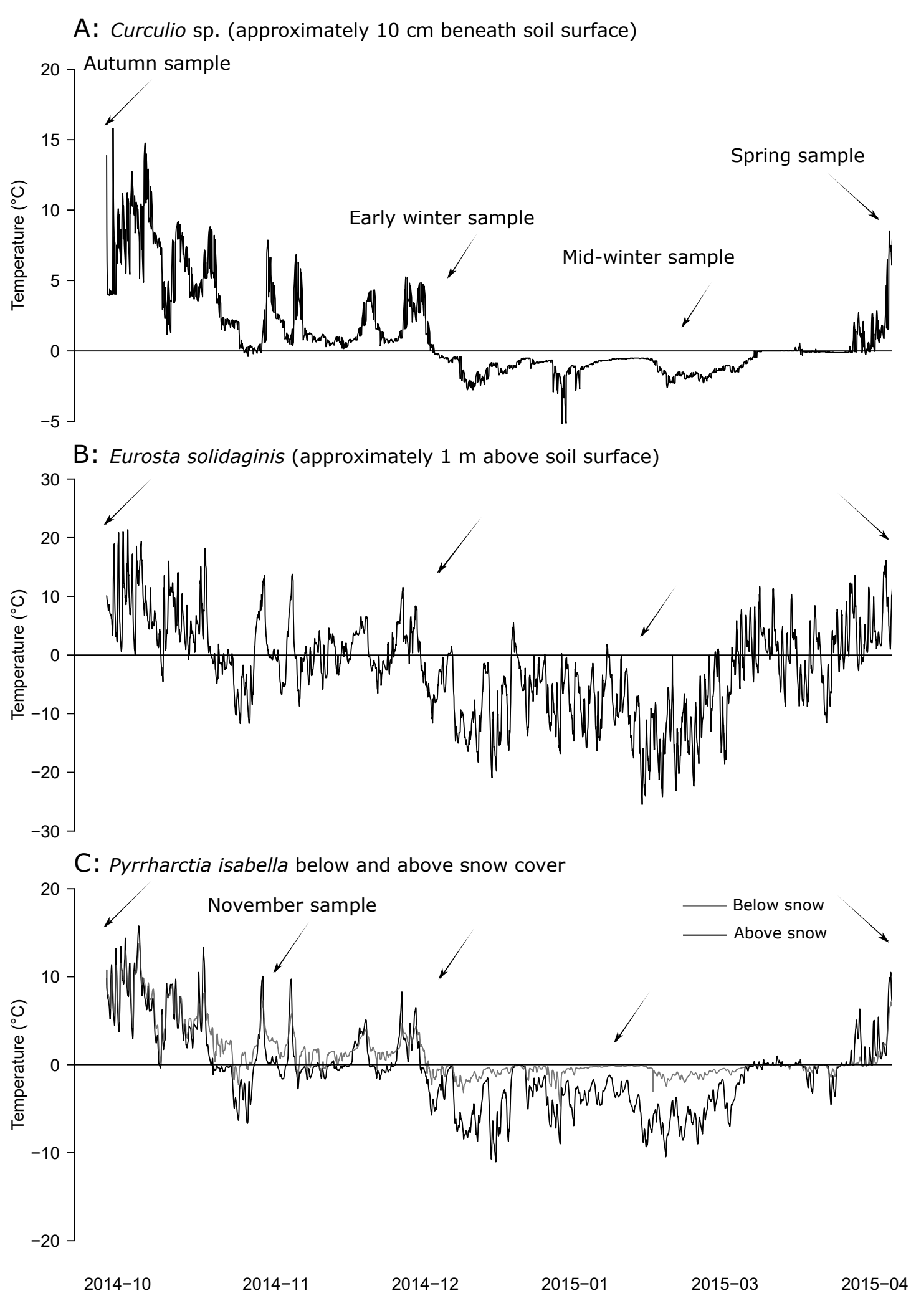
Fig. 6. Survival of infection with *Metarhizium brunneum* across seasons. Insects were infected topically with spores of *M. brunneum* suspended in 0.01% Tween80, and infection progressed at either 12 °C or 25 °C. Controls were inoculated with sterile 0.01% Tween80 and are shown as grey lines **A.**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

