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Interactive effects of temperature and time on cold tolerance and spring predation in overwintering soil predatory mites (*Gaeolaelaps aculeifer Canestrini*)

Kim Jensen*, Jesper G. Sørensen, Martin Holmstrup

a Department of Bioscience, Soil Fauna Ecology and Ecotoxicology, Aarhus University, Vejlsøvej 25, 8600 Silkeborg, Denmark

b Department of Bioscience, Genetics, Ecology and Evolution, Aarhus University, Ny Munkegade 116, 8000 Aarhus C, Denmark

c Arctic Research Center, Aarhus University, Ny Munkegade 114, 8000 Aarhus C, Denmark

* Corresponding author at: Department of Bioscience, Soil Fauna Ecology and Ecotoxicology, Aarhus University, Vejlsøvej 25, 8600 Silkeborg, Denmark

E-mail address: kj@bios.au.dk (K. Jensen).

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A B S T R A C T

Soil living mites have large potential as biocontrol agents against soil-dwelling pests, but little is known about their ecological and ecophysiological responses to cold. We investigated the interactive effects of acclimation temperature and time on cold tolerance in the laelapid mite *Gaeolaelaps aculeifer* Canestrini after exposure to 5, 10, 15, or 20 °C for 1, 4, or 8 days. Another group of mites were subjected to simulated winter by gradually lowering the temperature from 20 to 0.8 °C during three months, while measuring tolerance to -2 and -5 °C as well as supercooling point and melt onset temperature of body fluids at start (20 °C; “summer”), at 5 °C (“autumn”), and at 0.8 °C (“winter”). A third group was kept at constant 10 °C as a constant mild cold comparison. We found a strong interaction between exposure temperature and time on cold tolerance, with rapid cold hardening after 24 h at 5 °C but increasing cold acclimation at 10 °C. During simulated winter, tolerance to -2 °C was high after two months at 4.1 °C, but then decreased to intermediate levels after another month at 0.8 °C. The supercooling point did not change over the simulated winter, but melt onset temperature was lowered after 0.8 °C exposure. Mites preyed and reproduced readily following simulated winter, but at lower rates than if kept at constant 10 °C. Our study indicates that *G. aculeifer* can overwinter following release, and suggests that cold storage is advantageous before inoculative release in early spring.

**Keywords:** Cold exposure; Ecophysiology; Mesostigmata; Predation; Reproduction; Winter acclimation
Organisms have evolved adaptations to different temperatures (Angilletta, 2009; Prosser, 1955), and have in addition evolved phenotypic plasticity that allows individuals to thermally acclimate to their seasonal environment (DeWitt et al., 1998; Johnston and Bennett, 1996). Arthropods acclimate to thermal conditions by adjusting physiological parameters, and these changes can be highly beneficial for surviving stressful temperatures (Chown and Terblanche, 2007; Overgaard and MacMillan, 2017; Teets and Denlinger, 2013). This is particularly true for cold acclimation that can markedly alter thermal limits to cold exposures (Alemu et al., 2017; Klok and Chown, 2003; Sinclair and Roberts, 2005). However, acclimation mechanisms to prevent cold damage differ depending on whether thermal decline is abrupt or occurs gradually due to changing seasons (Teets and Denlinger, 2013), which has importance for survival across seasons for biological control agents. Furthermore, predation efficiency and reproduction of biological control agents is found to be negatively affected by cold (Helgadóttir et al., 2017; Jensen et al., 2017, 2019), but the interactive effects between exposure time and temperature have not been studied.

