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RESEARCH ARTICLE

Simulated winter warming negatively impacts survival of Antarctica's only endemic insect

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Abstract

1. Antarctic winters are challenging for terrestrial invertebrates, and species that live there have specialised adaptations to conserve energy and protect against cold injury in the winter. However, rapidly occurring climate change in these regions will increase the unpredictability of winter conditions, and there is currently a dearth of knowledge on how the highly adapted invertebrates of Antarctica will respond to changes in winter temperatures.
2. We evaluated the response of larvae of the Antarctic midge, *Belgica antarctica*, to simulated winters at three ecologically relevant mean temperature scenarios: warm (−1°C), normal (−3°C) and cold (−5°C). Within each scenario, larvae were placed into three distinct habitat types in which they are commonly observed (decaying organic matter, living moss, and *Prasiola crispa* algae). Following the simulated overwintering period, a range of physiological outcomes were measured, namely survival, locomotor activity, tissue damage, energy store levels and molecular stress responses.
3. Survival, energy stores and locomotor activity were significantly lower following the Warm overwintering environment than at lower temperatures, but tissue damage and heat shock protein expression (a proxy for protein damage) did not significantly differ between the three temperatures. Survival was also significantly lower in larvae overwintered in *Prasiola crispa* algae, although the underlying mechanism is unclear. Heat shock proteins were expressed least in larvae overwintering in living moss, suggesting it is less stressful to overwinter in this substrate, perhaps due to a more defined structure affording less direct contact with ice.
4. Our results demonstrate that a realistic 2°C increase in winter microhabitat temperature reduces survival and causes energy deficits that have implications for subsequent development and reproduction. While our Warm winter scenario was close to

the range of observed overwintering temperatures for this species, warmer winters are expected to become more common in response to climate change. Conversely, if climate change reduces the length of winter, some of the negative consequences of winter warming may be attenuated, so it will be important to consider this factor in future studies. Nonetheless, our results indicate that winter warming could negatively impact cold-adapted insects such as the Antarctic midge.

KEYWORDS
Antarctica, climate change, entomology, microclimate, physiological ecology

1 | INTRODUCTION

Winter presents distinct challenges for organisms. Consequently, species distributions, population dynamics, and responses to environmental change are often linked to winter conditions (Williams et al., 2015). Polar environments, characterised by long, harsh winters and highly seasonal resource availability, have considerably reduced terrestrial biodiversity relative to the rest of the planet (Convey, 2013). These environments are particularly challenging for terrestrial invertebrates, and polar species require unique adaptations to cope with short growing seasons and long harsh winters (Teets & Denlinger, 2014). However, these adaptations have been shaped over millions of years and, thus, these species may now be vulnerable to rapid environmental modifications, such as anthropogenically driven climate change.

In the Antarctic Peninsula, which harbours much of Antarctica's taxonomic diversity, winter air temperatures occasionally drop below −40°C (Bale & Hayward, 2010). In contrast, the microhabitats of many terrestrial invertebrates are buffered by snow and ice and typically remain between 0 and −5°C (Baust & Lee Jr., 1981; Elnitsky et al., 2008; Kawarasaki et al., 2014a). Temperatures as low as −10°C have been observed, but these are rare and considerably higher than temperatures above the snow layer (Convey et al., 2018). The Antarctic Peninsula and Scotia Arc, however, are warming more quickly than most of the globe, with macroclimate temperature increases up to 0.56°C per decade occurring between 1951–2000, with the fastest warming occuring during the winter (Steig et al., 2009; Turner & Overland, 2009). In these regions, annual snow accumulation has also increased over the last 100 years (Thomas, Melchior Van Wessem, et al., 2017). Elevated temperatures and increased snow depth will likely warm overwintering habitats, which can increase metabolic rates and influence winter acclimatisation for resident terrestrial invertebrates (Bale & Hayward, 2010; Marshall et al., 2020). Conversely, climate change may modify the length of winter, including delaying the onset of snow accumulation which, along with earlier snowmelt in spring, could make freezing events in early and late winter more stressful due to the absence of the insulating snow (Roberts et al., 2021; Williams et al., 2015).

Despite their buffered overwintering habitats, Antarctic invertebrates experience prolonged sub-zero temperatures that challenge metabolism and survival (Everatt et al., 2015), and successful overwintering requires multiple physiological and biochemical adjustments (see reviews by Hahn & Denlinger, 2011; Overgaard & MacMillan, 2017). The chironomid midge *Belgica antarctica* (Jacobs 1900, Diptera) has been a model system for investigating adaptations to terrestrial Antarctic environments (Kozeretska et al., 2021; Lee & Denlinger, 2015), and sequencing data indicate that this species has been evolving in isolation from its closest relative (*Eretmoptera murphyi*, endemic to sub-Antarctic South Georgia) for over 40 MYr (Allegrucci et al., 2006). The distribution of *B. antarctica* is highly heterogenous, with considerable variation in abiotic and biotic conditions on small spatial scales (Potts et al., 2020; Spacht et al., 2021). Larvae are generalist herbivores and detritivores, and are typically associated with moss beds, mats of the terrestrial algae *Prasiola crispa*, and decaying organic matter (Lee & Denlinger, 2015). This microhabitat diversity suggests the potential for behavioural regulation of their environment, as noted in other Antarctic invertebrates (Hayward et al., 2001, 2003); however, behavioural responses would only provide limited protection from winter cold. Larvae are freeze-tolerant year around but can also remain supercooled or undergo cryoprotective dehydration at low temperatures in dry environments (Elnitsky et al., 2008; Teets et al., 2020). In addition, seasonal cold acclimation and rapid cold hardening can cause plastic increases in freeze tolerance (Lee et al., 2006; Teets et al., 2019).

Cold tolerance and overwintering success in Antarctic midges may also be influenced by conditions in the surrounding soil. In other insects, soil characteristics such as moisture content, water potential, and texture can influence cold survival (Costanzo et al., 1997; Forge & MacGuidwin, 1992; Holmstrup, 2014), and indeed, in *B. antarctica* water and organic content affect whether larvae freeze or undergo cryoprotective dehydration in laboratory conditions (Elnitsky et al., 2008). Further, for polyphagous insects, plant type and quality can influence cold tolerance through changes in metabolism and cryoprotectant accumulation (Liu et al., 2009; Morey et al., 2016). Thus, in highly heterogenous environments such as terrestrial Antarctica, biotic conditions may have an unappreciated role in modifying cold tolerance, but the extent to which soil and plant composition influence overwintering survival in Antarctic insects has not been addressed.