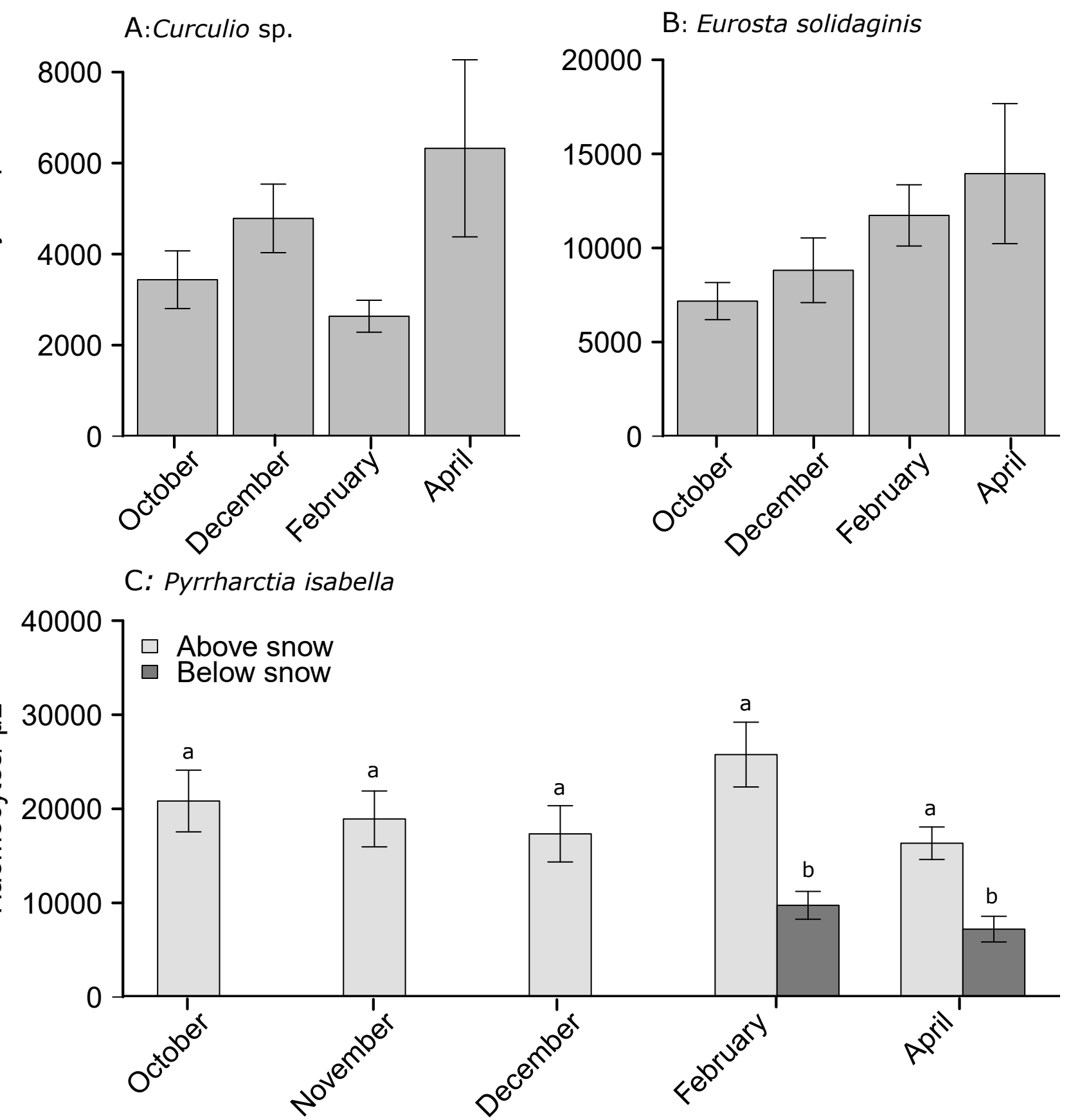
°C, December 12 °C; n = 25 October 12 °C; n = 17 December 25 °C; n = 14 February 25 °C; n = 15 February 12 °C; controls: n = 12-15). **C.** *Eurosta solidaginis* at 25 °C; **D.** *Eurosta solidaginis* at 12 °C; (n = 29 October 12 °C, n = 24 October 25 °C; n = 15 December; n = 18 February & April; controls: 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 idiosyncracy 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.