Microarthropods living in soils of temperate regions experience less extreme winter temperatures than animals living above ground because the soil is a thermally buffered habitat (Ashcroft and Gollan, 2013; Convey et al., 2015; Haruna et al. 2017). However, if no thermal insulation from snow cover or vegetation is present, they may occasionally be exposed to sub-zero temperatures with risks of winter mortality due to freezing (Henry, 2008; Holmstrup, 2018a). Most often, soil microarthropods survive sub-zero temperature using the strategy “freeze avoidance” in which the animal depends on the ability to remain unfrozen (supercooled) when the temperature of its habitat is below the freezing point (Cannon and Block, 1988; Holmstrup, 2014; Sømme, 1982). In general, freeze-avoiding species have physiological adaptations promoting the ability to supercool including the accumulation of cryoprotectants (e.g. polyols or sugar alcohols), the
masking or elimination of ice-nucleators and production of antifreeze proteins stabilizing the supercooled state (Lee, 2010; Zachariassen, 1985). Microarthropods are generally good supercoolers because they contain only small volumes of aqueous solutions with their minute body size (Cannon and Block, 1988; Sømme, 1982; Zachariassen et al., 2004).

During autumn, declining temperatures trigger a range of physiological acclimation processes that gradually prepare microarthropods for low winter temperatures and increase their cold tolerance. These processes often lead to better supercooling capacity, but also include homeoviscous adaptation of cellular membranes, increased oxidative stress defense systems and up-regulation of stress induced molecular chaperones such as heat shock proteins, altogether reducing the detrimental effects of low temperature (Teets and Denlinger, 2013). Seasonal biochemical and physiological changes need weeks to months to come into effect, but soil arthropods can also improve their cold tolerance on a much shorter time-scale to meet with short-term bouts of low temperature (Bahrndorff et al., 2009; Convey et al., 2015; Waagner et al., 2013). This phenomenon is termed rapid cold hardening requiring acclimation to mildly low temperatures for only a few days or even a few hours (Lee et al., 1987; Teets and Denlinger, 2013). The mechanisms by which rapid cold hardening works are not fully understood, but inhibition of apoptotic cell death, cellular calcium signaling and membrane restructuring are involved in this process (Teets and Denlinger, 2013).

The soil living mesostigmatid mite Gaeolaelaps aculeifer Canestrini (Acari: Laelapidae) is a generalist predator widespread in Europe (Usher and Davis, 1983). This mite lives in the upper soil layers (0-15 cm) where it feeds on a variety of soil invertebrates such as nematodes, springtails, enchytraeids, other mites, and insect larvae and pupae (Ignatowicz, 1974; Karg, 1961a,b; Moreira & de Moraes, 2015). It reproduces by arrhenotoky in which unfertilized females lay eggs that develop into haploid males, and fertilized females produce eggs developing into diploid females (De Jong,
1981; Usher and Davis, 1983). The egg-to-egg life cycle duration is approximately 25 days at 20 °C (Krogh, 1995). Females may live for more than 200 days and survive starvation for at least 100 days at 10 °C (Jensen et al., 2017; Murphy and Sardar, 1991).

Gaeolaelaps aculeifer is commercially available and successfully used as a biological control agent in augmentative releases against thrips and other soil-inhabiting pests in both greenhouses and agroecosystems (Gerson and Weintraub, 2007; van Lenteren, 2012). Mesostigmatid mites may be advantageous predatory control agents for inoculative releases in agriculture as their dispersal ability is limited, and they therefore are less likely to migrate away from the crop than most flying insect predators although some may disperse by phoresy (Koehler, 1997). However, inoculative releases are more likely to be successful if the species are able to establish permanently and tolerate the ambient abiotic conditions, and in particular, the thermal conditions during all seasons (Ghazy et al., 2016; Hart et al., 2002). Commercial biological control agents including G. aculeifer are often reared at 20-25 °C maximizing population growth rates and thereby production (e.g. Amin et al., 2014; Salehi et al., 2014), but if released to field conditions they encounter low temperatures during winter, which they must tolerate in order to avoid high winter mortality and establish permanent populations. Despite the importance of this species in various ecosystems, and as a biological control agent, little information about the cold tolerance of G. aculeifer is available (but see Jensen et al., 2017).

