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RESEARCH ARTICLE

Repeated freezing induces a trade-off between cryoprotection and egg production in the goldenrod gall fly, *Eurosta solidaginis*

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ABSTRACT

Internal ice formation leads to wholesale changes in ionic, osmotic and pH homeostasis, energy metabolism, and mechanical damage, across a small range of temperatures, and is thus an abiotic stressor that acts at a distinct, physiologically relevant, threshold. Insects that experience repeated freeze–thaw cycles over winter will cross this stressor threshold many times over their lifespan. Here, we examined the effect of repeatedly crossing the freezing threshold on short-term physiological parameters (metabolic reserves and cryoprotectant concentration) as well as long-term fitness-related performance (survival and egg production) in the freeze-tolerant goldenrod gall fly, *Eurosta solidaginis*. We exposed overwintering prepupae to a series of low temperatures (−10, −15 or −20°C) with increasing numbers of freezing events (3, 6 or 10) with differing recovery periods between events (1, 5 or 10 days). Repeated freezing increased sorbitol concentration by about 50% relative to a single freezing episode, and prompted prepupae to modify long-chain triacylglycerols to acetylated triacylglycerols. Long-term, repeated freezing did not significantly reduce survival but did reduce egg production by 9.8% relative to a single freezing event. Exposure temperature did not affect any of these measures, suggesting that threshold crossing events may be more important to fitness than the intensity of stress in overwintering *E. solidaginis*.

KEY WORDS: Thermal variability, Physiological thresholds, Freeze tolerance, Repeated stress, Tephritidae

INTRODUCTION

The relationship between physiological performance and body temperature in ectotherms is usually modelled as a continuous thermal performance curve (Huey and Stevenson, 1979; Schulte et al., 2011; Sinclair et al., 2016). These curves have been quantified for a broad array of organisms and traits (e.g. Edwards and Irving, 1943; Haugaard and Irving, 1943; Krog, 1954; Angilletta, 2006; Clusella-Trullas et al., 2011) and have been used to predict potential population growth (e.g. Luhring and DeLong, 2016), geographic ranges (e.g. Payne et al., 2016; Tuff et al., 2016) and responses to climate change (Woodin et al., 2013). However, it has become increasingly clear that important thresholds exist at which performance changes discontinuously, such that a very small change in temperature can cause a stepwise shift in the physiological parameter of interest. For example, the heat shock response is invoked after a shift of temperature of only 1 or 2°C and leads to rapid reorganizing of RNA splicing, damage to the cytoskeleton and the rapid upregulation of heat shock protein synthesis (reviewed by Richter et al., 2010), while the fruit flies *Drosophila melanogaster* and *Drosophila simulans* have sharp temperature thresholds where male sterility is induced (Chakir et al., 2002). Incorporating the existence and effects of these physiological thresholds is a key challenge for predicting ectotherm responses to climate change (Sinclair et al., 2016).

A clear example of such a physiological threshold is ice formation in freeze-tolerant ectotherms. Crossing this threshold reduces metabolic rate (Sinclair et al., 2013), dehydrates cells (Sinclair and Wharton, 1997) and induces cryoprotectant and heat shock protein production in some species (Churchill and Storey, 1989a; Teets et al., 2011). As a result, numerical models that incorporate the physiological effects of crossing these physiological thresholds provide more accurate predictions of the effect of temperature on organisms than those that do not. For example, incorporating the intensity and frequency of freezing events significantly improved the prediction of mangrove distribution (Cavanaugh et al., 2015), while incorporating metabolic rate depression associated with freezing events increased the accuracy of a model predicting overwinter energy use by a freeze-tolerant caterpillar (Marshall and Sinclair, 2012a).

Temperature varies on daily and weekly time scales, which means that temperate insects may experience repeated freezing events during autumn, winter and spring (Marshall and Sinclair, 2012b; Dillon et al., 2016). The physiological effects of repeated cold exposures on insects have been recently reviewed (Marshall and Sinclair, 2012b; Colinet et al., 2015). In *D. melanogaster*, repeated chilling (without freezing) leads to a reduction of both glycogen content and the production of female offspring (Marshall and Sinclair, 2010). Similarly, the effect of repeated cold exposure on glycerol production and glycogen depletion in freeze-avoidant spruce budworms (*Choristoneura fumiferana*) is much greater than the impact of more intense cold exposures (Marshall and Sinclair, 2015). In all these studies, repeated cold exposure was more costly than single cold exposures (even when matched for total time spent in the cold; Marshall and Sinclair, 2012b), but although they explored repeated cold exposure, they did not explicitly address the impact of crossing a physiological threshold.

Repeated ice formation damages the gut tissue of larvae of both the sub-Antarctic tineid *Pringleophaga marioni* (Sinclair and Chown, 2005) and the temperate erebid *Pyrrharctia isabella* (Marshall and Sinclair, 2011). Similarly, freeze-tolerant *Hydromedion sparsutum* beetles lose their freeze tolerance after repeated freeze–thaw cycles and are killed when subsequently frozen (Bale et al., 2001). Some cold hardening appears to occur during cycles of repeated freezing: the Antarctic midge, *Belgica antarctica*, increases heat shock protein abundance following

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repeated freezing events (Teets et al., 2012), while *P. isabella* elevates haemolymph concentrations of its main cryoprotectant, glycerol (Marshall and Sinclair, 2011).

We identify three broad categories of post-freezing physiological processes that could determine the impact of subsequent exposures. The first is stress-induced damage, which could reduce tolerance of subsequent exposures. Second, repair processes following injury could draw on finite energetic resources. Third, investment in preparation for subsequent exposures could increase physiological performance in following exposures. If energetic resources are diverted to fuel protective or repair processes, then repeated freezing could drive trade-offs with reproductive output. The processes may not be mutually exclusive: they may interact with each other depending on the underlying metabolic pathways involved (Zera and Harshman, 2001; Sokolova, 2013). Because these three processes feed back to determine subsequent tolerance of stress events, probing the relative importance of these processes and the dimensions of environmental stress that influence them is central to understanding the impacts of crossing physiological thresholds.

We used prepupae of the goldenrod gall fly, *Eurosta solidaginis*, to investigate the effects of repeated freezing on physiology and subsequent reproductive output. Because of its abundance and wide geographical range across North America, *E. solidaginis* has been used as a model for low-temperature physiology (Storey and Storey, 1990; Baust and Nishino, 1991; Irwin and Lee, 2003). *Eurosta solidaginis* is an obligate parasite of goldenrod plants (*Solidago* spp.), and spends the majority of its life (the entirety of winter) as a diapausing prepupa within a senesced gall that it induces on the plant (Uhler, 1951). During this overwintering phase, *E. solidaginis* is remarkably freeze tolerant, withstanding both intracellular and extracellular freezing of its body tissues in laboratory conditions at temperatures as low as −80°C (Lee et al., 1993; Mugnano et al., 1996; Yi and Lee, 2003). The prepupae experience relatively unbuffered air temperatures in their overwintering microclimate, and at ca. −8.5°C, their body water freezes (Layne, 1991; Mugnano et al., 1996; Irwin and Lee, 2003). As the adult flies cannot feed following the winter (Uhler, 1951), the lipid stores remaining upon spring emergence are an important determinant of egg production in the semelparous ovaries (Irwin and Lee, 2003). In the early autumn, *E. solidaginis* larvae accumulate large amounts of glycerol synthesized from glycogen stores as a cryoprotectant (Storey et al., 1981). Exposure to temperatures below 5°C induces the synthesis of sorbitol as a secondary cryoprotectant (Storey and Storey, 1988). *Eurosta solidaginis* also remodels its lipid membranes to increase fluidity (Pruitt and Lu, 2008), decreases saturation of triacylglycerols (long-chain triacylglycerols, lcTAGs; Bennett et al., 1997) and accumulates acetylated triacylglycerols (acTAGs; Marshall et al., 2014) from late August until late October, all of which are associated with freeze tolerance in this species.