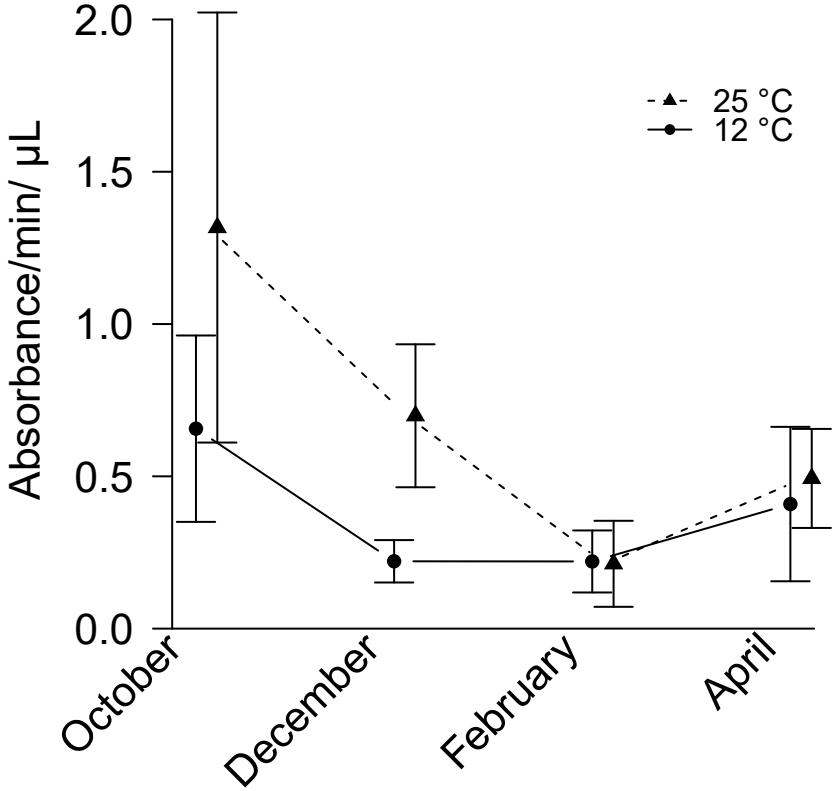
Table 1. Statistical results from lognormal models comparing survival curves. Survival was monitored at 12 °C and 25 °C for *Eurosta solidaginis* and *Curculio* sp. infected with the entomopathogenic fungus *Metarhizium brunneum*. Bolded p-values indicate significant differences.

Species	Temperature (°C)	Comparison	Z	P
E. solidaginis	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**
Curculio 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

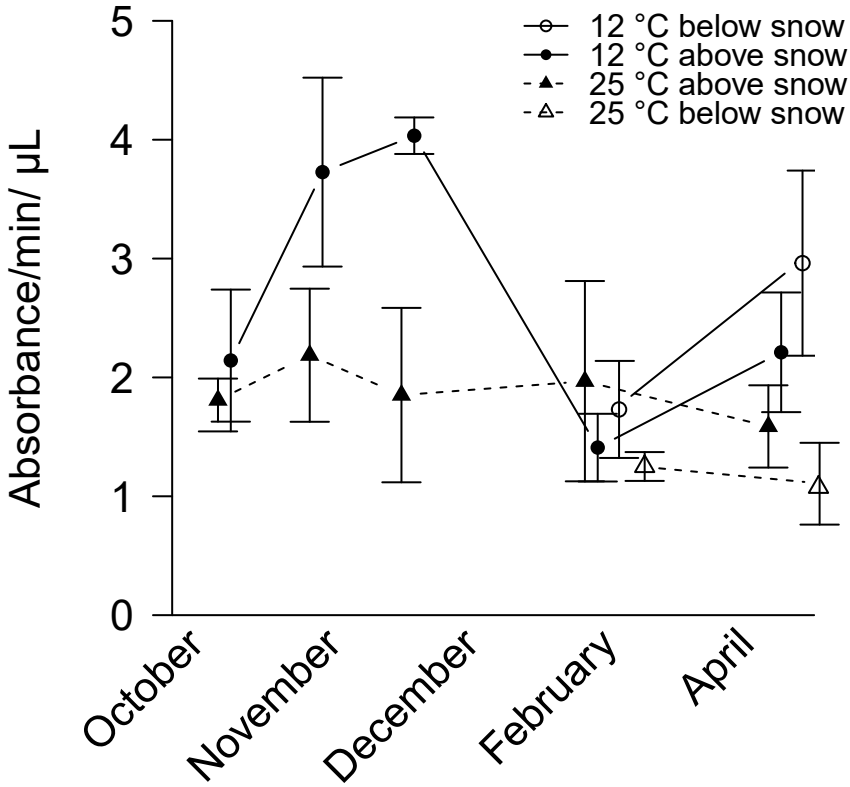

A: Curculio sp. (approximately 10 cm beneath soil surface)
Autumn sample
Early winter sample
Mid-winter sample
Spring sample
Temperature (°C)
20
15
10
5
0
−5
B: Eurosta solidaginis (approximately 1 m above soil surface)
30
20
10
0
−10
−20
−30
C: Pyrrharctia isabella below and above snow cover
November sample
Below snow
Above snow
2014−10
2014−11
2014−12
2015−01
2015−03
2015−04

