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

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21 A B S T R A C T

22 Soil living mites have large potential as biocontrol agents against soil-dwelling pests, but little is
23 known about their ecological and ecophysiological responses to cold. We investigated the
24 interactive effects of acclimation temperature and time on cold tolerance in the laelapid mite
25 *Gaeolaelaps aculeifer* Canestrini after exposure to 5, 10, 15, or 20 °C for 1, 4, or 8 days. Another
26 group of mites were subjected to simulated winter by gradually lowering the temperature from 20 to
27 0.8 °C during three months, while measuring tolerance to -2 and -5 °C as well as supercooling point
28 and melt onset temperature of body fluids at start (20 °C; “summer”), at 5 °C (“autumn”), and at 0.8
29 °C (“winter”). A third group was kept at constant 10 °C as a constant mild cold comparison. We
30 found a strong interaction between exposure temperature and time on cold tolerance, with rapid
31 cold hardening after 24 h at 5 °C but increasing cold acclimation at 10 °C. During simulated winter,
32 tolerance to -2 °C was high after two months at 4.1 °C, but then decreased to intermediate levels
33 after another month at 0.8 °C. The supercooling point did not change over the simulated winter, but
34 melt onset temperature was lowered after 0.8 °C exposure. Mites preyed and reproduced readily
35 following simulated winter, but at lower rates than if kept at constant 10 °C. Our study indicates
36 that *G. aculeifer* can overwinter following release, and suggests that cold storage is advantageous
37 before inoculative release in early spring.

38

39 *Keywords:* Cold exposure; Ecophysiology; Mesostigmata; Predation; Reproduction; Winter
40 acclimation

41

42 **1. Introduction**

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

56 Microarthropods living in soils of temperate regions experience less extreme winter
57 temperatures than animals living above ground because the soil is a thermally buffered habitat
58 (Ashcroft and Gollan, 2013; Convey et al., 2015; Haruna et al. 2017). However, if no thermal
59 insulation from snow cover or vegetation is present, they may occasionally be exposed to sub-zero
60 temperatures with risks of winter mortality due to freezing (Henry, 2008; Holmstrup, 2018a). Most
61 often, soil microarthropods survive sub-zero temperature using the strategy “freeze avoidance” in
62 which the animal depends on the ability to remain unfrozen (supercooled) when the temperature of
63 its habitat is below the freezing point (Cannon and Block, 1988; Holmstrup, 2014; Sømme, 1982).
64 In general, freeze-avoiding species have physiological adaptations promoting the ability to
65 supercool including the accumulation of cryoprotectants (e.g. polyols or sugar alcohols), the

66 masking or elimination of ice-nucleators and production of antifreeze proteins stabilizing the
67 supercooled state (Lee, 2010; Zachariassen, 1985). Microarthropods are generally good
68 supercoolers because they contain only small volumes of aqueous solutions with their minute body
69 size (Cannon and Block, 1988; Sømme, 1982; Zachariassen et al., 2004).

70 During autumn, declining temperatures trigger a range of physiological acclimation
71 processes that gradually prepare microarthropods for low winter temperatures and increase their
72 cold tolerance. These processes often lead to better supercooling capacity, but also include
73 homeoviscous adaptation of cellular membranes, increased oxidative stress defense systems and up-
74 regulation of stress induced molecular chaperones such as heat shock proteins, altogether reducing
75 the detrimental effects of low temperature (Teets and Denlinger, 2013). Seasonal biochemical and
76 physiological changes need weeks to months to come into effect, but soil arthropods can also
77 improve their cold tolerance on a much shorter time-scale to meet with short-term bouts of low
78 temperature (Bahrndorff et al., 2009; Convey et al., 2015; Waagner et al., 2013). This phenomenon
79 is termed rapid cold hardening requiring acclimation to mildly low temperatures for only a few days
80 or even a few hours (Lee et al., 1987; Teets and Denlinger, 2013). The mechanisms by which rapid
81 cold hardening works are not fully understood, but inhibition of apoptotic cell death, cellular
82 calcium signaling and membrane restructuring are involved in this process (Teets and Denlinger,
83 2013).

