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Overwintering Red Velvet Mites Are Freeze Tolerant

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1 **Overwintering red velvet mites are freeze tolerant**

2 Freeze-tolerant mite

3

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11

12 **What is already known:**

13 Freeze-tolerance, the ability to survive freezing, has evolved in many arthropod species
14 and in the arachnids, only in scorpions. All the microarthropods (mites and collembolans)
15 that have been studied have been freeze avoidant, and have been assumed to be
16 constrained to this strategy because of their small size. This has led researchers to
17 conclude that freeze avoidance is a basal trait.

18 **What this study adds:**

19 We find a mite that is freeze tolerant - the first known instance of this strategy in any mite or
20 microarthropod. This means that microarthropods are not, in fact, constrained to freeze
21 avoidance, and that our thought processes about the evolution of freeze tolerance might need
22 revision.

23 **Abstract**

24 Although many arthropods are freeze-tolerant (able to withstand internal ice), small-bodied
25 terrestrial arthropods such as mites are thought to be constrained to freeze avoidance. We field-
26 collected active adult red velvet mites, *Allothrombium* sp. (Trombidiidae), in winter in
27 Southwestern Ontario, Canada, where temperatures drop below -20°C . These mites froze
28 between -3.6 and -9.2°C and survived internal ice formation. All late-winter mites survived being
29 frozen for 24h at -9°C , and 50% survived one week. The LLT_{50} (the low temperature that kills
30 50% of mites) was c. -20°C in midwinter. Hemolymph osmolality and glycerol concentration
31 increased in midwinter, accompanied by decreased water content. Thus, this species is freeze
32 tolerant, demonstrating that there is neither phylogenetic nor size constraint to evolving this cold
33 tolerance strategy.

34

35

36 **Keywords**

37 Freeze tolerance, Acari, seasonality, cold hardiness, overwintering, cryoprotectant

38 **Introduction**

39 Winter temperatures drop below 0°C in terrestrial temperate, polar, and alpine
40 ecosystems. Arthropods in these habitats generally adopt one of two cold tolerance strategies:
41 freeze avoidance, preventing ice formation by depressing the temperature at which they freeze
42 (the supercooling point, SCP), or freeze tolerance, withstanding internal ice formation (Lee
43 2010). Freeze-tolerant insects accumulate low molecular weight cryoprotectants, such as glycerol
44 or proline (Lee 1991). They have high SCPs that likely facilitate control of the site or rate of ice
45 formation (Toxopeus and Sinclair in press). Finally, freeze tolerant insects sometimes produce
46 ice-binding proteins that initiate freezing at high temperatures (ice nucleating proteins) or control
47 the growth and distribution of ice crystals (thermal hysteresis and recrystallisation-inhibiting
48 proteins; Zachariassen and Kristiansen 2000).

49 Freeze tolerance appears to be a derived trait that has evolved multiple times in
50 arthropods (Sinclair et al. 2003; Toxopeus and Sinclair in press), including many Insecta,
51 Chilopoda, Crustacea, and in two Arachnids (both scorpions; Crawford and Riddle 1975;
52 Whitmore et al. 1985). Small arthropods contain a small volume of water and therefore supercool
53 easily (Sinclair et al. 2003). This propensity to supercool probably predisposes them towards
54 freeze avoidance (Cannon and Block 1988). In particular, there has been extensive work on the
55 cold tolerance of mites (Acari) and springtails (Collembola; collectively ‘microarthropods’) in
56 the Antarctic and Arctic (as well as non-polar regions; Cannon and Block 1988; Sjørnsen and
57 Sømme 2000), and thus far all have been freeze-avoidant or chill-susceptible. This is despite
58 many of those species having soft permeable bodies and occupying the ice-rich microhabitats
59 likely to promote inoculation by external ice and therefore freeze tolerance (Sinclair et al. 2003).
60 Thus, the general conclusion is that – whether due to their small size or phylogenetic constraints

61 – microarthropods do not evolve freeze tolerance (Cannon and Block 1988; Sinclair et al. 2003;
62 Toxopeus and Sinclair in press).

63

64 Here we report that overwintering red velvet mites are freeze tolerant, extending the
65 taxonomic breadth of incidence of this strategy and upending the expectation that all
66 microarthropods are freeze avoidant.