Whilst the physiological costs of short-term freezing are well-described (e.g. Kawarasaki et al., 2014b; Teets et al., 2011, 2019, 2020), the logistic practicalities of fieldwork in Antarctica prevent direct investigations of overwintering *B. antarctica* in their natural habitats. In the published literature, the maximum duration of experimental low

temperature exposure is 32 days (Kawarasaki et al., 2014a), which larvae readily survive, but there have been no attempts to simulate an entire overwintering period. In cold environments, it might be expected that warmer winters would reduce cold stress and increase survival, but polar invertebrate taxa appear to have varied responses to warming (see reviews by Bale & Hayward, 2010; Nielsen & Wall, 2013). Increased overwintering temperatures can elevate metabolic rate, leading to energy depletion in some temperate insects (Irwin & Lee, 2000, 2003; Williams et al., 2012). In the Antarctic mite *Alaskozetes antarcticus* metabolic rate increases rapidly with temperature, suggesting an inability to maintain energy balance at higher temperatures (Convey, 1992; Young & Block, 1980). On the other hand, cold winters (i.e. years with lower temperatures and/or less snowfall and reduced temperature buffering) may increase the risk of mortality from cold injury (Roberts et al., 2021). However, the extent to which winter temperatures influence the survival and energetics of Antarctic arthropods such as *B. antarctica* is unknown.

Here, we investigated the physiological effects of winter temperature on *B. antarctica larvae*, using three distinct thermal regimes that reflect ecologically relevant overwintering scenarios. We also provided three distinct substrate types, as physical characteristics of soil habitats can influence overwintering success in other insects (Costanzo et al., 1997; Holmstrup, 2014). Larvae were held in simulated overwintering environments for 180 days, after which we measured survival, locomotor activity, tissue damage, energy store levels and molecular stress responses. We hypothesised that warm winters would reduce energy stores and survival, due to the increased metabolic demands and the fact that the coldest winter environment was still well above the lower lethal limit. Additionally, while we predicted larvae would survive the coldest conditions, we hypothesised that cold winters would elicit sub-lethal cold injury, reflected by increased tissue damage and elevated heat shock protein expression, a well-established symptom of stress in *B. antarctica* (Teets et al., 2019, 2020; Teets, Peyton, et al., 2012). Finally, as we expected all larvae to undergo inoculative freezing in the three substrates used (Kawarasaki et al., 2014b), we predicted that we would see no difference across substrate type. Our results will inform predictions on how rapidly warming winter conditions may impact highly adapted invertebrate species that play important ecological roles in polar terrestrial ecosystems.

2 | MATERIALS AND METHODS

2.1 | Source of larvae

Larvae of *B. antarctica* were collected on islands within a 3 km radius of Palmer Station, Anvers Island (64°46′27″S, 64°03′10″W) in February 2020, from heterogeneous habitats that primarily consisted of decaying organic matter, moss beds and algal mats. These larvae were primarily 4th instar and were collected prior to their second Antarctic winter, after which they pupate and emerge as adults (Peckham, 1971). Following shipment to the home laboratory (University of Kentucky, Lexington KY, USA; ~1 month shipping time), larvae were maintained as a colony at 4°C on natural substrate containing a mix of rocks, decaying moss, and live *Prasiola crispa* algae until the experiment began. Larvae become significantly more cold-tolerant when they are returned to the laboratory, likely due to a combination of natural phenology and shipping at low temperatures, and hence can be considered winter-acclimated (Lee et al., 2006; Teets et al., 2020).

2.2 | Simulation of winter conditions

To create ecologically relevant winter conditions, environmental data from the following sources were used: two HOBO Water Temp Pro loggers from Torgersen Island (64°46′S 64°05′W), deployed from January 2005 to January 2006 (Elnitsky et al., 2008; Schulte et al., 2008); one HOBO Water Temp Pro logger from Anvers Island (64°45′S 64°05′W) and two Optic StowAway-TEMP(C)ONSET loggers deployed from January 2011 to January 2012 on Humble Island (64°45′S 64°05′W) (Kawarasaki et al., 2013, 2014a). We defined the Antarctic winter as the period when temperatures were consistently below −1°C (Figure S1), at which larvae are likely to be frozen due to inoculative freezing. During this period (approximately mid-April to mid-October), the mean temperature across all five datasets was −3.09°C (*SD* = 0.95, Figure 1a). The coldest winter had a mean temperature of −5.16°C (minimum temperature of −10.02°C), while the warmest winter had a mean temperature of −1.71°C (minimum temperature of −3.72°C). Accordingly, we chose the following conditions as our simulated winter regimes, with each condition lasting 180 days (Figure 1b): warm winter (mean temperature of −1°C), normal winter (mean temperature of −3°C) and cold winter (mean temperature of −5°C). Starting at 1°C, the temperature ramped down at approximately 0.5°C/day until the desired temperature was reached, following Kawarasaki et al. (2014a). Constant temperatures were chosen due to the snow-covered habitats providing a stable temperature during the winter (Convey et al., 2018). To simulate overwintering temperatures, three programmable incubators were used (Panasonic MIR-154), one per overwintering regime. One HOBO Water Temp Pro logger was placed in each incubator for 72 hr prior to the experiment to determine any discrepancies between the desired and recorded temperatures. After adjusting incubator temperatures, temperature was recorded at 5-min intervals for the duration of the experiment.

In addition, three substrates collected in Antarctica during the 2020 season were used to investigate the impact of habitat/substrate type on overwintering success: decaying organic matter, living moss (mix of *Polytrichum strictum* and *Chorisodontium aciphyllum*; Amesbury et al., 2017) and living algae (*Prasiola crispa*). Approximately 750 ml of each substrate was collected and extensively searched to remove any midge larvae prior to the experiment. To standardise moisture, each substrate was hydrated to saturation using deionised water, as these would likely be the conditions at the onset of the Antarctic winter in coastal areas (Kawarasaki et al., 2014b).