In galls that remain above the snow, *E. solidaginis* experiences regular freeze–thaw cycles over the northern part of its range. After repeated freezing, *E. solidaginis* have increased cryoprotectant concentrations; increased lipid peroxidation and oxidation protein products, indicative of oxidative damage; decreased adenylate charge; and increased long-term mortality (Churchill and Storey, 1989a; Doelling et al., 2014). Although this paints a picture of overall negative impacts of repeated freezing, being cold can be advantageous. *Eurosta solidaginis* overwintered in microclimates below the snow (where temperatures hover around 0°C rather than dropping below −20°C) produced fewer eggs in spring compared with their above-snow counterparts (Irwin and Lee, 2003), putatively because of the energetic consequences of the warmer conditions (reviewed by Sinclair, 2015). Thus, the impacts of thermal variability have not been dissected from those of crossing the ice-formation threshold, nor have the apparently negative immediate physiological consequences of repeated freezing been reconciled with the potential energy savings and long-term reproductive advantage of spending more of the winter frozen.

Here, we linked the short- and long-term effects of repeated freezing in *E. solidaginis* by measuring energy stores, cryoprotection, survival and egg production following single or repeated freezing events at three different temperature thresholds for three different periods of time between exposures in a framework that explicitly connects these measures. Our three non-mutually exclusive hypotheses were that: (1) repeated freezing causes significant injury that cannot be repaired and thus will lead to increased mortality relative to single freezing events, (2) the damage associated with repeated freezing can be repaired but exacts an energetic cost that will lead to unchanged survival but decreased fecundity, and (3) repeated freezing induces additional costs for cryoprotection that will lead to unchanged survival (because freeze-related damage has not occurred) but decreased fecundity.

MATERIALS AND METHODS

Insect collection and maintenance

We collected approximately 5000 *Solidago canadensis* galls containing *E. solidaginis* (Fitch 1855) from old-field habitats in London, ON, Canada (43°00′N, 81°15′W) in October and November 2009, following the senescence of the *S. canadensis* plants. We kept galls in an incubator at 15°C, for less than 2 weeks, until we extracted a total of 2700 prepupae from their galls. We placed the prepupae in perforated 0.2 ml microcentrifuge tubes, in constant darkness in an incubator cycling (12 h:12 h) between the normal high and low for London, ON (Environment Canada, http://www.weatheroffice.gc.ca; adjusted weekly), until the daily high reached 2°C and the daily low 0°C (late November). This cycle was then maintained for the remainder of the winter (see Marshall and Sinclair, 2012b, for examples of winter weather in this locale). We combined prepupae from all collection locations, and chose 60 individuals randomly for each experimental group. All prepupae were acclimated to laboratory conditions for at least 1 month prior to low-temperature exposure.

Experimental design

We conducted all low-temperature exposures during a 3 month period from late December 2009 to late March 2010. We investigated the effects of intensity, duration, period and frequency of freezing exposure, both separately and in interaction with each other (experimental design shown in Fig. 1). We examined the effects of intensity by exposing prepupae to one of three temperatures: −10, −15 or −20°C. We examined the effects of frequency of low-temperature exposure by subjecting prepupae either to 10, 12 h exposures at these temperatures, or to a single 120 h exposure. Period between exposures was examined by exposing flies to 12 h exposures on a daily, 5 day or 10 day cycle. The effect of number of exposures was studied by exposing flies to three, six or 10, 12 h low-temperature exposures. Finally, to control for the potential effects of diapause intensity, individuals were given a single 120 h low-temperature exposure at either the beginning or the end of the experimental period. We also sampled control individuals at the beginning (late December), middle (mid-February) and end (late March) of the experimental period.

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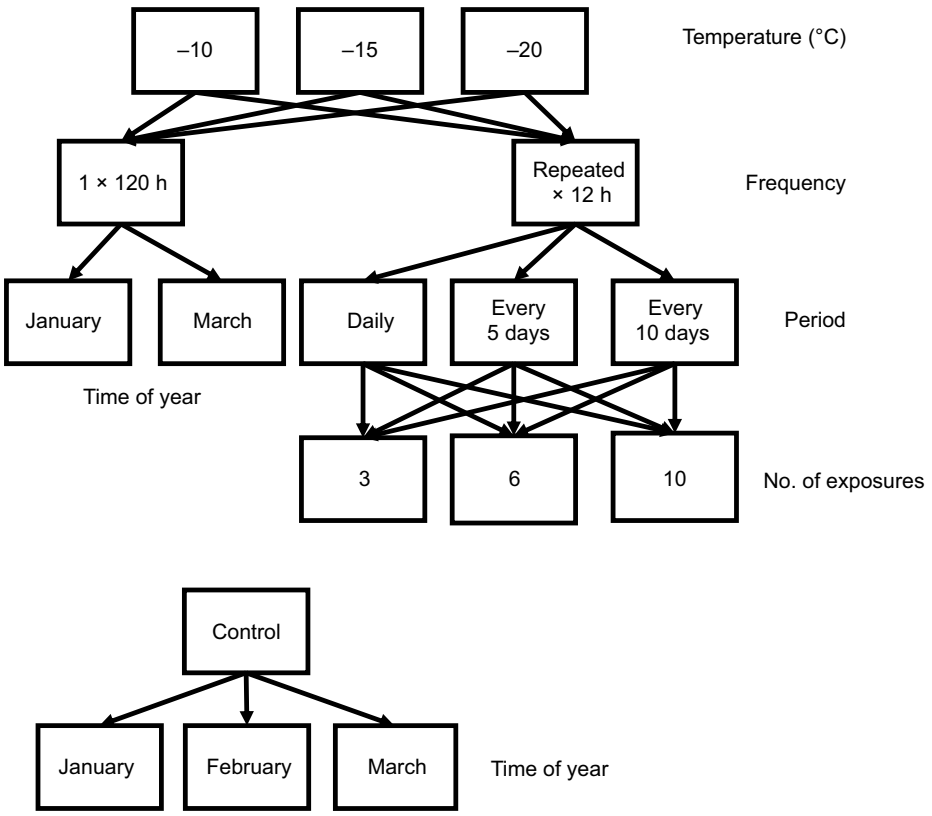

Fig. 1. Experimental design for testing the effects of repeated low-temperature threshold crossing on prepupae of *Eurosta solidaginis*. Control animals were sampled monthly from early January ('time of year' controls). Low-temperature exposures were conducted from late December to March and contrasted two different 'frequencies' of exposure: either a single 120 h exposure ('1×120 h') or repeated 12 h exposures ('Repeated×12 h') occurring at one of three exposure temperatures (−10, −15 or −20°C). For those prepupae that received repeated 12 h exposures, these occurred at one of three different periods: daily, every 5 days or every 10 days. Finally, for those animals that received repeated 12 h exposures, the number of exposures (3, 6 or 10 total) was varied across the differing periods of exposure in a fully crossed design to explore potential interactions. See Materials and Methods for the time line of extraction and incubator settings prior to low-temperature exposures. *n*=60 per experimental group.

Cold exposure

A programmable refrigerated circulator (Proline 3530C, Lauda, Würzburg, Germany) containing 30 l of 50:50 methanol:water was used to control temperature for all cold exposures. We placed groups of prepupae (still in 0.2 ml microcentrifuge tubes) in 50 ml centrifuge tubes, which were held upright in the circulator bath by an aluminium insert. The temperature within four representative tubes was monitored by 36 AWG Type T (copper–constantan) thermocouples (Omega, Laval, QC, Canada) interfaced to a computer running PicoLog Software for Windows (Pico Technology, Cambridge, UK) by Picotech TC-08 thermocouple interfaces (Pico Technology), and recording temperature at 0.5 s intervals. We began all low-temperature exposures at 0°C at 20:00 h local time, and cooled at a rate of 0.1°C min^{-1} to −10, −15 or −20°C. Rewarming similarly proceeded at 0.1°C min^{-1} until 0°C was reached. After the conclusion of a low-temperature treatment, all individuals were placed back in the incubator for recovery at 12 h:12 h 0:2°C. Twenty-four hours after each low-temperature exposure concluded, we transferred 15 individuals into 1.7 ml microcentrifuge tubes, and snap-froze them by direct immersion in liquid nitrogen vapour for at least 5 min. These were stored at −80°C for later analysis. A subset (20) of each group were placed back in the incubator at 12 h:12 h 0:2°C for later fitness assays.