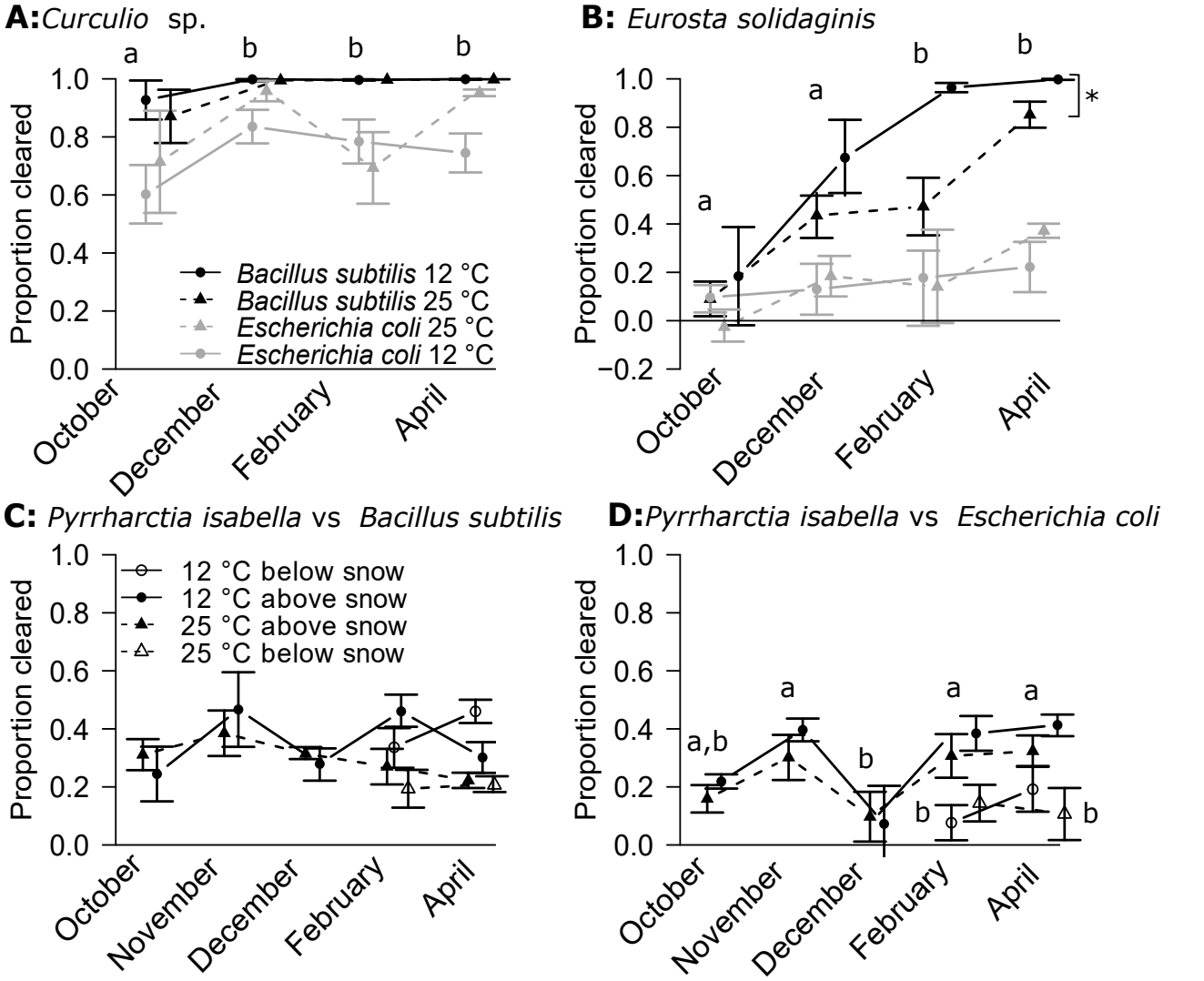

A: Curculio sp.
8000
6000
4000
2000
0
October
December
February
April
B: Eurosta solidaginis
20000
15000
10000
5000
0
October
December
February
April
C: Pyrrharctia isabella
40000
30000
20000
10000
0
Above snow
Below snow
a
a
a
a
b
a
b
October
November
December
February
April