For each treatment group (nine treatments total; three winter treatments by three substrate types), five replicate 50 ml tubes were

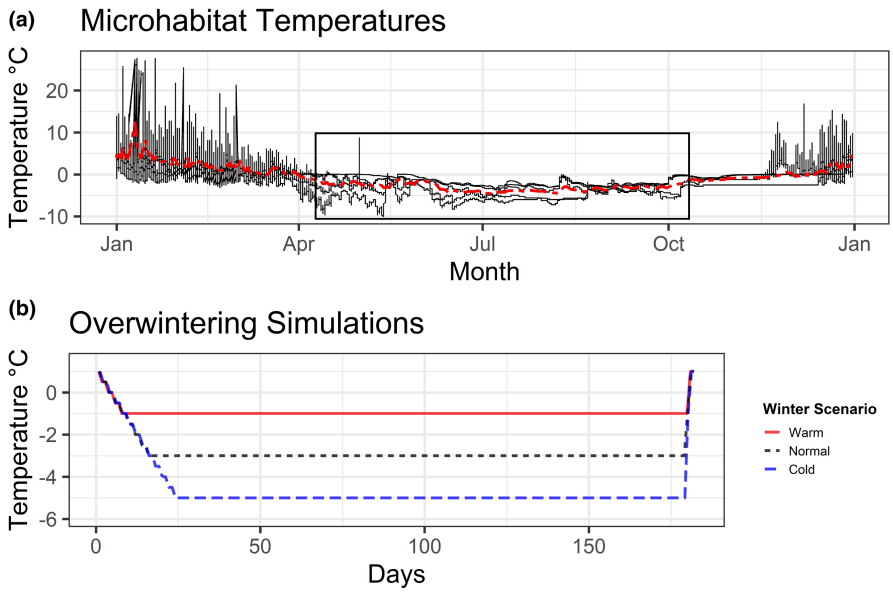

FIGURE 1 Temperature regimes across Antarctic winters and simulated winters. (a) Five available datalogger datasets were used to develop the experimental Antarctic winter microhabitat temperature regimes. Mean temperature across all five datasets is represented by the dotted red line, with the black rectangle denoting the approximate duration of the Antarctic winter, which was used to calculate average winter conditions. (b) The three overwintering temperatures generated for this study, all with a starting temperature of 1°C. Initial ramping took place at 0.5°C/day until the desired temperature was reached.

used, each filled with ~25 ml substrate and containing ~60 larvae (although due to counting errors and inability to recover all larvae, the mean number of recovered larvae per tube was 50 (range 21–80). Five 1 mm holes were made on each cap, with a cheesecloth placed between the tube and the cap to prevent escapes. After the simulated winter, samples were returned to 1°C for 24 hr, after which substrate was dispersed in ice water to separate larvae. Larvae were then given a recovery period of ~48 hr at 4°C on moist filter paper in a Petri dish, prior to physiological measurements. Fresh mass was measured prior to physiological measures. While larvae typically recover within 24 hr from short-term freezing (Teets et al., 2011, 2019), in these experiments, larvae were immobile until 72 hr after treatment. Thus, to ensure all our measurements were taken at the same time, and to avoid sampling dead larvae for molecular analyses, the total recovery time for all experiments was 72 hr (24 hr at 1°C, 48 hr at 4°C).

2.3 | Survival and recovery of locomotion

All larvae that were recovered following the overwintering simulations were assessed for survival by placing larvae in ice water and checking for either spontaneous movement or movement following gentle prodding. Locomotor activity was evaluated by recording the number of head capsule contractions in 60 s (n = 5 larvae per replicate, 25 per treatment). Larvae were placed in a plastic weighing dish and recorded using a Motic microscope camera with Motic Images Plus 2.0 (Motic, Shertz, TX). Previous studies have shown that this measure of locomotor activity is correlated with other metrics of freezing injury (Teets et al., 2019).

2.4 | Tissue damage/cell survival

Tissue damage was quantified using the LIVE/DEAD Sperm Viability Kit (ThermoFisher Scientific, Waltham, MA) according to Yi and Lee (2003). Midgut tissue samples were dissected in Coast's solution (Coast & Krasnoff, 1988) and incubated in SYBR-14 dye for 10 min, after which propidium iodide was added and the slide was incubated for a further 10 min. Tissues were imaged using a fluorescence microscope with FITC and Texas Red filters. The proportions of live (fluorescing green) and dead (fluorescing red) cells were assessed over areas with ~300 cells. For each treatment, N = 4–5 independent replicates.

2.5 | Energy store levels—Carbohydrates, lipids, and proteins

Larvae were maintained in the home laboratory in the same pan prior to experiments, so we assumed that the initial mean energy stores would be equal for each treatment group. Larvae were frozen at −80°C prior to energy store analysis. Each sample contained a pool of two larvae for proteins and lipids and four larvae for carbohydrates (n = 2–5 independent pools of larvae per treatment). For each treatment, a subset of two larvae was used to estimate water content, necessary as a correction factor in our analyses. Energy stores were measured using colorimetric assays, as previously described by Spacht et al. (2021). Briefly, total carbohydrates were quantified with the anthrone method, total lipid stores with vanillin-phosphoric acid reagent and total protein stores were measured with the Pierce BCA protein assay kit, according to the manufacturer's protocol (ThermoFisher).

2.6 | Stress gene expression

Larvae were frozen at −80°C prior to assessing stress gene expression. Specifically, we measured the mRNA expression of heat shock proteins, which have well-established stress response roles in *B. antarctica* (Lopez-Martinez et al., 2008; Teets et al., 2019; Teets, Peyton,

et al., 2012). Five genes, each from a major heat shock protein family, were assessed (Table 1). Primers sequences for heat shock proteins were obtained from Teets et al. (2019), whilst primers for ribosomal protein l19 (rpl19) were as per Teets et al. (2013).

RNA was extracted from pools of five larvae per treatment using Tri Reagent (ThermoFisher), resuspended in Buffer RLT (Qiagen, Germantown, MD, USA) and further purified using RiboPure RNA purification kit (ThermoFisher). RNA quantity and purity were assessed with a spectrophotometer. First-strand cDNA synthesis was conducted with the qScript cDNA synthesis kit (Quanta bio, Beverley, MA, USA), and the cDNA samples were diluted 10-fold. Two microlitres of cDNA were then used for qPCR reactions, which also contained the following components: 10 µl 2x PerfeCTa SYBR Green Fast Mix, 2 µl of both forward and reverse primer (2.5 µmol/L concentration), 4 µl nuclease-free water. Using a QuantStudio 6 Flex real-time PCR system (ThermoFisher), reactions were run for a total of 40 cycles and cycle threshold (Ct) values were calculated. Following Teets et al. (2013, 2020), gene expression was calculated using $2^{-\Delta Ct}$ method, with the expression of each gene normalised to rpl19, and fold changes were scaled relative to the treatment group *normal winter, decaying organic matter*. We used this group as the reference because the normal winter was considered standard, and decaying organic matter is the most common habitat in which larvae are typically found in the field.