67

68 **Methods**

69 We hand-collected a total of 340 adult red velvet mites [*Allothrombium* sp.
70 (Trombidiidae), voucher CNC871154, Canadian National Collection], mean \pm SEM fresh mass
71 4.3 ± 0.2 mg, from the soil surface and leaves of *Cirsium arvense* at the Environmental Sciences
72 Western farm in Ilderton, Ontario (43.1°N, 81.3°W) between March 2016 and May 2017. To
73 access mites under snow in winter, we collected c.100 individuals in November 2016 and buried
74 them in ‘field cages’ – 600mL plastic containers containing 2cm of soil and *C. arvense* leaves,
75 such that the surface of the soil in the containers was level with the surrounding soil. After
76 collecting them from the field or removing them from the field cages (the mites always remained
77 on the surface of the soil), we housed mites in 600mL containers at 4°C (winter - January,
78 February, March) or room temperature (spring - April, May and autumn - November) for up to
79 five days. We were unable to find mites between May and September, likely because juvenile
80 trombindinid red velvet mites are ectoparasitic (Zhang 1998). We determined the SCP, cold
81 tolerance strategy, and low temperature at which 50% of individuals are killed (LLT₅₀) using
82 methods outlined elsewhere (Sinclair et al. 2015).

83 We measured SCP in March, April, September, October, and November 2016, and
84 January, February, March, and May 2017. We placed mites individually in 1.7mL
85 microcentrifuge tubes in contact with a 36-AWG type-T thermocouple and recorded temperature
86 at 2 Hz via a TC-08 interface and PicoLog software (Pico Technology, Cambridge, UK). We
87 placed tubes in an aluminium block cooled by fluid circulated from a Proline RP855 bath
88 (Lauda, Würzburg, Germany; before July 2016) or by a custom-built Peltier-effect device. We
89 cooled mites from 4°C (winter) or 20°C (spring and autumn) to 0°C at 1.0°C·min⁻¹, and
90 0.5°C·min⁻¹ (or 0.25 °C ·min⁻¹ before July 2016) thereafter.

91
92 To determine cold tolerance strategy in March, September, October, and November 2016,
93 and January 2017, we cooled groups of mites until half the individuals had frozen (i.e. produced
94 exotherms), then removed all of them to 4°C (winter) or ~20°C (spring and autumn). After 24h,
95 mites that were upright and moving were recorded as alive.

96
97 We estimated LLT₅₀ in March and November 2016, and January 2017 in groups of four
98 mites exposed for 1h to a range of temperatures below the SCP. We began cooling at 21°C (for
99 March measurements), 15°C (November), or 4°C (January; we based these temperatures on
100 approximate maximum air temperatures for each month), and cooled and rewarmed at 0.25°C
101 min⁻¹ (March measurements), or to 0°C at 1°C·min⁻¹, and 0.5°C·min⁻¹ thereafter, and rewarmed
102 at 0.5°C·min⁻¹. We assessed survival after 24h at 20°C (March, November) or 4°C (January).

103
104 We examined survival after prolonged freezing at temperatures below the SCP (but that
105 yielded 100% survival of brief exposures) in April 2016 and March 2017. We cooled groups of

106 7-10 mites from 21°C to -8.6°C at 0.25°C·min⁻¹, held them for 1, 8, and 24h, rewarmed at
107 0.25°C·min⁻¹ and assessed survival (as above). In March 2017, we cooled three groups of four
108 mites from 0°C to -9.0 ±0.7°C at 0.5°C·min⁻¹, and held one group for each of 1h, 12h, and one
109 week, before rewarming at 0.5°C·min⁻¹.

110

111 To measure hemolymph osmolality, we amputated the front right leg under immersion
112 oil, extracted c. 20 nL of hemolymph, and determined osmolality and thermal hysteresis (Otago
113 Osmometers, Dunedin, New Zealand) as previously described (Crosthwaite et al. 2011). We
114 measured whole-body water content gravimetrically, as the difference between fresh mass and
115 mass after drying to a constant mass at 60°C (Sjursen and Sømme 2000). We rehydrated and
116 crushed these mites in 0.05% Tween 20, and measured glycerol concentration
117 spectrophotometrically (details in Crosthwaite et al. 2011).

118

119 We compared SCP, osmolality, water content, and glycerol content using ANOVA in R
120 (version 3.2.2). We calculated LLT₅₀ from a generalized linear model in R and used non-
121 overlapping 95% confidence intervals to compare months.