In a recent study, we found that cold tolerance of commercially reared G. aculeifer can improve following short-term acclimation to mildly low temperature (4 days at 10 °C), which potentially can be exploited during mass rearing to increase survival and predation efficacy under low temperature field conditions (Jensen et al., 2017). However, we also found that cold tolerance was not improved after 4 days at 5 °C, indicating detrimental effects of abrupt exposure to this temperature (Jensen et al., 2017). It is therefore important to elucidate how acclimation temperature
and acclimation time interact under short-term cold acclimation, and further, to describe the effects of seasonal acclimation to winter conditions. Rapid cold hardening has been studied in a few other predatory mites (Broufas and Koveos, 2001; Ghazy and Amano, 2014), but more ecologically relevant long-term acclimation treatments and associated cost-benefit trade-offs of relevance for the efficiency of predatory mites as biological control agents have not been studied before. We also found lower predation rate and egg production after cold exposure in previous studies (Jensen et al., 2017, 2019). However, time of cold exposure time was constant within these studies and did not include temperatures below 10 °C. Furthermore, no effect of gradual thermal decline was included, which would theoretically allow stepwise acclimation to increasing cold.

Our main hypothesis was that beneficial effects of cold-acclimation on cold tolerance correlate positively with acclimation time at mild cold (10 and 15 °C), but that exposure would become increasingly detrimental over time at very low temperature (5 °C and below). We also hypothesized that a gradual cooling would acclimate mites to tolerate and survive low temperature (5 °C) that is detrimental at abrupt exposure. Accordingly, we here report the effects on cold tolerance of both short-term cold acclimation (1-8 days) upon abrupt exposure and long-term seasonal cold acclimation simulating a 5-month autumn and winter course with a gradual thermal decline in *G. aculeifer*. We used adult females for these studies since only this life stage and sex overwinters in northern Europe (Karg, 1961a). Lastly, we tested the predation efficiency and egg production of females after the simulated overwintering to determine their state of fitness and biological control potential, and to compare whether simulated winter exposure had reduced voracity and fitness compared to long-term storage at mildly low temperature (10 °C).
2. Materials and Methods

2.1. Animals and maintenance

A culture of predominantly adult *G. aculeifer* were purchased from Öre Bio-Protect GmbH (Schwentinental, Germany). This strain was originally collected in 1995 from compost soil near Schwentinental, Germany, and has since then been cultured at room temperature in moist vermiculite and fed mould mites (*Tyrophagus putrescentiae* Schrank) as prey. After receipt, we collected adult *G. aculeifer* using Tullgren extraction into plastic beakers (diameter 6 cm; height 4 cm) with a bottom of moistened plaster of Paris/charcoal (8:1 w/w). We then kept ca. 100 adult females in each beaker at 20 ± 0.5 °C for two months and fed them early-instar *Folsomia candida* Willem (Isotomidae; Collembola) *ad libitum* until onset of the experiment. We used this springtail as prey since it has high nutritional value for *G. aculeifer* ( Heckmann et al., 2007; Krogh, 1995).

2.2. Effects of short-term cold acclimation on cold tolerance

To test the effects of short-term acclimation, we exposed mites taken directly from 20 °C to acclimation at 5, 10, 15 or 20 °C for 1, 4 or 8 days, respectively. Following the exposure, we allocated groups of ten mites (*N* = 5-15 replicates per treatment, Table 1) in 2 mL Eppendorf tubes, and acutely exposed the mites to −2 ± 0.1 °C for 24 h by submerging the tubes in a precooled water bath (Lauda Eco Gold, RE 1050, Lauda-Königshofen, Germany). After the cold exposure, the mites from each tube were placed for 24 h at 20 °C in a 5 cm Petri dish with a water-saturated Plaster of Paris bottom to recover. Percentage survival was scored under a dissection microscope by counting the proportion of individuals that were able to walk after gentle stimulation with a fine paintbrush.
2.3 Effects of seasonal cold acclimation on cold tolerance