2.7 | Statistical analysis

Larval and cell survival were analysed using a binomial generalised linear mixed effect model (GLMM) within the R package LME4 (Bates et al., 2015), with both winter temperature and substrate type as independent variables and replicate tube nested within the interaction between winter temperature and substrate type as a random effect. Pairwise comparisons for each independent variable were then made using Tukey's test of means. Locomotion data were also assessed using a GLMM, but with a Poisson error family. To estimate effect sizes for models, the function *F_to_eta2* within the package EFFECTSIZE (Ben-Shachar et al., 2020) was used. Model theoretical R^2 was calculated using the package MuMIn (Barton, 2020).

Energy store levels (proteins, lipids and carbohydrates) were analysed in a general linear model (GLM) using the independent variables winter temperature and substrate type, with dry mass of each sample included as a covariate. Stress gene expression (ΔCt value) was investigated using a GLM for each gene, with winter temperature and substrate type used as the independent variables in each case. To determine estimated effect sizes for each GLM, the function *effectsize* within the package EFFECTSIZE was used following variance calculation using the function *anova*.

For each analysis, overall model performance, the error family and the link function for models were assessed using the package PERFORMANCE (Lüdecke et al., 2021), which ranks models based on five model indices (AIC, BIC, R2, RMSE and Sigma). To assess model fit, we used visual confirmation of standardised residuals plotted against model fitted values, as well as the production of residual distributions that best satisfied the model assumptions (Thomas, Lello, et al., 2017). All data used to generate the figures in this paper are available from the Dryad Digital Repository (https://doi.org/10.5061/dryad.d7wm37q3k).

3 | RESULTS

3.1 | Overwintering temperatures

Incubator temperature closely tracked the desired temperature. Excluding ramp down and ramp up procedures, mean temperatures achieved across the warm, normal and cold winter conditions were −1.33°C (*SD* = 0.28), −2.97°C (*SD* = 0.16) and −4.94°C (*SD* = 0.28), respectively (Figure S2).

3.2 | Survival

Median survival for each winter condition, calculated from three substrate types, was: warm winter = 33.33% (*SD* = 25.78), normal winter = 48.72% (*SD* = 29.65), cold winter = 74.07% (*SD* = 27.31). Survival in the warm winter was significantly lower than in either the normal winter (GLMM, Tukey, *z*-value = −4.832, $p < 0.001$) or the cold winter (GLMM, Tukey, *z*-value = −7.410, $p < 0.001$). Survival in the cold was also higher than in the normal winter, although this difference was less distinct than in comparison with the warm winter (GLMM, Tukey, *z*-value = 2.801, $p = 0.014$). Effect sizes for both winter temperature and substrate indicated that survival was more strongly affected by substrate than winter

TABLE 1 Primers for stress gene expression measurements. Ribosomal protein l19 (rpl19) was used a control gene

Gene	GenBank accession no.	Forward primer	Reverse primer
sHsp	GAAK01009816	5′-GACACCCTTATCAGACGACTAC-3′	5′-CTTCTCGTTCTTCGTGCTTTG-3′
hsp40	GAAK01004380	5′-ACTCTGACCGGAGAAGTGATA-3′	5′-CTCGCTTTGTTGGCTCTTTG-3′
hsp60	GAAK01010161	5′-GTTGCAGGGAGTTGACATAC-3′	5′-GGCAACAGTTACACCATCTT-3′
hsp70	GAAK01011953	5′-CTGCTTTGGCTTACGGTTTG-3′	5′-CCTTCGTCGATGGTCAAGATAG-3′
hsp90	GAAK01011429	5′-CCGGTGGTAGCTTTATCATCTC-3′	5′-GGTAACGATGGCCTTGATCTTA-3′
rpl19	JX462670	5′-ACATCCACAAGCGTAAGGCTGAGA-3′	5′-TTCTTGTTTCTTGGTGGCGATGCG-3′

temperature (partial eta^2; 0.76 and 0.39, respectively, binomial GLMM, deviance = 269.4, residual degrees of freedom = 38, theoretical R^2 = 0.85). Across all three temperatures, survival in living algae was significantly lower than in decaying organic matter (GLMM, Tukey, z-value = −10.05, $p < 0.001$) or living moss (GLMM, Tukey, z-value = −11.06, $p < 0.001$), and no larvae survived on algae in the warm winter (Figure 2a).

3.3 | Locomotor activity

Locomotion rate (number of head contractions in 60 s) was more strongly affected by substrate than winter temperature (Figure 2b, partial eta^2, 0.32 and 0.04, respectively, Poisson GLMM, deviance = 1,545.1 residual degrees of freedom = 191, theoretical R^2 = 0.549). Locomotion was significantly lower in the warm winter