84 The soil living mesostigmatid mite *Gaeolaelaps aculeifer* Canestrini (Acari: Laelapidae) is a
85 generalist predator widespread in Europe (Usher and Davis, 1983). This mite lives in the upper soil
86 layers (0-15 cm) where it feeds on a variety of soil invertebrates such as nematodes, springtails,
87 enchytraeids, other mites, and insect larvae and pupae (Ignatowicz, 1974; Karg, 1961a,b; Moreira &
88 de Moraes, 2015). It reproduces by arrhenotoky in which unfertilized females lay eggs that develop
89 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

114 and acclimation time interact under short-term cold acclimation, and further, to describe the effects
115 of seasonal acclimation to winter conditions. Rapid cold hardening has been studied in a few other
116 predatory mites (Broufas and Koveos, 2001; Ghazy and Amano, 2014), but more ecologically
117 relevant long-term acclimation treatments and associated cost-benefit trade-offs of relevance for the
118 efficiency of predatory mites as biological control agents have not been studied before. We also
119 found lower predation rate and egg production after cold exposure in previous studies (Jensen et al.,
120 2017, 2019). However, time of cold exposure time was constant within these studies and did not
121 include temperatures below 10 °C. Furthermore, no effect of gradual thermal decline was included,
122 which would theoretically allow stepwise acclimation to increasing cold.

123 Our main hypothesis was that beneficial effects of cold-acclimation on cold tolerance
124 correlate positively with acclimation time at mild cold (10 and 15 °C), but that exposure would
125 become increasingly detrimental over time at very low temperature (5 °C and below). We also
126 hypothesized that a gradual cooling would acclimate mites to tolerate and survive low temperature
127 (5 °C) that is detrimental at abrupt exposure. Accordingly, we here report the effects on cold
128 tolerance of both short-term cold acclimation (1-8 days) upon abrupt exposure and long-term
129 seasonal cold acclimation simulating a 5-month autumn and winter course with a gradual thermal
130 decline in *G. aculeifer*. We used adult females for these studies since only this life stage and sex
131 overwinters in northern Europe (Karg, 1961a). Lastly, we tested the predation efficiency and egg
132 production of females after the simulated overwintering to determine their state of fitness and
133 biological control potential, and to compare whether simulated winter exposure had reduced
134 voracity and fitness compared to long-term storage at mildly low temperature (10 °C).

135

136 2. Materials and Methods

137

138 2.1. Animals and maintenance

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

148

149 2.2 Effects of short-term cold acclimation on cold tolerance

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

158

159 2.3 Effects of seasonal cold acclimation on cold tolerance

160 In order to simulate the temperature decline during Danish autumn and winter (seasonal acclimation
161 treatment) we placed 20 beakers with up to 100 adult female *G. aculeifer* per beaker for 10 days at
162 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
163 for 35 days at 0.8 ± 0.2 °C. After this gradual thermal decline and winter simulation, the beakers
164 with mites were placed for 11 days at 5 °C followed by 30 days at 10 °C in order to simulate rising
165 temperatures during spring. Throughout this experiment, mites were provided *ad libitum* with early-
166 instar *F. candida*. Another group of female *G. aculeifer* were kept in similar beakers and density at
167 10 °C (constant 10 °C treatment) from two months before the start of the seasonal cold acclimation
168 treatment and similarly provided *ad libitum* with early-instar *F. candida* as a long-term “constant
169 mild cold” acclimation treatment. At intervals, mites from both acclimation treatments were
170 sampled and assayed for cold tolerance as described in section 2.2, using groups of ten mites after
171 acute exposure to either -2 or -5 ± 0.1 °C for 24 h ($N = 6$ replicates per treatment). No progeny
172 appeared and completed development within any of the two treatments, so all assays were
173 performed on adult females that were placed in the beakers at experiment start.