In order to simulate the temperature decline during Danish autumn and winter (seasonal acclimation treatment) we placed 20 beakers with up to 100 adult female *G. aculeifer* per beaker for 10 days at 15.7 ± 0.2 °C, then for 16 days at 9.4 ± 0.2 °C, followed by two months at 4.1 ± 0.2 °C and finally for 35 days at 0.8 ± 0.2 °C. After this gradual thermal decline and winter simulation, the beakers with mites were placed for 11 days at 5 °C followed by 30 days at 10 °C in order to simulate rising temperatures during spring. Throughout this experiment, mites were provided *ad libitum* with early-instar *F. candida*. Another group of female *G. aculeifer* were kept in similar beakers and density at 10 °C (constant 10 °C treatment) from two months before the start of the seasonal cold acclimation treatment and similarly provided *ad libitum* with early-instar *F. candida* as a long-term “constant mild cold” acclimation treatment. At intervals, mites from both acclimation treatments were sampled and assayed for cold tolerance as described in section 2.2, using groups of ten mites after acute exposure to either −2 or −5 ± 0.1 °C for 24 h (*N* = 6 replicates per treatment). No progeny appeared and completed development within any of the two treatments, so all assays were performed on adult females that were placed in the beakers at experiment start.

2.4 Effects of seasonal cold acclimation on supercooling point and melt onset temperature

Mites from the seasonal acclimation treatment were sampled at intervals and assayed for supercooling point and melt onset temperature using differential scanning calorimetry (DSC), including mites from the constant 10 °C treatment only at the final collection point (*N* = 6 samples of 5 mites per treatment, Table 1). This analysis provided the supercooling point of each individual and subsequently the mean melt onset temperature of all individuals within a sample as an indication of the melting point of body fluids. For DSC analysis, we used a DSC4000 calorimeter (Perkin Elmer, Waltham, MA, USA). Four to five mites were transferred to a 50 μL aluminium
DSC-pan, which was immediately hermetically sealed. Samples of mites were subjected to a DSC program consisting of six steps: (1) hold for 1 min at 5 °C; (2) cool to –35 °C at a rate of –5 °C min⁻¹; (3) hold for 1 min at –35 °C; (4) heat to –20 °C at a rate of 5 °C min⁻¹; (5) hold for 1 min at –20 °C (6) heat to 5 °C at a rate of 1 °C min⁻¹. The melting endotherm (enthalpy change during melting of ice formed in the animal) of the heating scan curve was analyzed and the melt onset temperature estimated using Pyris Software (Perkin Elmer, Waltham, MA, USA) as described by Block (1994).

2.5. Effects of “overwintering” on predation and fecundity

After the seasonal cold acclimation and following re-warming to 10 °C for 30 days, we tested kill rate and reproduction during 8 days at 20 °C on *F. candida* as described by Jensen et al. (2019). In brief, we allocated 24 adult female mites from each overwintering treatment (seasonal acclimation and constant 10 °C) to individual wells in two 24-well cell culture plates distributing treatments evenly across plates. The well bottoms were covered with 5 mm water-saturated plaster of Paris/charcoal, which was kept saturated throughout the experiment. The tops were closed with rubber plugs. We supplied 10 early-instar *F. candida* to each mite. Killed *F. candida* and numbers of eggs laid by each female mite were counted daily. Killed springtail were replaced and their remains removed daily.

2.6. Statistical Analysis

For the short-term acclimation experiment, we analyzed the effects of temperature, time, and their interaction on cold survival using a linear mixed model. Proportions were arcsin-sqrt transformed to account for heterogeneity of variances. Due to strong non-linear responses, the model was fitted using second degree polynomials for the two predictor variables. As we found a strong significant interaction between acclimation time and temperature and non-linear temperature effects on cold
tolerance, effects of acclimation time on cold tolerance were further analyzed within each
temperature using time as a linear predictor variable.

For the simulated winter acclimation experiment, we analyzed the effects of sampling time
point, overwintering treatment, and their interaction on the proportion of surviving individuals
using linear mixed models within each test temperature (-2 or -5 °C). Proportions were arcsin-sqrt
transformed to account for heterogeneity of variances. Differences in cold tolerance between
individual time points were analyzed using a Tukey HSD test. Supercooling points and melt onset
temperature, numbers of *F. candida* killed and numbers of eggs laid per female *G. aculeifer* after
overwintering, and numbers of eggs laid per killed *F. candida* were analysed using linear mixed
models. All analyses were performed in the lme4 package (Bates et al., 2015) in R (v. 3.4.2) (R-
Development-Core-Team, 2017).