Supercooling point

The day following the last cold exposure of each experimental group, we measured supercooling points in a subsample of 20 prepupae. We placed the thermocouple in direct contact with each prepupa, which were placed into individual microcentrifuge tubes that were placed into an aluminium block cooled by the bath fluid. Bath temperature was cooled from 0°C to −25°C at a rate of 0.1°C min^{-1}. Supercooling point was recorded as the lowest temperature before the latent heat of crystallization was detected (Sinclair et al., 2015).

Survival and egg production

At the conclusion of the low-temperature treatments, all remaining prepupae (20 per group) were kept in constant darkness at 12 h:12 h 0:2°C until 30 April 2010. We then transferred prepupae from individual 0.2 ml microcentrifuge tubes to groups of 20 in 100 mm diameter Petri dishes lined with moistened filter paper, and incubated them in constant light at a constant 20°C to induce pupation (Sanyo Scientific MIR-153, Bensenville, IL, USA). Petri dishes were checked daily for eclosed adults, and females were placed into a new Petri dish to allow development of eggs (males were kept separately; Irwin and Lee, 2003). Adults were frozen 3 days after eclosion and stored at −20°C until dissection. We then weighed all adults, measured thorax width and length of females under 15× magnification, and dissected females and counted the eggs in both ovaries (Irwin and Lee, 2003).

Metabolite and energy content assays

We homogenized prepupae using an ice-cold 1 ml glass homogenizer in pools of three individuals in 0.1% butylated hydroxytoluene (BHT) in 125 µl of ice-cold Coast's solution (Coast, 1988). We poured the homogenate into a 1.7 ml microcentrifuge tube, then rinsed the homogenizing tube four times with 125 µl of Coast's solution. We mixed the sample vigorously, and froze a 70 µl aliquot at −80°C for lipid analysis. We centrifuged the remaining liquid for 15 min at 15,000 ***g***, removed the supernatant (~420 µl) and mixed it thoroughly. We split the supernatant into four 100 µl aliquots which were frozen at −80°C for later metabolite analysis.

We measured sorbitol content of supernatant samples diluted 1:99 with 0.05% Tween 20 using a Sorbitol Assay Kit with sorbitol standards (Megazyme International Ireland, Bray, Ireland). Protein, glycogen (both diluted 1:99 with 0.05% Tween 20) and glycerol (diluted 1:199 with 0.05% Tween 20) content were all measured

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spectrophotometrically (after Gefen et al., 2006) using bovine serum albumin, Type II glycogen from oyster and glycerol standards, respectively. Briefly, soluble protein concentration was measured using a bicinchoninic acid kit (BCA1, Sigma-Aldrich, Oakville, ON, Canada). Glycogen mass was measured using a hexokinase-based glucose assay kit (GAHK20, Sigma-Aldrich) following overnight amyloglucosidase (A9228, Sigma-Aldrich) digestion at room temperature. Glycerol concentration was measured using Free Glycerol Reagent (F6425, Sigma-Aldrich).

We extracted all lipids from undiluted homogenate as in Williams et al. (2011). We added a 50 µl aliquot of homogenate to 2.5 ml 0.1% BHT in 2:1 chloroform:methanol, with an additional 100 µg of 1 mg ml^{-1} 1-stearoyl-*rac*-glycerol added as an internal standard. We centrifuged the samples at 2000 ***g*** for 10 min, then added 1 ml 0.25% KCl. We heated the mixture at 70°C for 10 min to allow separation of aqueous and organic layers. We then removed the lower organic layer (ca. 1.5 ml) and placed it into a glass vial, and dried the samples under nitrogen at 70°C. After resuspension in 800 µl chloroform, samples were stored at −20°C until analysis.

To separate and quantify neutral lipid classes, we spotted 1.5 µl of each sample in triplicate using a glass syringe on silica-coated Chromarods that had been repeatedly blank-scanned in an Iatroscan MK-6 TLC-FID (thin-layer chromatography coupled to flame ionization detector; Shell-USA, Spotsylvania, VA, USA). We then developed rods in a mixture of 70:30:05 benzene:chloroform:formic acid for 35 min to allow separation of neutral lipid classes (Williams et al., 2011). We removed rods from the solvent mixture and dried them (70°C, 5 min) before they were scanned at 3 cm s^{-1} on the Iatroscan. We identified individual peaks by comparing the retention time with known standards, and quantified them using PeakSimple software (SRI Instruments, Torrance, CA, USA) against standard curves of known concentrations of lcTAGs and free fatty acids (FFAs) with a fatty acid profile of 60:20:20 oleic acid:palmitic acid:palmitoleic acid (Pruitt and Lu, 2008). acTAGs (Marshall et al., 2014) were quantified against a standard curve of known concentration from acTAGs purified from other prepupae.

Statistical analysis

We began all statistical analyses by first examining the effects of period, number of exposures and intensity of exposure in the individuals that received repeated 12 h exposures. Then, the effects of month exposed, month sampled and intensity of temperature exposure were compared in individuals that received a single 120 h exposure. Control individuals were compared among sampling months. A final model including all three exposure types was then used to investigate differences among treatments. Maximal models were first fitted, including all potential terms and interactions. Then, using the step() algorithm implemented in R (Venables and Ripley, 2003), we simplified to the model with the lowest Akaike's information criterion (AIC) by sequentially removing the highest-order interaction terms. As the step function will halt when removing a term increases the AIC regardless of its significance (i.e. ΔAIC between the simplified and original model <2; Crawley, 2005), we compared AIC values between the best-fit model from the step function with the next-simplest model (i.e. the best-fit model from the step function with the highest-order interaction term removed) using the extractAIC function in R. If the increase in AIC at this point was not significant (ΔAIC<2), we restarted the step function with the next simplest model (Crawley, 2005).

To compare survival to eclosion among flies that had received different low-temperature exposures, we used generalized linear models with a binomial error distribution. To test for differences in the number of eggs among flies that had received different low-temperature exposures, we used generalized linear models with a quasi-Poisson distribution. We conducted all cryoprotectant and metabolic fuel analyses on a mass per individual fly basis after scaling up from the concentration of each aliquot then dividing by the number of individuals in each sample (i.e. 3), and we included soluble protein content as a covariate. We report means and s.e.m. throughout. For plots that represent data from models with covariates, the predict function in the base package of R was used to estimate response values that took into account the value of the covariate. Alpha was set to 0.05 in all statistical tests.

RESULTS

Survival and egg production

Mass of the adult fly best predicted the number of eggs (AIC=1988, versus 2095 or 2083 for thorax width or length), so the number of eggs was compared among females with wet fly mass as a covariate. There was no effect of exposure temperature, number of exposures or period between exposures on the number of eggs produced in female flies that received a repeated exposure (Table S1). Similarly, there was no impact of exposure temperature or time of year exposed on the number of eggs in flies that received a prolonged exposure (Table S1). Finally, when we compared flies from all frequencies of exposure (control, repeated and prolonged), those that had received repeated freeze exposures had significantly fewer eggs than either of the other two groups (Table S1; Fig. 2). On average, repeatedly frozen flies produced 195.5±4.6 eggs each, while flies that received a single prolonged exposure produced 214.6±11.0 eggs each, a difference of 9.8%.