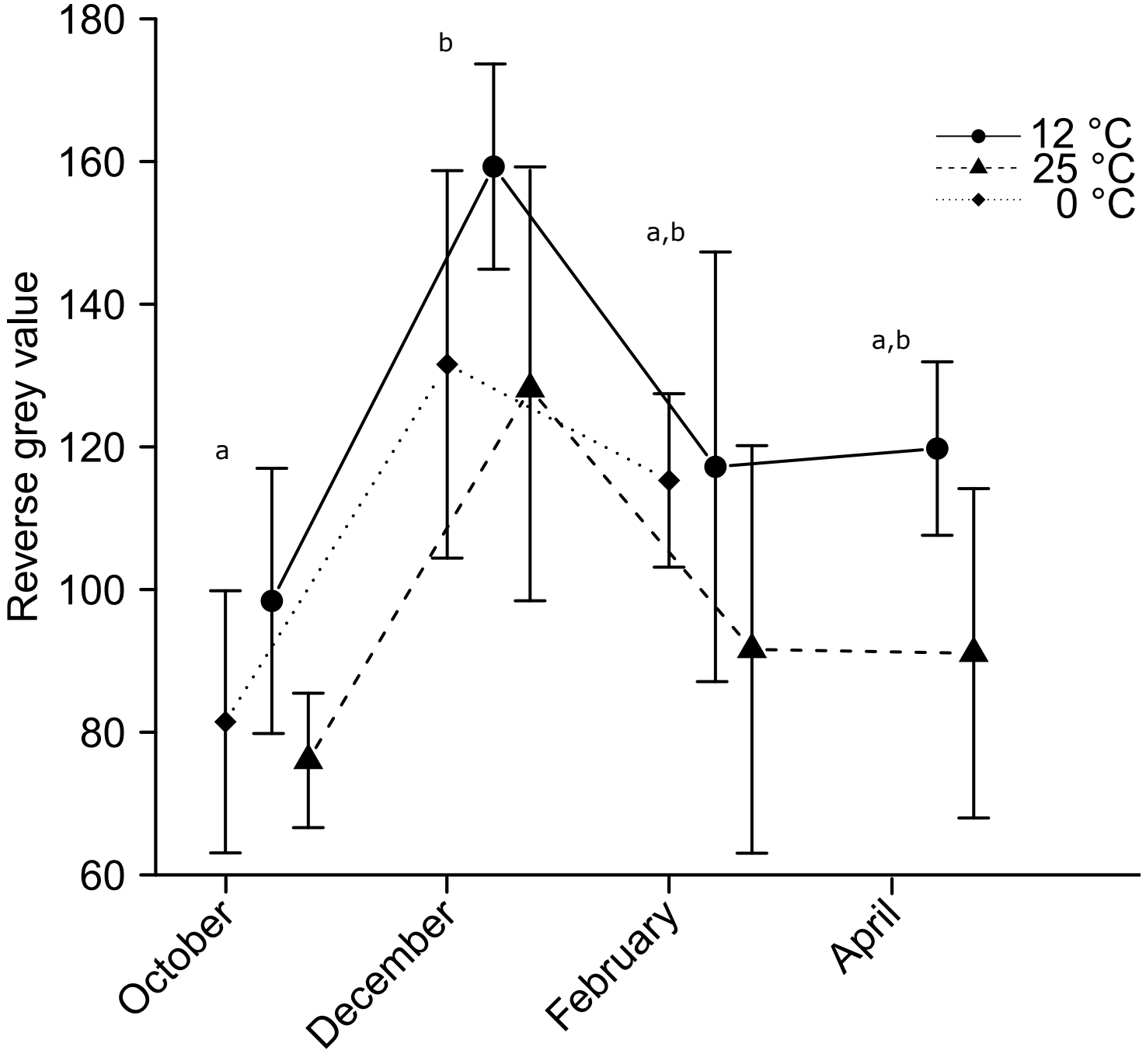
A: *Curculio* sp.