3. Results

3.1. Effects of short-term cold acclimation on cold tolerance

Acclimation temperature significantly affected survival at -2 °C for 24 h (*F*<sub>2,107</sub> = 23.3, *P* < 0.001),
showing that cold tolerance was generally highest after acclimation at low temperature (Fig. 1).
Furthermore, the interaction between temperature and acclimation time significantly affected cold
tolerance (*F*<sub>2,107</sub> = 16.8, *P* < 0.001), reflecting opposing effects of acclimation time on survival to
cold exposure between temperatures (Fig. 1). Acclimation time alone did not have significant
influence on cold tolerance across temperatures (*F*<sub>1,107</sub> = 0.03, *P* = 0.87; Fig. 1). When analyzing the
effect of acclimation time on cold tolerance separately within each acclimation temperature, we
found a significant negative linear effect of exposure time at 5 °C on survival to -2 °C exposure
(\(F_{1,25} = 43.0, P < 0.001\), estimate ± sem = -0.12 ± 0.01), while longer exposure time at 10 °C had a significantly positive linear effect on survival to cold (\(F_{1,25} = 17.9, P < 0.001\), estimate ± sem = 0.10 ± 0.02). At 15 and 20 °C, acclimation time had no significant effect on cold tolerance (\(P > 0.05\); Fig. 1), although there was a tendency towards higher cold tolerance after more than one day of acclimation at 15 °C (Fig. 1).

3.2. Effects of seasonal cold acclimation on cold tolerance

Survival at -2 °C for 24 h was significantly affected by acclimation treatment (constant 10 °C or simulated seasonal cold acclimation) (\(F_{1,30} = 218.9, P < 0.001\)), sampling time point (\(F_{2,30} = 66.9, P < 0.001\)) and their interaction (\(F_{2,30} = 70.3, P < 0.001\)). Whereas the proportion surviving -2 °C exposure was nearly 100% at all time points for the mites that were maintained at constant 10 °C (Fig. 2), cold tolerance changed over time in the mites exposed to seasonal cold as they were exposed to different temperatures (Fig. 2). Survival to -2 °C exposure was lowest at the outset of the experiment before any cold acclimation, highest after seasonal cooling including the period at 5 °C, and intermediate late in winter after an additional period at 0.8 °C, with significant differences between all three time points (Tukey HSD test at \(\alpha = 0.05\); Fig. 2).

Survival to -5 °C exposure was low within all treatments. The linear model showed no effect of acclimation treatment (constant 10 °C or simulated winter acclimation) (\(F_{1,30} = 1.1, P = 0.30\)) and no interaction (\(F_{2,30} = 2.4, P = 0.11\)), but a significant effect of sampling time point (\(F_{2,30} = 4.2, P = 0.025\)). This main effect was caused by the poor cold tolerance in the first sampling time point by mites in the simulated winter treatment, where animals came directly from 20 °C and no cold acclimation had occurred (Fig. 2).
3.3. Effects of seasonal cold acclimation on supercooling point and melt onset temperature

A linear model of the effect of simulated winter acclimation showed no significant effect on supercooling point ($F_{1,81} = 1.3$, $P = 0.26$; Fig. 3). Furthermore, simulated winter and constant 10 °C acclimation did not lead to a difference in supercooling point at the end of the experiments ($F_{1,54} = 0.3$, $P = 0.58$; Fig. 3).

Simulated winter acclimation did significantly decrease the body fluid melt onset temperature during the course of the experiment ($F_{1,16} = 15.7$, $P = 0.001$; Fig. 4). However, the effect size was modest with a linear model estimate of -0.003 °C per day throughout the simulated winter acclimation treatment. The constant 10 °C treatment had a minor effect on melt onset temperature, with a significantly higher melt onset temperature than that of the simulated winter treatment at the end of the experiment ($F_{1,10} = 5.5$, $P < 0.05$; Fig. 4).