A total of 549 flies emerged from the 849 prepupae set aside for measuring adult characteristics (overall survival rate=65%). Of these, a total of 247 were female (overall female:male sex

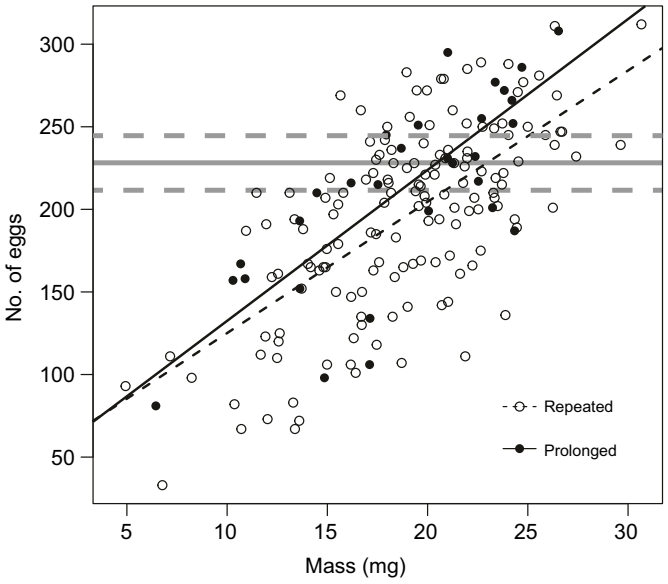

Fig. 2. Effects of freezing temperature exposure during overwintering on the number of eggs produced by *E. solidaginis* after spring emergence as adults. Exposure type was either a single (prolonged) 120 h freezing event or repeated 12 h freezing events. Mass reported is adult mass 3 days after eclosion. Repeatedly exposed individuals had significantly lower egg production (body mass: P<0.001, effect of cold exposure type: P=0.048, N=50–148). All statistics are presented in Table S1. Mean and s.e.m. egg production by controls maintained at 0°C is indicated by the solid and dashed grey lines, respectively.

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ratio=0.45). In flies that received repeated low-temperature freezing events, there was a significant three-way interaction between temperature and period between events on survival (Table S2). Flies that received repeated freezing events every 10 days had increased survival relative to other periods between freezing events. In flies that received a prolonged freezing event, there was a significant two-way interaction between freezing temperature and time of year frozen (Table S2; Fig. 3). Finally, when we compared flies from all frequencies of freezing events (control, repeated and prolonged), there was a significant two-way interaction between freezing temperature and freezing regime (i.e. repeated or single matched for time; Table S2), which was driven by high survival in flies that received 12 h freezing events every 10 days at higher temperatures relative to all other freezing groups (Fig. 3).

Supercooling point

The average supercooling point of prepupae was −8.6±0.1°C (n=964), and while there were statistically significant effects due to

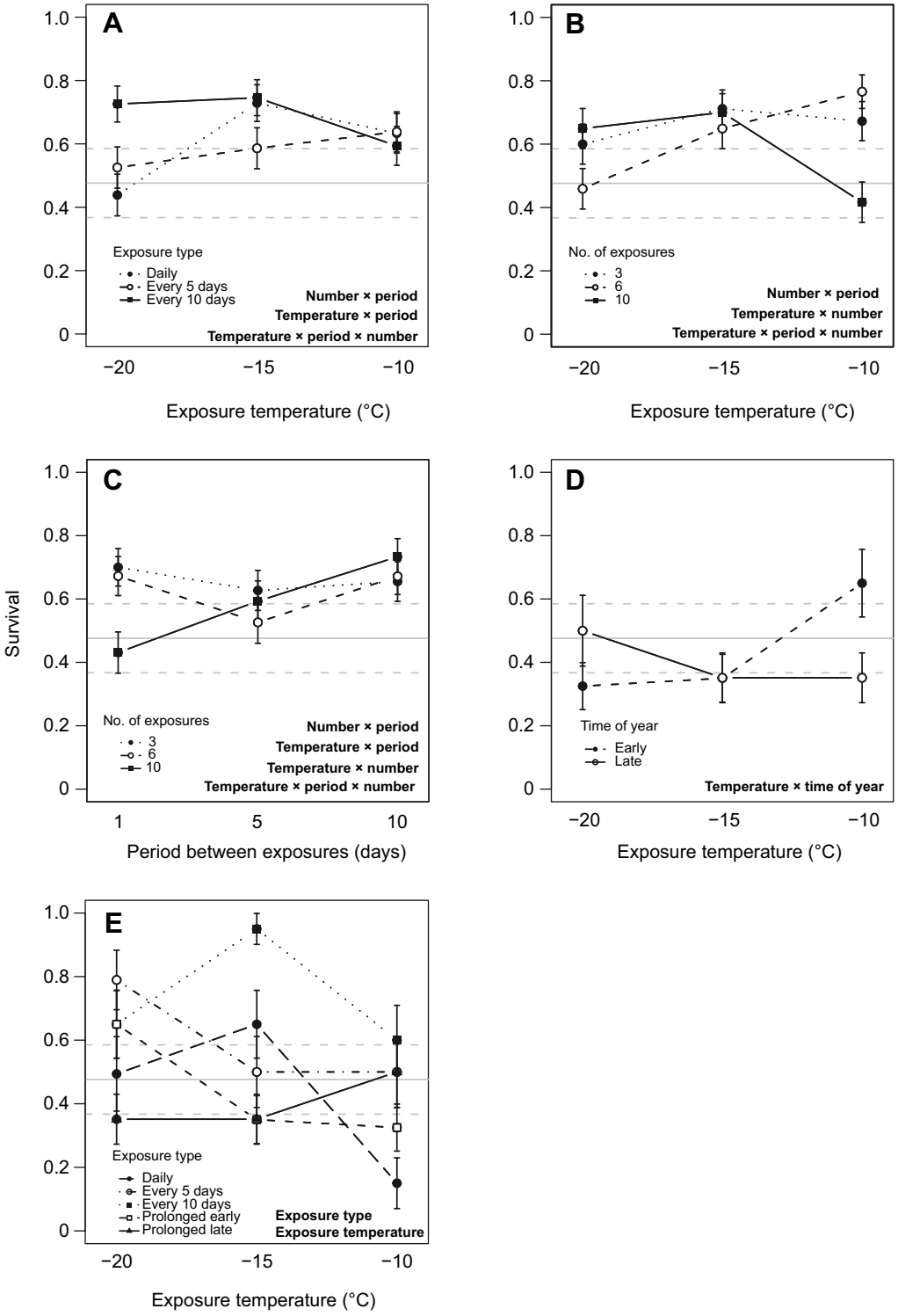

Fig. 3. Effects of freezing temperature exposure during overwintering on the survival of *E. solidaginis* to eclosion. Solid and dashed horizontal grey lines represent mean and s.e.m. survival of control flies, respectively. Points represent the mean (and s.e.m.) proportion of flies that survived. Bold terms refer to significant effects in the most parsimonious ANOVA model, as tested by Akaike's information criterion (AIC; all statistics are presented in Table S2). (A) Flies that received 10, 12 h freezing events as prepupae at three different time intervals. (B,C) Flies that received repeated 12 h freezing events as prepupae. (D) Flies that received a single, 120 h cold exposure in January ('Early') or March ('Late'). (E) Flies that received repeated 12 h bouts at three different time intervals ('Daily', 'Every 5 days' or 'Every 10 days') or a single 120 h bout of freezing either in January ('Early') or March ('Late').

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freezing treatment, the effect sizes were small (all <1.61°C; Table S3). The final comparison between repeated and prolonged freezing exposure showed a depression of 0.77°C due to repeated freezing exposures, which is close to the accuracy of Type T thermocouples (0.5°C). Of the measured prepupae, 111 (11.4% of the total) froze at temperatures below −10°C, 16 froze at temperatures below −15°C (1.7% of the total) and five froze at temperatures below −20°C (0.5% of the total).