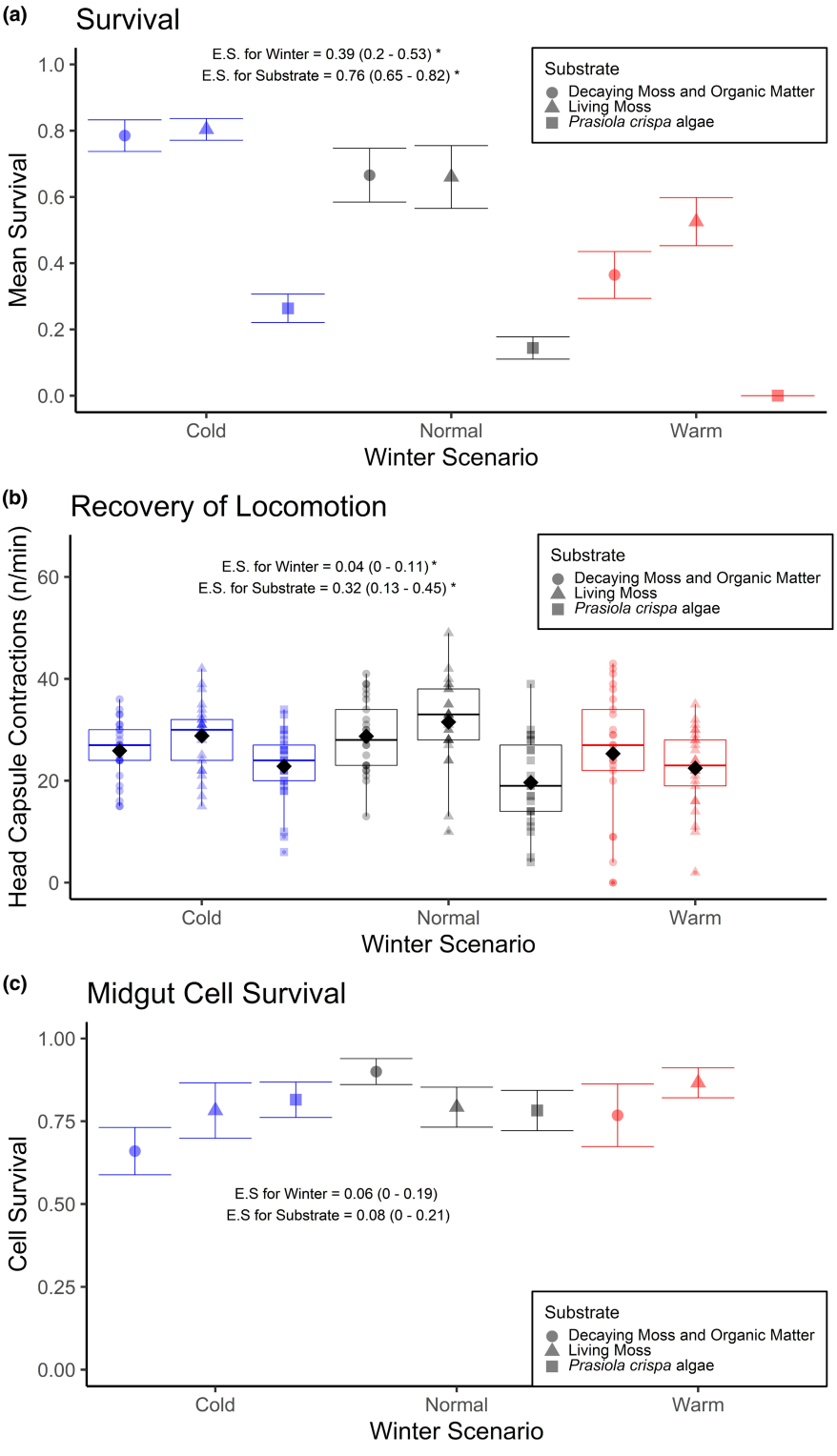

FIGURE 2 Effects of overwintering conditions on (a) Survival of overwintered larvae (b) Locomotor activity and (c) Proportion of surviving midgut cells. Larvae were exposed to either cold (−5°C), normal (−3°C), or warm (−1°C) winters for 180 days in each of three substrates (Decaying Organic Matter, Living Moss and *Prasiola crispa* algae). In panels (a) and (c), mean value with associated standard error bars is shown. In (b), each symbol represents an individual larva, with mean value in each group plotted as a black solid diamond. Partial eta^2 values are shown (effect size (E.S.), calculated using the package *effectsize*), where * denotes a significant contribution to overall model structure and brackets contain 90% confidence intervals. Given 100% mortality observed among larvae exposed to Warm winter condition in *Prasiola* algae, no data were available for this group in (b) and (c).

regime than the normal (Poisson GLMM, Tukey, *z*-value = −2.511, $p = 0.032$) and cold (Poisson GLMM, Tukey, *z*-value = −2.403, $p = 0.043$). Larvae overwintered on living algae had lower locomotion rates than either decaying organic matter (Poisson GLMM, Tukey, *z*-value = −3.863, $p < 0.001$) or living moss (Poisson GLMM, Tukey, *z*-value = −4.238, $p < 0.001$).

3.4 | Tissue damage/cell survival

Midgut tissue viability (Figure 2c) was 81.7% (*SD* = 16.46%), 83.1% (*SD* = 11.47%) and 75.3% (*SD* = 14.5%), in the warm, normal and cold winter regimes, respectively. In this model (Binomial GLMM, deviance = 1,166, residual degrees of freedom = 28, theoretical $R^2 = 0.15$), differences across winters were not significantly different (GLMM, Tukey, $p > 0.05$, partial eta$^2 = 0.06$). Similarly, the effect of substrate type was also not statistically significant (GLMM, Tukey, $p > 0.05$, partial eta$^2 = 0.08$).

3.5 | Energy store analyses—Total carbohydrates, lipids and proteins

Energy stores were analysed following 72 hr of recovery time following the overwintering scenarios. Due to the relatively high rate of mortality observed in larvae that overwintered on living algae, we were unable to include data from this substrate groups across all simulated winter conditions in this analysis.

For carbohydrates (Figure 3a), the model (GLM, $F_{4,20} = 30.31$, $p < 0.001$, adjusted $R^2 = 0.83$), indicated that dry mass and winter conditions had greater effects than substrate type (dry mass: *F*-value = 102.08, $p < 0.001$, partial eta$^2 = 0.84$; winter scenario: *F*-value = 8.55, $p = 0.002$, partial eta$^2 = 0.46$; substrate: *F*-value = 2.065, $p = 0.166$, partial eta$^2 = 0.09$). Carbohydrate levels were positively correlated with dry mass, indicating that larger larvae had higher carbohydrate levels (GLM, $t = 10.103$, $p < 0.001$). Carbohydrate stores were significantly higher in the Normal winter compared to the Cold winter (GLM, Tukey, $t = 2.676$, $p = 0.037$).

Total lipids were lowest in larvae overwintered in living moss substrate under the warm winter regime (Figure 3b). In this model (GLM, $F_{4,20} = 34.24$, $p < 0.001$, adjusted $R^2 = 0.873$), dry mass had the greatest effect on lipid stores (*F*-value = 99.856, $p < 0.001$, partial eta$^2 = 0.83$), followed by winter (*F*-value = 13.145, $p < 0.001$, partial eta$^2 = 0.57$) and substrate (*F*-value = 10.798, $p = 0.003$, partial eta$^2 = 0.35$). Larvae from the cold winter had significantly higher lipid levels than those under warm conditions (GLM, Tukey, $t = 2.954$, $p = 0.02$). Lipid stores did not vary significantly between substrate types ($t = -0.315$, $p = 0.756$).