3.4. Effects of “overwintering” on predation and fecundity

Following the simulated winter, mites killed significantly fewer *F. candida* prey ($F_{1,46} = 19.7$, $P < 0.0001$; Fig. 5A), and laid significantly fewer eggs ($F_{1,46} = 11.7$, $P = 0.0013$; Fig. 5B) than mites that had been maintained at a constant 10 °C throughout the winter. However, the mites that had been exposed to constant 10 °C did not exploit killed prey significantly more efficiently for egg production than the simulated winter exposed mites ($F_{1,46} = 2.6$, $P = 0.11$; Fig. 5C).

4. Discussion

Soil predatory mites have great potential as biological control agents in agroecosystems (Koehler, 1997; Moreira and de Moraes, 2015), but more knowledge on their ecology and ecophysiology including overwintering potential would be advantageous for successful use in a temperate environment (Knapp et al., 2018). We show a distinct interaction between effects of temperature and exposure time on cold tolerance following abrupt cold exposure (Fig. 1). Under gradual thermal
decline, mites gradually acclimated to decreasing temperature and only near the freezing point did
low thermal exposure have a negative effect on tolerance to -2 °C (Fig. 2). Mites readily killed prey
and resumed egg production following the simulated winter, but the low temperatures in late winter
caused lower predation and reproduction compared to mites only exposed to mild cold (10 °C) over
the entire period. Our results show that the time vs. temperature regime of cooling significantly
affects cold tolerance in adult female *G. aculeifer*, with gradual thermal decline and temperatures
not too close to freezing minimizing the damaging effects of cold.

A sudden shift in temperature from 20 to 5 °C was evidently stressful if the mites were kept
at 5 °C for several days (Fig. 1). However, if exposure to 5 °C was preceded by a gradual decrease
in temperature, survival to -2 °C exposure was nearly 100% (Fig. 2). Interestingly, mites that had
been kept at 10 °C for several months were equally or more cold-tolerant than mites subjected to
simulated winter at temperatures of 0-5 °C (Fig. 2). It is therefore clear from our study that efficient
cold acclimation must be gradual or at least not represent such a large and abrupt change in
temperature that the detrimental effects overshadow the beneficial effects for cold survival. The
highly beneficial effect of acclimation to 5 °C for just one day is a clear effect of rapid cold
hardening (Teets and Denlinger, 2013), whereas the decreasing benefit of acclimation over time at
this temperature could in part be due to low-temperature stress of the hardening treatment in itself.
Thus, it is possible that protective responses initiated during the longer acclimation times are over-
ridden by the stress accumulated during the longer hardening treatment as observed in other
arthropods such as fruit flies (Overgaard et al., 2006).

The supercooling capacity of *G. aculeifer* was as good as is normally seen for soil mites and
other microarthropods (Sømme, 1982). On average, female mites had supercooling points around -
18 °C and rarely above -10 °C. This implies that the observed lethality at -2 or -5 °C was not
associated with freezing of body fluids. Accordingly, *G. aculeifer* is categorized as chill susceptible
possessing moderately chill tolerant characteristics (Bale, 1993, 1996). Supercooling point did not decrease during winter acclimation as seen in some arthropods including mites (Hodkova and Hodek, 1997; Ma et al., 2006; Sjursen and Sømme, 2000), but not in all (Bennett and Lee, 1989; Sinclair, 1997; Turnock et al., 1983). The supercooling point seems in this case not to be associated with cold tolerance, but rather is a simple physical phenomenon resulting from the small volume of hemolymph that these mites contain (Renault et al., 2002).