Carbohydrates and cryoprotectants

None of the temperatures, periods between freezing events or number of freezing events impacted glycogen mass in flies that received repeated low-temperature freezing events (Table S4). There was no impact on glycogen content due to sampling time in flies that received prolonged freezing exposure, but as temperature decreased, the amount of glycogen also decreased significantly (Table S4). Therefore, temperature of the freezing event was retained in the model when comparing single and repeated freezing events. In control flies, glycogen content dropped significantly through the experiment (Table S4). Finally, there were no significant differences in glycogen content among any of the freezing regimes (Table S4).

Glycerol content decreased with decreasing temperature in prepupae that received freezing events daily or every 10th day, while prepupae that received freezing events every 5 days maintained high glycerol concentration (Table S5; Fig. 4A,C). In addition, increasing the number of freezing events decreased glycerol content at decreasing temperature (Fig. 4B,C). In prepupae frozen in January and March that were sampled immediately, there was a negative relationship between freezing temperature and glycerol mass, while prepupae that were frozen in January but sampled in March had the same glycerol content regardless of freezing temperature (Table S5; Fig. 4D). In control prepupae, there was no impact of time of year sampled on glycerol mass (Table S5; Fig. 4E). When prepupae from each freezing regime (repeated, prolonged and control) were compared, there was a significant effect of freezing regime on glycerol mass (Table S5; Fig. 4F). This was further simplified, and prepupae were pooled within each freezing regime. There was a significant impact of freezing regime on glycerol mass ($F_{2,101}$=8.325, P<0.001). Prepupae that had received repeated freezing events had significantly increased glycerol mass relative to that of prepupae that received prolonged freezing events (P=0.023) or control prepupae (P<0.001), while prepupae that received prolonged freezing events did not have significantly elevated glycerol mass relative to control prepupae (P=0.153).

Sorbitol content was reduced in prepupae that received freezing events every 10 days for a total of 10 exposures (Table S6; Fig. 5A–C), but for other numbers of freezing events there were complicated interactions (Fig. 5A–C). There was a significant interaction between freezing temperature and time of year frozen on sorbitol content in prepupae that received a prolonged freezing event (Table S6; Fig. 5D). Prepupae frozen in January had a higher sorbitol content than prepupae frozen in March at almost all temperatures (Fig. 5D), while in prepupae sampled immediately after a prolonged freeze, sorbitol mass decreased with decreasing temperature (Fig. 5D). Control prepupae had a higher sorbitol content early in the year, and a lower content in February and March (Table S6; Fig. 5E). When all prepupae were compared against each other, there was a significant impact of freezing regime on sorbitol mass, with prepupae that received repeated 12 h freezing events having a higher sorbitol content than that of control and prepupae that had a prolonged freezing event (Table S6; Fig. 5F).

Lipid reserves

The predominant energy reserves in *E. solidaginis* were lipids (8.61±0.19 mg out of a total fresh body mass of 47.17±0.51 mg), and these were shared among three distinct classes of nearly equal mass: lcTAGs (2.50±0.05 mg), FFAs (2.74±0.05 mg) and acTAGs (3.38±0.07 mg).

Preliminary model exploration showed that total lipid mass was a better predictor of the mass of each neutral lipid component than protein mass (R^2=0.846 for model regression of total lipid mass, R^2=0.141 for total protein content), so total lipid mass was retained as a covariate in all analyses of lipid content. Lower temperature and increased number of freezing events decreased lcTAG mass in prepupae that received repeated 12 h freezing events (Table S7; Fig. 6A–C). Similarly, time of year frozen and sampled and temperature interacted to affect lcTAG mass in prepupae that received a single 120 h freezing event (Table S7; Fig. 6D). In control flies, lcTAG mass did not significantly change through the winter (Table S7; Fig. 6E). Finally, there was a significant decrease in lcTAG mass in flies that received repeated freezing events relative to that of flies that received prolonged freezing events (P=0.014, all other statistics in Table S7; Fig. 6F).

Increasing temperature significantly increased acTAG mass in prepupae that received repeated 12 h freezing events (Table S8; Fig. 7A–C). By contrast, there was no effect of temperature on acTAG mass in prepupae that received prolonged freezing events, nor was there an effect of time of year frozen or sampled (Table S8; Fig. 7D). Finally, when all freezing regimes were compared (while retaining the temperature term for the prepupae with repeated freezing events), there was a significant difference in acTAG mass in prepupae among the groups (Table S8; Fig. 7F). Prepupae that had repeated freezing events had significantly greater acTAG mass than control prepupae (P<0.001) or prepupae that received a prolonged freezing event (P=0.041). Prepupae that had received a prolonged freezing event did not have significantly increased acTAG relative to control prepupae (P=0.180).

DISCUSSION

Studies of the fitness costs of cold exposure in insects have generally focused on the effects of intensity and duration, despite the fact that frequency and period of temperature fluctuations also vary in natural environments (Chown and Terblanche, 2007; Marshall and Sinclair, 2012a), and increasing evidence that temperature fluctuations can drive ectotherm responses to the abiotic environment (Colinet et al., 2015; Marshall and Sinclair, 2015; Williams et al., 2016). While a broad negative effect of repeated cold exposure has been shown previously (Marshall and Sinclair, 2010, 2012b, 2015), and more specifically for repeatedly frozen insects (Marshall and Sinclair, 2011; Doelling et al., 2014), here, we tested the relative importance of intensity of stress and time on both short-term physiological responses and long-term fitness effects. Repeated freezing events did not cause additional mortality, so we found no evidence for our first hypothesis that repeated freezing would cause increased unrepaired damage that could lead to mortality. However, in support of our second and third hypotheses, we did find evidence that repeated freezing led to both increased investment in polyol cryoprotection and a shift in lipid resources away from storage triacylglycerols towards acTAGs. This increased cryoprotection was accompanied by a significant decrease in egg production, a trade-off induced only by repeated freezing events and not by a single freezing event matched for time and intensity.

We found that repeated internal ice formation decreased egg production. *Eurosta solidaginis* larvae cease feeding in late summer

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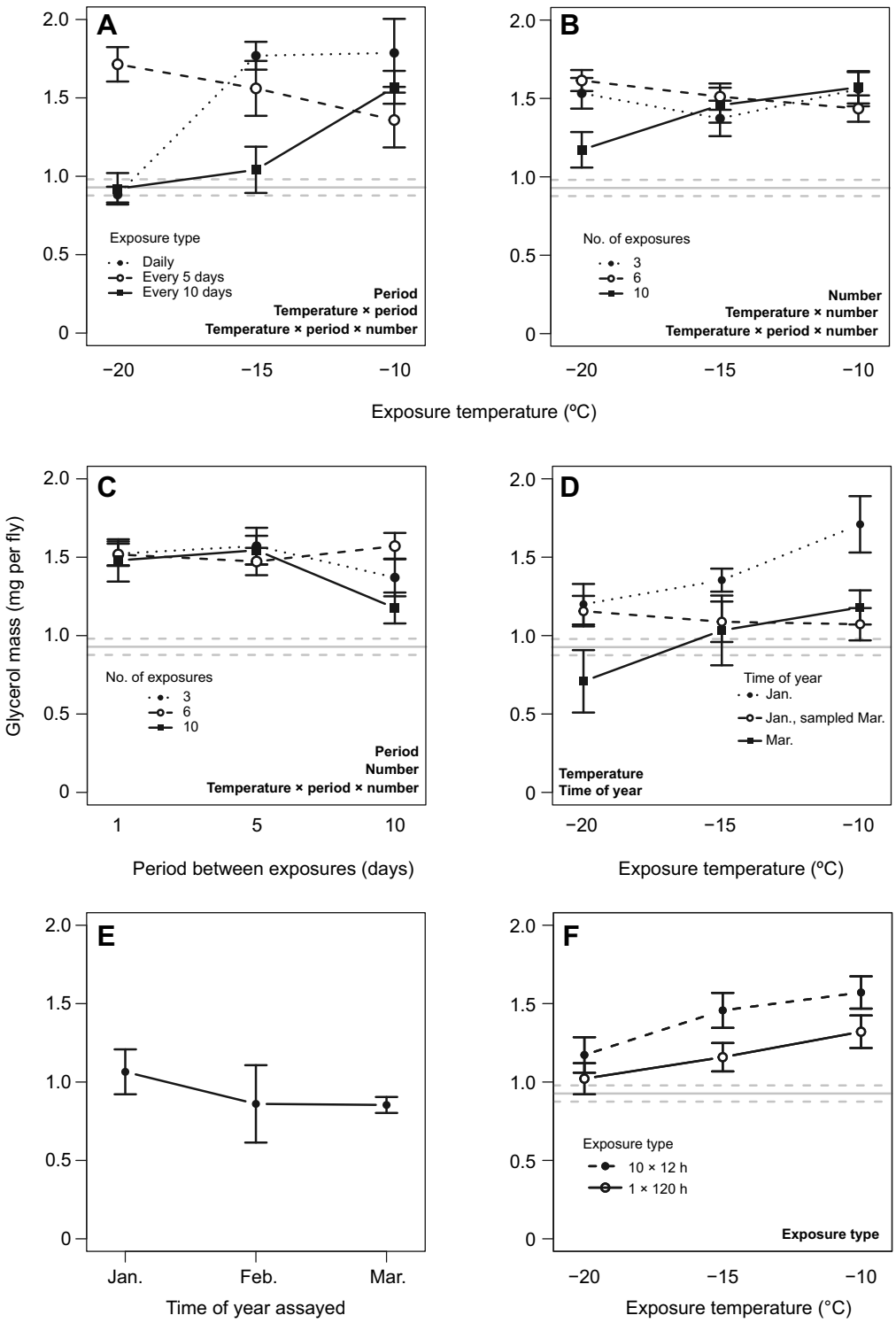