174

175 2.4. Effects of seasonal cold acclimation on supercooling point and melt onset temperature

176 Mites from the seasonal acclimation treatment were sampled at intervals and assayed for
177 supercooling point and melt onset temperature using differential scanning calorimetry (DSC),
178 including mites from the constant 10 °C treatment only at the final collection point ($N = 6$ samples
179 of 5 mites per treatment, Table 1). This analysis provided the supercooling point of each individual
180 and subsequently the mean melt onset temperature of all individuals within a sample as an
181 indication of the melting point of body fluids. For DSC analysis, we used a DSC4000 calorimeter
182 (Perkin Elmer, Waltham, MA, USA). Four to five mites were transferred to a 50 µL aluminium

183 DSC-pan, which was immediately hermetically sealed. Samples of mites were subjected to a DSC
184 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[–]
185 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
186 °C (6) heat to 5 °C at a rate of 1 °C min^{–1}. The melting endotherm (enthalpy change during melting
187 of ice formed in the animal) of the heating scan curve was analyzed and the melt onset temperature
188 estimated using Pyris Software (Perkin Elmer, Waltham, MA, USA) as described by Block (1994).

189

190 2.5. Effects of “overwintering” on predation and fecundity

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

200

201 2.6. Statistical Analysis

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

207 tolerance, effects of acclimation time on cold tolerance were further analyzed within each
208 temperature using time as a linear predictor variable.

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

218

219

220 **3. Results**

221

222 *3.1. Effects of short-term cold acclimation on cold tolerance*

223 Acclimation temperature significantly affected survival at -2 °C for 24 h ($F_{2,107} = 23.3$, $P < 0.001$),
224 showing that cold tolerance was generally highest after acclimation at low temperature (Fig. 1).
225 Furthermore, the interaction between temperature and acclimation time significantly affected cold
226 tolerance ($F_{2,107} = 16.8$, $P < 0.001$), reflecting opposing effects of acclimation time on survival to
227 cold exposure between temperatures (Fig. 1). Acclimation time alone did not have significant
228 influence on cold tolerance across temperatures ($F_{1,107} = 0.03$, $P = 0.87$; Fig. 1). When analyzing the
229 effect of acclimation time on cold tolerance separately within each acclimation temperature, we
230 found a significant negative linear effect of exposure time at 5 °C on survival to -2 °C exposure

231 ($F_{1,25} = 43.0$, $P < 0.001$, estimate $\pm$ sem = -0.12 ± 0.01), while longer exposure time at 10 °C had a
232 significantly positive linear effect on survival to cold ($F_{1,25} = 17.9$, $P < 0.001$, estimate $\pm$ sem = 0.10
233 ± 0.02). At 15 and 20 °C, acclimation time had no significant effect on cold tolerance ($P > 0.05$;
234 Fig. 1), although there was a tendency towards higher cold tolerance after more than one day of
235 acclimation at 15 °C (Fig. 1).

236

237 3.2. Effects of seasonal cold acclimation on cold tolerance

238 Survival at -2 °C for 24 h was significantly affected by acclimation treatment (constant 10 °C or
239 simulated seasonal cold acclimation) ($F_{1,30} = 218.9$, $P < 0.001$), sampling time point ($F_{2,30} = 66.9$, P
240 < 0.001) and their interaction ($F_{2,30} = 70.3$, $P < 0.001$). Whereas the proportion surviving -2 °C
241 exposure was nearly 100% at all time points for the mites that were maintained at constant 10 °C
242 (Fig. 2), cold tolerance changed over time in the mites exposed to seasonal cold as they were
243 exposed to different temperatures (Fig. 2). Survival to -2 °C exposure was lowest at the outset of the
244 experiment before any cold acclimation, highest after seasonal cooling including the period at 5 °C,
245 and intermediate late in winter after an additional period at 0.8 °C, with significant differences
246 between all three time points (Tukey HSD test at $\alpha = 0.05$; Fig. 2).

247 Survival to -5 °C exposure was low within all treatments. The linear model showed no effect
248 of acclimation treatment (constant 10 °C or simulated winter acclimation) ($F_{1,30} = 1.1$, $P = 0.30$) and
249 no interaction ($F_{2,30} = 2.4$, $P = 0.11$), but a significant effect of sampling time point ($F_{2,30} = 4.2$, $P =$
250 0.025). This main effect was caused by the poor cold tolerance in the first sampling time point by
251 mites in the simulated winter treatment, where animals came directly from 20 °C and no cold
252 acclimation had occurred (Fig. 2).