The melt onset temperature decreased slightly from ca. -1.7 °C in warm acclimated animals over time and as ambient temperature was lowered, and ended at ca. -2.1 °C (at ambient temperature of 0.8 °C). The melt onset temperature of intact mites estimated by DSC analysis is not the same as the melting point of their hemolymph, but there is a proportionality between the two (Holmstrup, 2018b). Our results therefore indicate that hemolymph osmolality increased significantly during long-term cold acclimation likely by accumulation of low-molecular weight cryoprotectants. Cold tolerant mites typically accumulate glycerol and some sugar alcohols (e.g. mannitol) during cold acclimation as their principal cryoprotectant (Cannon, 1987; Sømme, 1982), and this is thought to improve cold-tolerance by stabilizing protein and membrane integrity at low temperatures (Crowe, 2002; Koštál et al., 2001, 2012; Lee, 2010). However, the increase in osmolality of cold acclimated *G. aculeifer* did not correlate with cold tolerance. On the contrary, mites acclimated at constant 10 °C had a better survival at -2 °C than mites acclimated at 0.8 °C despite the fact that these cold acclimated mites had a higher osmolality.

Karg (1961a) reported that *G. aculeifer* in fields near Berlin, Germany, where soil temperature was around 0 °C or just below overwintered solely as adult females. Densities in early spring were very low and populations peaked in August-September, suggesting that regrowth of populations occurred quickly during spring and summer despite high mortality during winter (Karg, 1961a). Our results supplement this picture and indicate that inoculative releases in north-west
Europe can successfully establish populations of *G. aculeifer* that are able to overwinter and proliferate in these regions. Our results, showing that overwintering females were able to prey on springtails and reproduce following the simulated winter, illustrate that *G. aculeifer* can indeed overwinter and proliferate (Fig. 5). Negative effects of cold exposure on predation and reproduction have been demonstrated before in biological control agents including *G. aculeifer* (Helgadóttir et al., 2017; Jensen et al., 2017, 2019). Here, we show that low thermal exposure rather than longer time of mild cold exposure negatively affected fitness and predation in *G. aculeifer* (Fig. 5). Thus, adult female *G. aculeifer* can overwinter under conditions resembling winters in the temperate region. However, as the species is not highly cold tolerant, it needs refuges where temperatures do not drop far below the freezing point.

Cold storage of biological control agents is common practice, but long term storage typically causes cold damage (Colinet and Boivin, 2011; Luczynski et al., 2008). Our study shows that storage of *G. aculeifer* at 10 °C produces mites with high cold tolerance after few days of storage. Furthermore, mites can be kept over long term without deleterious effects. This is advantageous if releasing the mites in early spring where night temperatures may drop below freezing. Furthermore, 10 °C storage also increases starvation tolerance in *G. aculeifer* (Jensen et al., 2017, 2018). If used preventatively in spring when temperatures fluctuate and pests have not yet established in the crop, storage at 10 °C might therefore be advantageous as mites are tolerant to low temperature and can sustain with little prey. If stored at lower temperature, we recommend a gradual thermal decline to the target storage temperature to allow acclimation and minimize deleterious effects of cold shock even though this induces high cold tolerance at a short term.
5. Conclusion

We found that adult female *G. aculeifer* gradually acclimate to increasing cold under overwintering, but that they are unable to tolerate -5 °C regardless of acclimation temperature. The species is thus robust to typical winter soil temperatures in northern Europe, but not highly cold tolerant. If cold storage is preferred before use in biological control, both storage temperature and cooling rate is of importance. Mites reared at 20 °C can thus at advantage be given 24 h of exposure to 5 °C before use under cold conditions. However, we recommend a longer term maintenance at 10 °C before use in cold as mites have induced proper acclimation responses to sustain under cold. If storing at temperatures below 10 °C, we recommend a gradual thermal decline to facilitate acclimation and prevent cold damage. Our study shows that *G. aculeifer* can overwinter and sustain under cold temperatures through rapid cold hardening and seasonal acclimation, but that predatory performance and reproduction are highest if mites are protected from severe cold. We encourage actively cold acclimating biological control agents at mild cold prior to release when used in cold or thermally fluctuating environments to improve their tolerance to intermittent cold shocks while maintaining high preying and reproductive rates.