Fig. 4. Effects of low-temperature exposure on glycerol mass in *E. solidaginis* prepupae. Solid and dashed horizontal grey lines represent mean and s.e.m. of control flies, respectively. Points represent means (±s.e.m.). Bold terms refer to significant effects in the most parsimonious ANOVA model, as tested by AIC (n=5 pools of three individuals per group; all statistics are presented in Table S5). (A) Flies that received 10, 12 h freezing events as prepupae at three different time intervals. (B,C) Flies that received repeated 12 h freezing events as prepupae. (D) Flies that received a single, 120 h cold exposure in January or March and were sampled immediately, or received a bout in January but were sampled in March. (E) Control flies maintained at 0ºC that were sampled in January, February, then March. (F) Flies that received 10, 12 h bouts or a single 120 h bout of freezing exposure.

and overwinter on the lipid and carbohydrate stores acquired through the feeding season; thus, egg production in the spring is directly related to the overwintering conditions they experience (Irwin and Lee, 2002, 2003). The prepupae in our study spent 120 h frozen, which may appear brief relative to a 3–6 month overwintering period in nature. However, we predicted the freeze-tolerant woolly bear caterpillar *P. isabella* spends a total of 362 h frozen during a 5 month winter in the same habitat from which we sourced the animals for this study (Marshall and Sinclair, 2012a). Although spending additional time at a low temperature might be expected to increase egg production as a result of the energetic savings (Irwin and Lee, 2003; Sinclair, 2015), flies that received prolonged low-temperature exposure produced no more eggs than controls (Fig. 2). By contrast, individuals that received cold exposures of 120 h (matched for duration and intensity), but in 12 h bouts, had reduced egg production (Fig. 2). *Drosophila melanogaster* also reduced production of (female) offspring after repeated low temperature exposures (Marshall and Sinclair, 2010).

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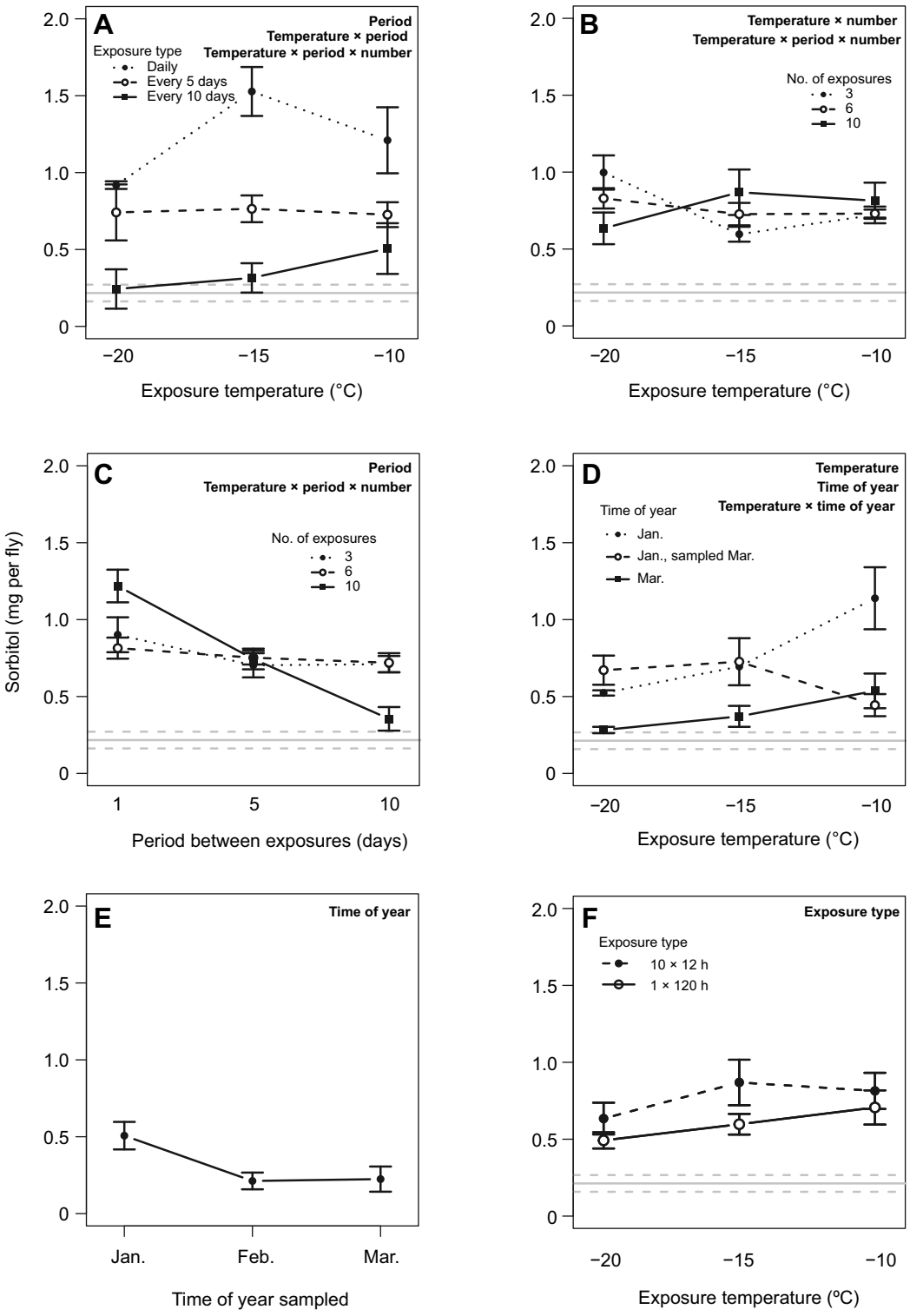

Fig. 5. Effects of low-temperature exposure on sorbitol mass in *E. solidaginis* prepupae. Solid and dashed horizontal grey lines represent means and s.e.m. of control flies, respectively. Points represent means (±s.e.m.). Bold terms refer to significant effects in the most parsimonious ANOVA model, as tested by AIC (n=5 pools of three individuals per group; all statistics are presented in Table S6). (A) Flies that received 10, 12 h freezing events as prepupae at three different time intervals. (B,C) Flies that received repeated 12 h freezing events as prepupae. (D) Flies that received a single, 120 h cold exposure in January or March and were sampled immediately, or received a bout in January but were sampled in March. (E) Control flies maintained at 0ºC that were sampled in January, February, then March. (F) Flies that received 10, 12 h bouts or a single 120 h bout of freezing exposure.