253

254 3.3. *Effects of seasonal cold acclimation on supercooling point and melt onset temperature*

255 A linear model of the effect of simulated winter acclimation showed no significant effect on
256 supercooling point ($F_{1,81} = 1.3$, $P = 0.26$; Fig. 3). Furthermore, simulated winter and constant 10 °C
257 acclimation did not lead to a difference in supercooling point at the end of the experiments ($F_{1,54} =$
258 0.3, $P = 0.58$; Fig. 3).

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

265

266 3.4. *Effects of “overwintering” on predation and fecundity*

267 Following the simulated winter, mites killed significantly fewer *F. candida* prey ($F_{1,46} = 19.7$, $P <$
268 0.0001; Fig. 5A), and laid significantly fewer eggs ($F_{1,46} = 11.7$, $P = 0.0013$; Fig. 5B) than mites
269 that had been maintained at a constant 10 °C throughout the winter. However, the mites that had
270 been exposed to constant 10 °C did not exploit killed prey significantly more efficiently for egg
271 production than the simulated winter exposed mites ($F_{1,46} = 2.6$, $P = 0.11$; Fig. 5C).

272

273 **4. Discussion**

274 Soil predatory mites have great potential as biological control agents in agroecosystems (Koehler,
275 1997; Moreira and de Moraes, 2015), but more knowledge on their ecology and ecophysiology
276 including overwintering potential would be advantageous for successful use in a temperate
277 environment (Knapp et al., 2018). We show a distinct interaction between effects of temperature
278 and exposure time on cold tolerance following abrupt cold exposure (Fig. 1). Under gradual thermal

279 decline, mites gradually acclimated to decreasing temperature and only near the freezing point did
280 low thermal exposure have a negative effect on tolerance to -2 °C (Fig. 2). Mites readily killed prey
281 and resumed egg production following the simulated winter, but the low temperatures in late winter
282 caused lower predation and reproduction compared to mites only exposed to mild cold (10 °C) over
283 the entire period. Our results show that the time vs. temperature regime of cooling significantly
284 affects cold tolerance in adult female *G. aculeifer*, with gradual thermal decline and temperatures
285 not too close to freezing minimizing the damaging effects of cold.

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

299 The supercooling capacity of *G. aculeifer* was as good as is normally seen for soil mites and
300 other microarthropods (Sømme, 1982). On average, female mites had supercooling points around -
301 18 °C and rarely above -10 °C. This implies that the observed lethality at -2 or -5 °C was not
302 associated with freezing of body fluids. Accordingly, *G. aculeifer* is categorized as chill susceptible

303 possessing moderately chill tolerant characteristics (Bale, 1993, 1996). Supercooling point did not
304 decrease during winter acclimation as seen in some arthropods including mites (Hodkova and
305 Hodek, 1997; Ma et al., 2006; Sjørnsen and Sømme, 2000), but not in all (Bennett and Lee, 1989;
306 Sinclair, 1997; Turnock et al., 1983). The supercooling point seems in this case not to be associated
307 with cold tolerance, but rather is a simple physical phenomenon resulting from the small volume of
308 hemolymph that these mites contain (Renault et al., 2002).

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

322 Karg (1961a) reported that *G. aculeifer* in fields near Berlin, Germany, where soil
323 temperature was around 0 °C or just below overwintered solely as adult females. Densities in early
324 spring were very low and populations peaked in August-September, suggesting that regrowth of
325 populations occurred quickly during spring and summer despite high mortality during winter (Karg,
326 1961a). Our results supplement this picture and indicate that inoculative releases in north-west

327 Europe can successfully establish populations of *G. aculeifer* that are able to overwinter and
328 proliferate in these regions. Our results, showing that overwintering females were able to prey on
329 springtails and reproduce following the simulated winter, illustrate that *G. aculeifer* can indeed
330 overwinter and proliferate (Fig. 5). Negative effects of cold exposure on predation and reproduction
331 have been demonstrated before in biological control agents including *G. aculeifer* (Helgadóttir et
332 al., 2017; Jensen et al., 2017, 2019). Here, we show that low thermal exposure rather than longer
333 time of mild cold exposure negatively affected fitness and predation in *G. aculeifer* (Fig. 5). Thus,
334 adult female *G. aculeifer* can overwinter under conditions resembling winters in the temperate
335 region. However, as the species is not highly cold tolerant, it needs refuges where temperatures do
336 not drop far below the freezing point.