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Table 1
Number of replicates and adult female *Gaeolaelaps aculeifer* per replicate in the experimental assays.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Assay</th>
<th>Replicates (#)</th>
<th>Mites per replicate (#)</th>
</tr>
</thead>
<tbody>
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<td>Short term exposure</td>
<td>-2°C tolerance after 1 day at 5°C</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>-2°C tolerance after 4 days at 5°C</td>
<td>10</td>
<td>10</td>
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<tr>
<td></td>
<td>-2°C tolerance after 1 day at 10°C</td>
<td>5</td>
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**Figure legends**

**Fig. 1.** Proportional survival (mean ± S.E.) to -2 °C exposure for 24 h in adult female *Gaeolaelaps aculeifer* pre-exposed to 5, 10, 15, or 20 °C for 1, 4, or 8 days. As mites were maintained at 20 °C prior to experiment, the 20 °C treatment functions as a constant temperature control.

**Fig. 2.** Proportional survival (mean ± S.E.) to either -2 or -5 °C exposure for 24 h in adult female *Gaeolaelaps aculeifer* subjected to simulated winter temperatures (solid gray line) or constant 10 °C (dashed gray line) at 20, 96, and 138 days since initiation of the winter simulation. Seasonally acclimated mites (circles) were thus exposed to temperatures following the solid gray line prior to the cold assays, while mites kept at constant 10 °C (triangles) had followed the dashed gray line.

**Fig. 3.** Supercooling temperatures (mean ± S.E. and all individual points) of adult female *Gaeolaelaps aculeifer* subjected to simulated winter temperatures (solid gray line) or constant 10 °C (dashed gray line) at 20, 96, and 138 days since initiation of the winter simulation. Seasonally acclimated mites (circles) were thus exposed to temperatures following the solid gray line prior to the supercooling assay, while mites kept at constant 10 °C (triangles) had followed the dashed gray line. These were only tested at 138 days.

**Fig. 4.** Melt onset temperatures (mean ± S.E.) of adult female *Gaeolaelaps aculeifer* subjected to simulated winter temperatures (solid gray line) or constant 10 °C (dashed gray line) at 20, 96, and 138 days since initiation of the winter simulation. Seasonally acclimated mites (circles) were thus exposed to temperatures following the solid gray line prior to the melt onset assay, while mites kept at constant 10 °C (triangles) had followed the dashed gray line. These were only tested at 138 days.
Fig. 5. Numbers (mean ± S.E.) of (A) juvenile *Folsomia candida* prey killed, (B) eggs produced, and (C) eggs produced per killed prey by adult female *Gaeolaelaps aculeifer* previously subjected to simulated winter followed by re-warming to 10 °C (winter treatment, solid gray line in Fig. 2-4) or constant 10 °C (dashed gray line in Fig. 2-4).
Fig. 1

![Graph showing the proportion surviving at different temperatures (5°C, 10°C, 15°C, 20°C) over days of exposure (1, 4, 8).](image)
Fig. 2

![Graph showing the proportion of organisms surviving at different acclimation times and temperatures. The graph is divided into two parts, one for -2°C and another for -5°C. The x-axis represents acclimation time (days), ranging from 20 to 138, and the y-axis represents the proportion surviving, ranging from 0 to 1.0. Symbols indicate different treatments: Winter acclimated (○) and Constant 10°C (△). The dashed line indicates a temperature threshold.](image-url)
Fig. 3

![Graph showing supercooling point vs. acclimation time and temperature.](graph.png)

- **Winter acclimated**: Represented by circles.
- **Constant 10°C**: Represented by triangles.

Temperature (°C) vs. Acclimation time (days).
Fig. 4

![Graph showing the relationship between acclimation time and melt onset temperature. The graph includes two lines: one for winter acclimated and another for constant 10°C. The x-axis represents acclimation time (days), and the y-axis represents melt onset temperature (°C).](image-url)
Fig. 5

(A)  $F.\ candida$ killed (#)

(B)  Eggs laid (#)

(C)  Eggs laid per killed $F.\ candida$ (#/#)

Previously experienced cold regime