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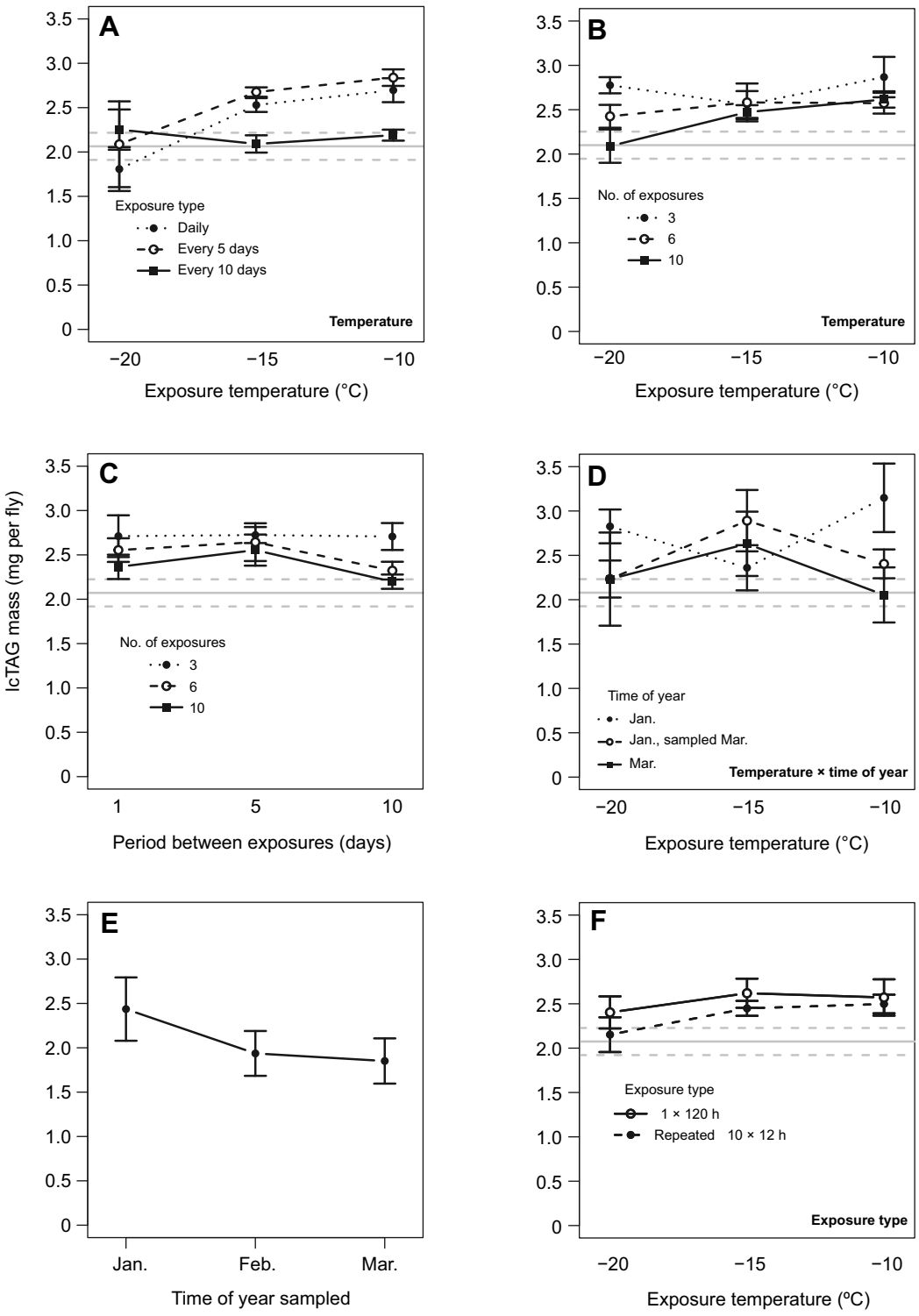

Fig. 6. Effects of low-temperature exposure on long-chain triacylglycerol (lcTAG) mass in *E. solidaginis* prepupae. Solid and dashed horizontal grey lines represent mean and s.e.m. of control flies, respectively. Points represent means (±s.e.m.). Bold terms refer to significant effects in the most parsimonious ANOVA model, as tested by AIC (n=5 pools of three individuals per group; all statistics are presented in Table S7). (A) Flies that received 10, 12 h freezing events as prepupae at three different time intervals. (B,C) Flies that received repeated 12 h freezing events as prepupae. (D) Flies that received a single, 120 h cold exposure in January or March and were sampled immediately, or received a bout in January but were sampled in March. (E) Control flies maintained at 0ºC that were sampled in January, February, then March. (F) Flies that received 10, 12 h bouts or a single 120 h bout of freezing exposure.

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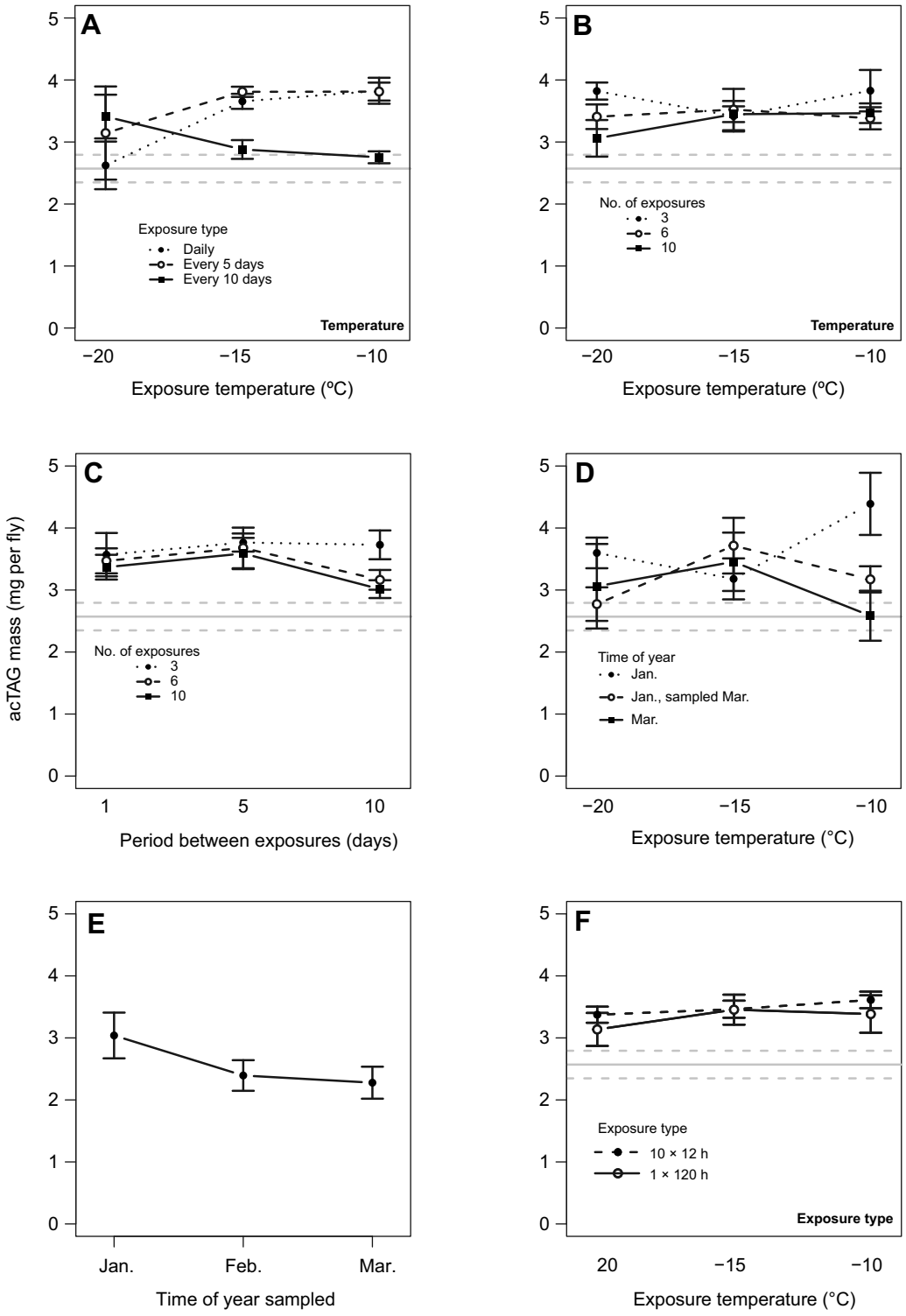