Total protein stores exhibited a clear trend related to winter temperature, being highest in the Cold winter (Figure 3c). In this model (GLM, $F_{4,20} = 71.89$, $p < 0.001$, adjusted $R^2 = 0.92$), protein stores were more affected by larval dry mass (*F*-value = 245.1, $p < 0.001$, partial eta$^2 = 0.92$) than either substrate (*F*-value = 20.861, $p < 0.001$, partial

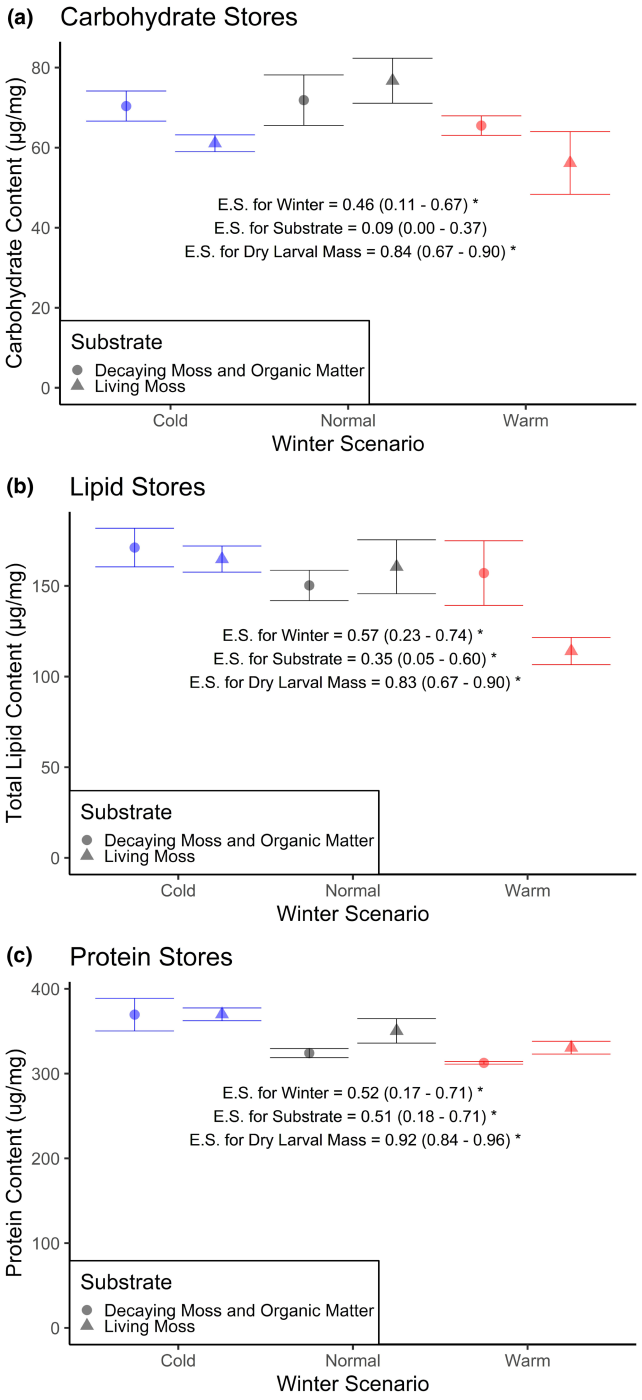

FIGURE 3 Energy store levels of surviving *Belgica antarctica* larvae following exposure to different simulated overwintering conditions. (a) Total carbohydrate stores assessed using Anthrone reagent. (b) Total lipid stores assessed using vanillin-phosphoric acid reagent. (c) Total protein stores assessed using BCA Protein Assay. Larvae were exposed to either Cold (−5°C), Normal (−3°C), or Warm (−1°C) winters for 180 days in each of three substrates (Decaying Organic Matter, Living Moss and *Prasiola crispa* algae). In each plot, nutrient store value was corrected by dry mass of each sample. For each treatment, $n = 2–5$ independent samples. In each plot, mean value with associated standard error is shown, with partial eta^2 values shown (effect size (E.S.), calculated using the package *effectsize*), where * denotes a significant contribution to overall model structure and brackets contain 90% confidence intervals.

TABLE 2 GLM analyses for expression of five stress genes from *Belgica antarctica* larvae following a 180-day overwintering period within different substrates across three biologically relevant Antarctic winter temperature scenarios. Winter and substrate *F*-values were calculated using the function *anova*. Asterisks denote significance ($p < 0.05$).

Gene	Model *F*-value	Model *p*-value	Model adjusted R^2	Winter *F*-value	Substrate *F*-value
Hsp40	$11.14_{3,20}$	<0.001	0.569	2.147	29.123*
Hsp60	$1.293_{3,20}$	0.304	0.037	1.634	0.611
Hsp70	$1.695_{3,20}$	0.2	0.083	0.373	4.339
Hsp90	$2.625_{3,20}$	0.079	0.175	1.255	5.364*
sHsp	$6.313_{3,20}$	0.003	0.409	3.318	12.302*

eta^2 = 0.51) or winter (*F*-value = 10.793, $p < 0.001$, partial eta^2 = 0.52). Total protein stores were higher under the cold winter conditions than either the normal (GLM, Tukey, $t = 3.939$, $p = 0.002$) or warm winter (GLM, Tukey, $t = 4.968$, $p < 0.001$). Protein stores were positively associated with increasing dry mass (GLM, $t = 15.656$, $p < 0.001$). Protein stores were also significantly higher in living moss than decaying organic matter (GLM, Tukey, $t = 2.851$, $p = 0.009$).

3.6 | Stress gene expression

Due to lack of surviving larvae, we were unable to include larvae from living algae in this analysis. Following normalisation to the reference gene, *rpl19*, transcript abundance (ΔCt value) of the five stress genes was analysed by separate GLM analyses. All models highlighted that winter temperature did not significantly impact gene expression (Table 2; Figure 4). For Hsp60 and Hsp70, transcript abundance did not significantly vary across substrate type (Table 2; Figure 4, $p > 0.05$). Hsp40 exhibited higher transcript abundance in decaying organic matter when compared to living moss (GLM, Tukey, $t = 5.397$, $p < 0.001$), as did Hsp90 (GLM, Tukey, $t = 2.316$, $p = 0.031$) and sHsp (GLM, Tukey, $t = 3.507$, $p = 0.002$).

4 | DISCUSSION

Belgica antarctica is the only endemic insect in Antarctica and considering the low invertebrate diversity, it likely plays a key ecological role within these simple soil ecosystems (Bokhorst et al., 2019; Chown & Convey, 2016). Understanding how rapidly occurring anthropogenically driven climate change will impact endemic fauna uniquely adapted to polar environments is key to predicting the broader consequences of climate change in these regions (Kozeretska et al., 2021). In this study, we assessed physiological responses to three ecologically relevant overwintering conditions in larvae of *B. antarctica*, by simulating three 180-day winter regimes in three distinct natural substrates. Survival was significantly lower following the warm winter regime (mean temperature of −1.33°C), indicating that an increase in mean winter temperature of as little as 2°C can lead to a considerable reduction in survival (Figure 2). Survival was highest in the Cold winter (mean temperature of −4.94°C), which was also associated with higher lipid and protein stores, suggesting that the benefits of reduced metabolic costs at lower temperatures perhaps outweigh any increased risk of cold injury. Indeed, our results may indicate that the 'normal' overwinter temperatures used in this study (−2.97°C) may already be warmer than optimal for *B. antarctica*. Our results are consistent with previous work on the goldenrod fly *Eurosta solidaginis*, another highly cold-tolerant species, where warmer overwintering temperatures were deleterious for survival (Irwin & Lee, 2000, 2003). Also, substrate type had an unexpectedly strong effect on overwintering outcomes, and specifically, the abundant terrestrial algae, *Prasiola crispa*, appears to be a poor overwintering substrate, with greatly reduced survival and recovery of locomotion. Our results also suggest that overwintering in living moss was least costly, as larvae from these groups had comparatively lower heat shock protein expression and higher post-winter protein levels.