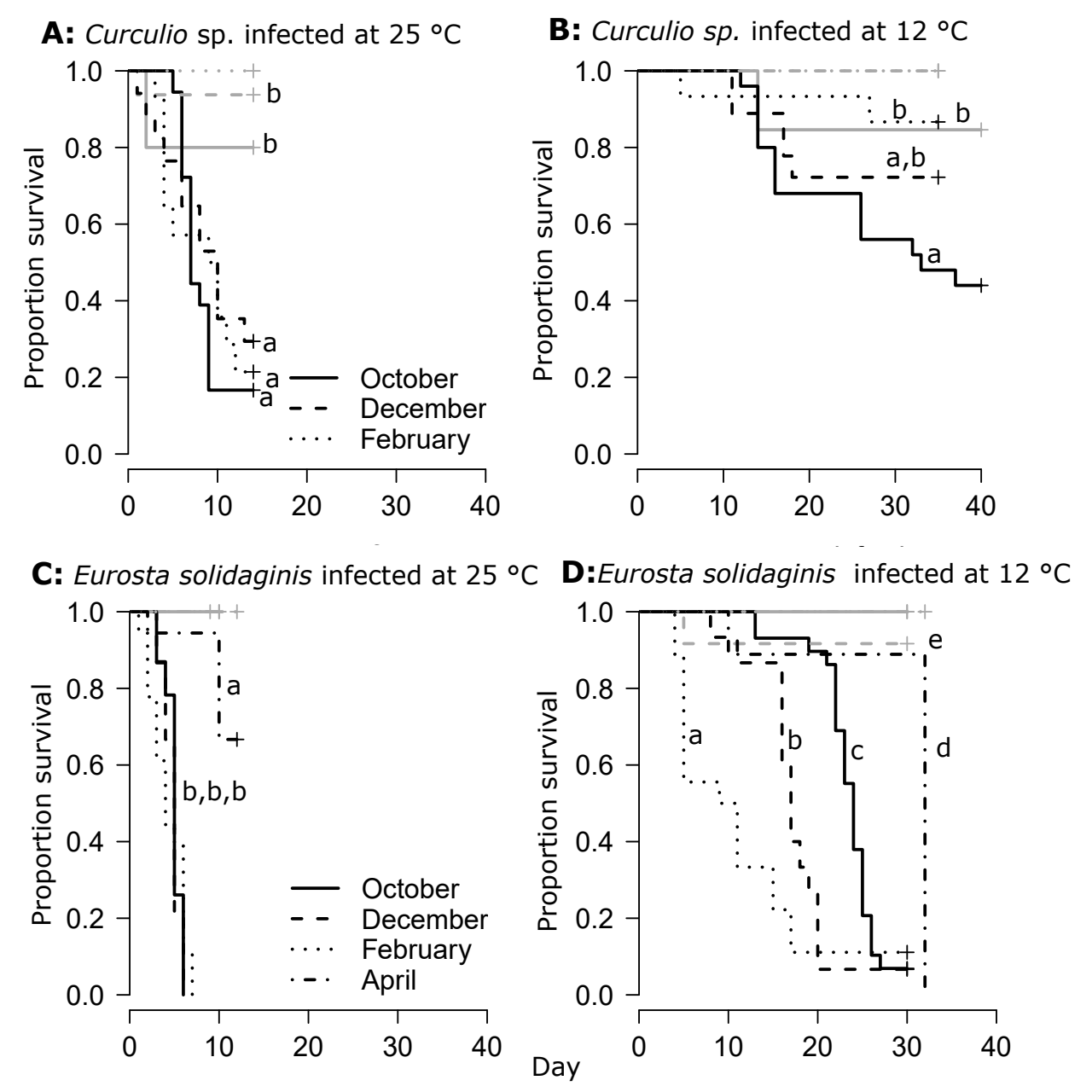


B: *Pyrrharctia isabella*




A: Curculio sp.
B: Eurosta solidaginis
C: Pyrrharctia isabella vs Bacillus subtilis
D: Pyrrharctia isabella vs Escherichia coli
Proportion cleared
Bacillus subtilis 12 °C
Bacillus subtilis 25 °C
Escherichia coli 25 °C
Escherichia coli 12 °C
12 °C below snow
12 °C above snow
25 °C above snow
25 °C below snow
October
November
December
February
April
a
b
a,b
*


180
160
140
120
100
80
60
Reverse grey value
a
b
a,b
a,b
12 °C
25 °C
0 °C
October
December
February
April


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
Proportion survival
Day
October
December
February
April
a
b
b
a
a
a
a,b
b
b
a
b,b,b
a
b
c
d
e