337 Cold storage of biological control agents is common practice, but long term storage typically
338 causes cold damage (Colinet and Boivin, 2011; Luczynski et al., 2008). Our study shows that
339 storage of *G. aculeifer* at 10 °C produces mites with high cold tolerance after few days of storage.
340 Furthermore, mites can be kept over long term without deleterious effects. This is advantageous if
341 releasing the mites in early spring where night temperatures may drop below freezing. Furthermore,
342 10 °C storage also increases starvation tolerance in *G. aculeifer* (Jensen et al., 2017, 2018). If used
343 preventatively in spring when temperatures fluctuate and pests have not yet established in the crop,
344 storage at 10 °C might therefore be advantageous as mites are tolerant to low temperature and can
345 sustain with little prey. If stored at lower temperature, we recommend a gradual thermal decline to
346 the target storage temperature to allow acclimation and minimize deleterious effects of cold shock
347 even though this induces high cold tolerance at a short term.

348

349 **5. Conclusion**

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

364

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368

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 525

526 **Table 1**

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

Treatment	Assay	Replicates (#)	Mites per replicate (#)
Short term exposure	-2°C tolerance after 1 day at 5°C	5	10
	-2°C tolerance after 4 days at 5°C	10	10
	-2°C tolerance after 8 days at 5°C	12	10
	-2°C tolerance after 1 day at 10°C	5	10
	-2°C tolerance after 4 days at 10°C	10	10
	-2°C tolerance after 8 days at 10°C	12	10
	-2°C tolerance after 1 day at 15°C	5	10
	-2°C tolerance after 4 days at 15°C	10	10
	-2°C tolerance after 8 days at 15°C	12	10
	-2°C tolerance after 1 day at 20°C	5	10
	-2°C tolerance after 4 days at 20°C	15	10
	-2°C tolerance after 8 days at 20°C	12	10
Seasonal acclimation	-2°C tolerance before cooling (at 20°C)	6	10
	-2°C tolerance at 96 days (at 4.1°C)	6	10
	-2°C tolerance at 138 days (at 0.8°C)	6	10
	-5°C tolerance before cooling (at 20°C)	6	10
	-5°C tolerance at 96 days (at 4.1°C)	6	10
	-5°C tolerance at 138 days (at 0.8°C)	6	10
Constant 10°C	-2°C tolerance at 22 days	6	10
	-2°C tolerance at 96 days	6	10
	-2°C tolerance at 138 days	6	10
	-5°C tolerance at 22 days	6	10
	-5°C tolerance at 96 days	6	10
	-5°C tolerance at 138 days	6	10
Seasonal acclimation	Supercooling and melt onset at 20°C	6	5
	Supercooling and melt onset at 4.1°C	6	5
	Supercooling and melt onset at 0.8°C	6	5
Constant 10°C	Supercooling and melt onset at 135 days	6	5
Seasonal acclimation	Subsequent kill rate and reproduction	24	1
Constant 10°C	Subsequent kill rate and reproduction	24	1

528

529

530 **Figure legends**

531

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

535

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

541

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

548

549 **Fig. 4.** Melt onset temperatures (mean $\pm$ S.E.) of adult female *Gaeolaelaps aculeifer* subjected to
550 simulated winter temperatures (solid gray line) or constant 10 °C (dashed gray line) at 20, 96, and
551 138 days since initiation of the winter simulation. Seasonally acclimated mites (circles) were thus
552 exposed to temperatures following the solid gray line prior to the melt onset assay, while mites kept
553 at constant 10 °C (triangles) had followed the dashed gray line. These were only tested at 138 days.

554

555 **Fig. 5.** Numbers (mean $\pm$ S.E.) of (A) juvenile *Folsomia candida* prey killed, (B) eggs produced,
556 and (C) eggs produced per killed prey by adult female *Gaeolaelaps aculeifer* previously subjected
557 to simulated winter followed by re-warming to 10 °C (winter treatment, solid gray line in Fig. 2-4)
558 or constant 10 °C (dashed gray line in Fig. 2-4).

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561 Fig. 1

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564 Fig. 2

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570 Fig. 4

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