While we assume that all larvae froze in all treatments due to inoculative freezing, a possible explanation for our results is that some midges in the warm winter remained unfrozen and therefore had a greater metabolic rate, as observed in *E. solidaginis* (Irwin & Lee, 2002). However, larvae are highly susceptible to inoculative freezing, especially in moist substrates (Elnitsky et al., 2008; Kawarasaki et al., 2014b). Avoiding freezing in the warm winter (which had an average measured temperature of −1.3°C) would require a haemolymph osmolality of ~715 mOsm, which is considerably higher than has ever been measured in fully hydrated larvae, even after prolonged freezing (Kawarasaki et al., 2013, 2014a; Teets, Kawarasaki, et al., 2012). Thus, it appears unlikely that larvae would resist inoculative freezing, so the negative consequences of warmer winters can likely be attributed to differences in temperature, and not whether the larvae were frozen or unfrozen. At the other extreme, larvae in the Cold winter would have a higher ice fraction and thus be at higher risk of cellular dehydration, in addition to direct injury from low temperature. However, we did not see any evidence of increased freezing injury following the Cold winter with regards to cell viability, locomotor function, or stress gene expression. In previous work with short-term freezing, we observed differential heat shock protein expression after 12–24 hr (Teets et al., 2011, 2019), but here, larvae took 72 hr to recover, and were then sampled for qPCR to avoid inadvertently including dead larvae in our analysis. Thus, we may have seen different results had we been able to sample larvae earlier in recovery.

Warm winters negatively impacted protein stores, with values being ~9% lower compared to the Cold winter scenario (Figure 3c). Many larval Diptera use storage proteins (particularly hexamerins) as an energy source (Burmester, 1999) and, in *B. antarctica*, stressors that reduce storage proteins in larvae reduce adult reproductive output (Finch et al., 2020). Lipids are one of the primary energy sources for larvae (Teets et al., 2019), and our results indicate that larvae under the Cold winter regime had the highest lipid stores. Finally, in contrast to lipids and proteins, carbohydrate content was

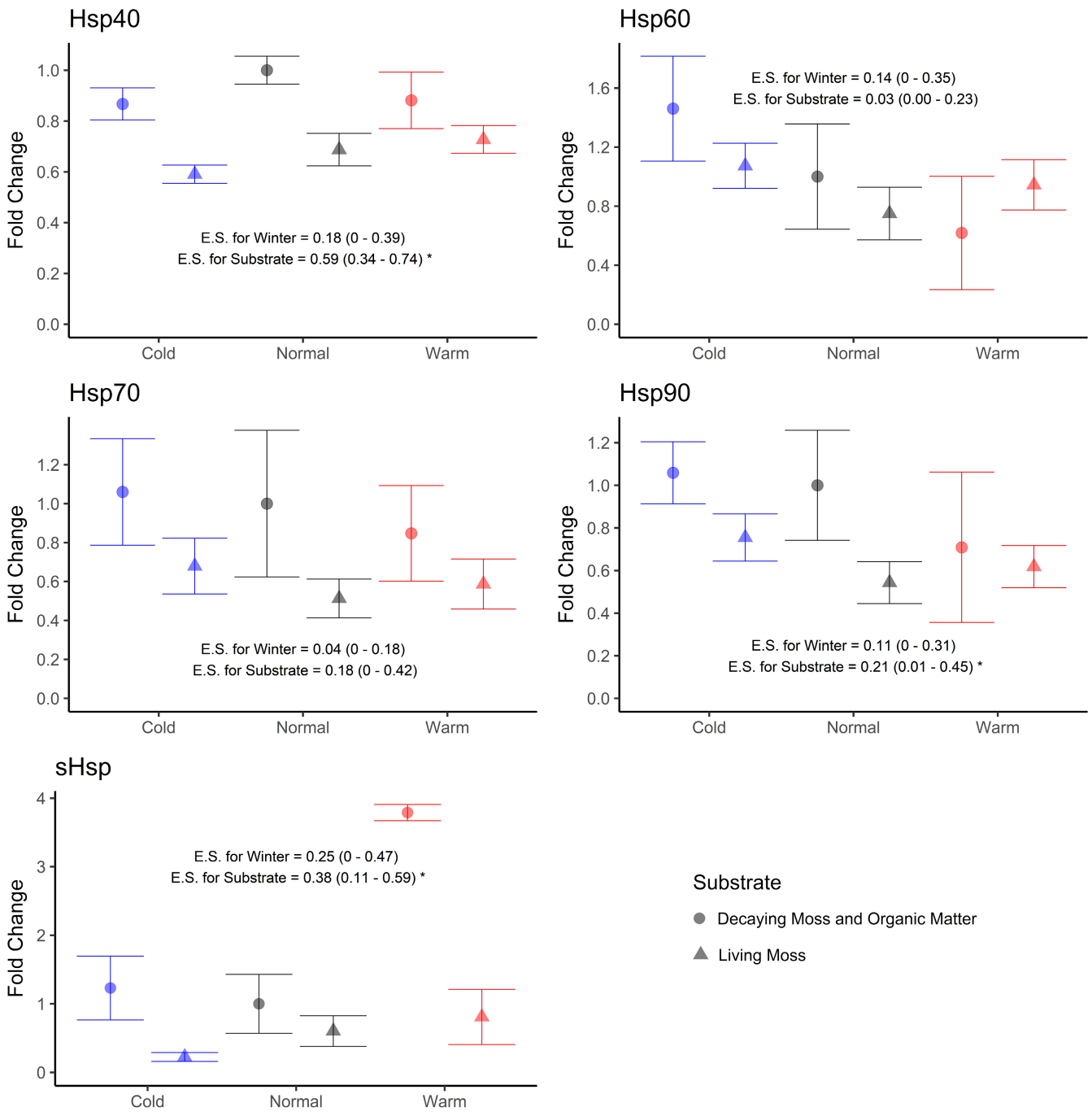

FIGURE 4 Transcript abundance of five heat shock proteins (Hsp40, Hsp60, Hsp70, Hsp90 and sHsp) following three different overwintering temperature scenarios, measured after 72 hr recovery. Fold changes are scaled to the group *Normal winter, Decaying Organic Matter*. Larvae were exposed to either Cold (−5°C), Normal (−3°C), or Warm (−1°C) winters for 180 days in each of three substrates (Decaying Organic Matter, Living Moss and *Prasiola crispa* algae). Due to the relatively high rate of mortality observed in larvae that overwintered on *Prasiola crispa* algae, we were unable to include data from this substrate groups. For each treatment, $n = 2–5$ samples (with 5 larvae per sample). In each plot, mean value with associated standard error is shown, with partial eta^2 values shown (effect size (E.S.), calculated using the package *effectsize*), where * denotes a significant effect on fold change ($p < 0.05$).