Fig. 7. Effects of low-temperature exposure on acetylated triacylglycerol (acTAG) mass in *E. solidaginis* prepupae. Solid and dashed horizontal grey lines represent means and s.e.m. of control flies, respectively. Points represent means (±s.e.m.). Bold terms refer to significant effects in the most parsimonious ANOVA model, as tested by AIC (n=5 pools of three individuals per group; all statistics are presented in Table S8). (A) Flies that received 10, 12 h freezing events as prepupae at three different time intervals. (B,C) Flies that received repeated 12 h freezing events as prepupae. (D) Flies that received a single, 120 h cold exposure in January or March and were sampled immediately, or received a bout in January but were sampled in March. (E) Control flies maintained at 0ºC that were sampled in January, February, then March. (F) Flies that received 10, 12 h bouts or a single 120 h bout of freezing exposure.

This suggests that the phenotype of increased somatic investment at the cost of reproductive output is a general response to repeated low-temperature stress in insects.

Both glycerol and sorbitol concentrations significantly increased after repeated cold exposures, which probably improves cold tolerance in the face of repeated freezing events. Surprisingly, there was less accumulation at lower temperature exposures relative to higher temperature exposures. As described previously (Churchill and Storey, 1989a; Doelling et al., 2014), repeatedly frozen *E. solidaginis* prepupae mobilize sorbitol; unlike these previous studies, we also detected increased glycerol in repeatedly frozen prepupae. Glycerol is easily catabolized (Churchill and Storey, 1989b) and, because glycerol is not readily recycled into glycogen (Storey and Storey, 1986), the decreased total glycerol mass with increasing period and number of exposures suggests direct catabolism of glycerol over time. By contrast, sorbitol is easily recycled to glycogen by *E. solidaginis* (Storey and Storey, 1983), which may explain why all exposure groups had similar glycogen concentration by the conclusion of the experiment (Table S4).

Repeated freezing appears to increase haemolymph glycerol concentration in other freeze-tolerant species (Marshall and Sinclair, 2011; Teets et al., 2011), suggesting it is a common response to repeated freezing exposure. Improved cold hardiness following low-temperature exposure ('rapid cold-hardening') is well characterized in chill-susceptible insects (Chen et al., 1987; Bale et al., 2002) and a few freeze-tolerant species (Lee et al., 2006; Teets et al., 2011). However, we do not interpret the increased glycerol and sorbitol with repeated freezing in *E. solidaginis* as rapid cold hardening, because repeated freezing induces additional glycerol and sorbitol synthesis and induces a fitness cost – neither of which is the case for rapid cold hardening.

Repeated freezing did not appear to consume glycogen and lipid reserves in *E. solidaginis*, which is in contrast to other studies on repeated freezing in insects (Churchill and Storey, 1989a; Teets et al., 2011). This is surprising, given we observed increased cryoprotectant accumulation which should cause a decrease in glycogen content of approximately 0.60 mg per prepupa (based on 1 mol of glucose units being consumed for every 1 mol of sorbitol and every 2 mol of glycerol) as was observed in *C. fumiferana* following repeated cold events (Marshall and Sinclair, 2015). Instead, prepupae shifted their lipid allocation from lcTAGs to acTAGs with repeated freezing in an equimolar fashion, as we have reported previously (Marshall et al., 2014). Acetylated triacylglycerols have two, rather than three, fatty acid moieties, and are thus lower in energy density than lcTAGs (Durrett et al., 2010), so it is possible that this translates to lower energy density in *E. solidaginis* and that cryoprotectant accumulation was fuelled by catabolism of the third fatty moiety. This conversion may be especially important when acTAGs are rapidly converted back to lcTAGs during metamorphosis (Marshall et al., 2014). We note other potential costs of repeated cold exposure; for example, *hsp70* expression increases with repeated freezing in *B. antarctica* (Teets et al., 2011) and lipid peroxidation significantly increases with repeated freezing in *E. solidaginis* (Doelling et al., 2014). These factors that we did not measure could contribute to reduced egg production.

We also note that we made our metabolite measurements during winter to obtain a snapshot during the overwintering period, so it is also possible that differences in energy reserves were not detectable until the end of winter. The biochemistry of acTAGs remains under investigation, but it is clear that biosynthesis of glycerol and sorbitol from glycogen is not 100% efficient (Storey and Storey, 1990). Thus, the increased energetic allocation to cryoprotection by the prepupae that received repeated exposures is probably a non-reversible ATP investment. Indeed, repeated freezing events reduce available ATP for *E. solidaginis* (Churchill and Storey, 1989a). This increased expenditure could explain the reduced egg production in flies that received repeated exposures, and provides a mechanistic link between the stress induced by repeated low-temperature exposures and the resulting reduction in fitness. Caloric restriction induces many insects to shift investment from reproduction to survival, and this switch is believed to be mediated by insulin signalling, at least in *D. melanogaster* (reviewed by Rion and Kawecki, 2007). In overwintering insects with a limited energetic budget, the conditions for a trade-off between reproduction and survival are met (van Noordwijk and de Jong, 1986; Zera and Harshman, 2001), although the signalling that mediates this transition is still unknown.

This study of altering the frequency and period of an easily manipulated variable (temperature), in a species with fitness proxies and physiology that are relatively easily measured demonstrates three important points: (1) laboratory measures of the capacity of phenotypic plasticity following a single stress exposure or acclimation are large underestimates (Churchill and Storey, 1989a; Lee et al., 2006; Teets et al., 2011), (2) stress accumulates along dimensions other than just intensity and duration, and (3) this stress can mediate life history trade-offs between somatic maintenance and reproduction. Thus, in the case of *E. solidaginis*, models that only incorporate the results from intensity and duration would overestimate fitness as the costs of cold exposure accumulate along the axes of frequency and period.

This demonstrates the importance of crossing physiological thresholds, a neglected parameter in many ecophysiological studies, and we suggest this paradigm could be extended to other abiotic stressors such as water availability, pH, wind and wave energy that similarly fluctuate and may cross similar physiological thresholds (Gaines and Denny, 1993; Chown and Terblanche, 2007; Gunderson et al., 2016; Williams et al., 2016). To understand how repeated threshold crossing influences populations, we propose two distinct steps. First, detailed laboratory studies (such as ours) to identify potential physiological thresholds and link their effects to population parameters such as survival and reproductive output. Second, species distribution or population models that incorporate abiotic stressors should investigate the impact of these threshold-crossing events. In the case of temperature, using predictors like number of threshold-crossing temperatures in addition to maximum, mean and minimum temperature in species distribution models should be routine, but would require incorporating environmental data with sufficient time resolution to detect threshold events (e.g. Kearney, 2012).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: K.E.M., B.J.S.; Methodology: K.E.M., B.J.S.; Validation: K.E.M.; Formal analysis: K.E.M.; Investigation: K.E.M.; Resources: K.E.M., B.J.S.; Data curation: K.E.M.; Writing - original draft: K.E.M.; Writing - review & editing: K.E.M.,

B.J.S.; Visualization: K.E.M.; Supervision: B.J.S.; Project administration: B.J.S.; Funding acquisition: B.J.S.

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Supplementary information
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