significantly higher in the Normal winter scenario relative to the cold winter regime (Figure 3a). We speculate that larvae in the colder winter may have mobilised carbohydrates for cryoprotectant production or to provide energy for repairing cold injury. While both protein and lipid energy stores were comparable to summer-acclimatised larvae from previous studies (Spacht et al., 2021; Teets et al., 2020), carbohydrate levels were markedly lower, suggesting that overwintering in a frozen state affects carbohydrate energy stores more than other energy stores, as seen in some freeze-tolerant vertebrates (Storey & Storey, 1986). We were unable to take prewinter baseline measurements in these experiments, so additional experiments are needed to track energy levels across the entire winter

and characterise the trajectory of energy depletion. Taken together, however, our results indicate that larvae that experience a warm winter tend to have the lowest levels of energy stores. These results correspond with locomotor activity levels, where larvae from the warm winter regime were slowest, potentially due to energy drain. With limited time prior to pupation after winter (Peckham, 1971), and as adult *B. antarctica* lack functional mouthparts, energy store depletion during late larval instars would likely have irreversible consequences on the energy available for reproduction.

While we experimentally manipulated winter temperature in this study, climate change is also expected to change the duration of winter, dependent on snow-cover and sea ice/temperature dynamics at a given location. Surface and sub-surface temperatures are highly heterogeneous (Convey et al., 2018; Spacht et al., 2021) and, consequently, adult emergence time varies spatially, with colder sites showing delayed emergence (Spacht et al., 2021). Potentially, midges that experience warmer winters could withstand the stressors associated with elevated winter temperatures if the duration were shorter. Thus, future work will need to simultaneously account for variation in winter duration and temperature, as both parameters are important for fully understanding the consequences of variable winter environments (Roberts et al., 2021). Additionally, we did not attempt to account for any possible influence of thermal variability on overwintering success, and we hypothesize that increased temperature variability would reduce survival and energy stores through a combination of both energy drain and cold stress (Marshall et al., 2020).

The clear contrast in survival across different substrates was unexpected. While overwintering in decaying organic matter and living moss led to comparable survival and locomotor activity, larvae in living algae (*Prasiola crispa*) consistently had the lowest survival and locomotor activity (Figure 2). *Prasiola crispa* synthesises a range of phytosterols that show contact toxicity to *D. melanogaster* (Zemolin et al., 2014), and while *B. antarctica* has evolved to feed on *P. crispa*, perhaps long-term exposure to these compounds in sealed vials might explain the mortality and slow movement observed. In natural habitats, these chemicals may leach away, or larvae could behaviourally avoid constant exposure to them. We also observed that substrate type, rather than winter temperature, had a stronger effect on stress gene expression, but due a lack of surviving larvae we could not include *P. crispa*. Three heat shock proteins exhibited significantly higher transcript abundance in decaying organic matter compared to living moss and while the underlying mechanism of this result is unclear, perhaps the distinct physical structure of each substrate influences rates of ice formation (for discussion of cold tolerance in different habitat types, see Costanzo et al., 1997; Holmstrup, 2014; Kawarasaki et al., 2014b). Differential ice growth may extend the period before inoculation occurs and allow larvae to partially dehydrate, which can enhance freezing tolerance (Hayward et al., 2007). Another possibility is that variation in decomposition rates across three substrates could influence microhabitat conditions, but we did not observe any clear signs of decay in our substrate samples, likely due to limited microbial activity while substrates are frozen. Thus, while we hypothesised that cold winters would elicit the highest levels of stress gene expression due to increased freezing injury, substrate was the only factor that influenced expression. Across all our experiments, it is clear that substrate type plays a greater role in overwintering success than previously recognised. With moss productivity increasing during the last ~50 years across *B. antarctica*'s range (Amesbury et al., 2017), it is possible that suitable overwintering habitats for invertebrates will increase as Antarctic terrestrial ecosystems continue to warm.

5 | CONCLUSIONS

Climate change in Antarctica is likely to have profound consequences for the highly adapted species that exist there. In the Antarctic Peninsula, increased snowfall will locally increase thermal buffering, and when combined with increased air temperature, will likely lead to higher subnivean temperatures. To our knowledge, this is the first study to simulate a full-length winter and determine physiological costs of variable winter environments in an Antarctic arthropod. An ecologically-relevant increase of 2°C significantly reduced survival and energy stores, which likely has implications for population size and reproductive capacity (Finch et al., 2020). While a 2°C increase is within the range of current temperatures, warmer winters are expected to become more common and widespread in response to climate change, with macroclimate increasing up to 0.56°C per decade (Turner & Overland, 2009). However, reduced winter length may negate some impacts of climate warming, so it will be important to include this factor in future studies. The abundant algae, *Prasiola crispa*, while being an important food resource for *B. antarctica*, does not appear to provide a suitable overwintering substrate, possibly due to different mechanics of freezing within this substrate or perhaps due to the release of toxic phytosterols. Rather, more heterogenous substrates, such as mosses, appear to be more suitable for overwintering. Together, our results suggest that continued winter warming in the Antarctic Peninsula may negatively impact cold-adapted invertebrates and their associated soil communities.

AUTHORS' CONTRIBUTIONS

J.J.D. and N.M.T.: originally conceived the idea, formulated methodology; J.D.G., S.H.: collected insects and substrate; J.J.D., N.M.T., E.A.M., L.U., M.C.L.: performed laboratory work; J.J.D. and N.M.T.: performed statistical modelling; J.J.D. and N.M.T.: assembled figures and wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository https://doi.org/10.5061/dryad.d7wm37q3k (Devlin et al., 2022).

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