122 **Results**

123 All mites collected between November and March survived internal ice formation; a
124 smaller proportion survived freezing at other times (Table 1). SCP ranged from -3.9°C (March
125 2016) to -9.2°C (March 2017), and mean SCPs ranged from $-6.2 \pm 0.2^\circ\text{C}$ in March 2016 to -8.4
126 $\pm 0.2^\circ\text{C}$ in January 2017 (Fig. 1a). SCP differed significantly among sampling points (Fig. 1a),
127 but not in a manner that was associated with freeze tolerant mites having consistently higher or
128 lower SCPs than their freeze intolerant counterparts. LLT_{50} ($\pm 95\% \text{CI}$) was lower in January ($-$
129 $20.0 \pm 2.7^\circ\text{C}$), than in March (-7.4 ± 3.2) or November ($-12.1 \pm 1.8^\circ\text{C}$; Fig. 1b). All mites in April
130 and March survived being frozen at -8.6°C or $-9.0 \pm 0.7^\circ\text{C}$ for 24h, and 8/12 of March-collected
131 mites survived frozen for one week at -9.0°C .

132 Hemolymph osmolality ranged from 462 (March 2017) to 1997mOsm (February 2017;
133 Fig. 2a). Mean osmolality was highest in mites collected in January and February (Fig. 2a; Table
134 2). Water content was significantly lower in February 2017 than at other times of year, except
135 November 2016 (Figure 2a). We did not observe thermal hysteresis in any hemolymph sample
136 and saw no spicular ice crystal growth suggestive of ice-binding activity (Crosthwaite et al.
137 2011). Glycerol concentration was highest in midwinter (Fig. 2b).

138

139

140 **Discussion**

141 To our knowledge, this is the first report of freeze tolerance in a microarthropod. Not all
142 individuals survive internal ice formation in autumn, but by midwinter (January and February),
143 *Allothrombium* sp. can survive at least one week in a frozen state.

144

145 The supercooling point was lowest in midwinter, but there did not appear to be a strong
146 association between SCP and cold tolerance, unlike in many freeze-tolerant insects, and the total
147 range of mean SCP is only c. 2°C in *Allothrombium* sp., compared to seasonal shifts of 10°C or
148 more in other freeze tolerant species (Duman 2001). The supercooling point was c. 1.5°C higher
149 in March 2016 than in March 2017 (Fig. 1A). We identify two possible explanations for this that
150 may not simply be due to inter-annual variation. First, the March 2016 sample was collected on
151 March 26th, much later in the month than the March 2017 sample (collected on March 6th); the
152 2016 sample may perhaps be better reflective of SCP in the spring (we note it does not differ
153 from the April and May timepoints). Second, the March and April 2016 SCPs were measured by
154 cooling the mites at 0.25 °C·min⁻¹, whereas the 2017 mites were cooled at 0.5°C·min⁻¹. Slower
155 cooling rates do lead to higher SCPs (Salt 1966), so this could explain the discrepancy, although
156 the likely presence of ice nucleating agents would be expected to at least partially mitigate this
157 effect. Either way, a difference in mean SCP of a few °C is unlikely to be biologically
158 significant, and reflects the overall small range of SCPs in adult *Allothrombium* sp., and suggests
159 selection for a consistently high supercooling point.

160

161 *Allothrombium* sp. is a large mite (fresh mass 4.1 ± 1.4 mg in the animals in our study),
162 about twenty times the size of the c. 0.2 mg *Alaskozetes antarcticus* (Block 1977). This relatively

163 large size does not explain the high SCP nor imply an inability to depress the SCP in the winter;
164 non-cold-hardy insects of comparable size to *Allothrombium* sp., such as *Drosophila*, have SCPs
165 below -15 °C without special adaptations (Strachan et al. 2011), and much larger insects can
166 maintain very low SCPs (e.g. the 70-100 mg emerald ash borer *Agilus planipennis* has a mean
167 SCP below -30 °C in winter; Crosthwaite et al. 2011). SCP depression may not be possible for
168 soft-bodied species that (like *Allothrombium* sp.) are routinely exposed to ice nucleators from the
169 habitat. We speculate that this high probability of freezing (alongside year-round activity) could
170 have favored evolution of freeze tolerance in this species, as has been postulated for freeze
171 tolerance more generally (Toxopeus and Sinclair in press). Interestingly, these mites appear to
172 have adopted a divergent strategy to the cryoprotective dehydration used by Collembola in
173 similar habitats to avoid freezing (Sørensen and Holmstrup 2011).

174

175 Hemolymph osmolality was highest in midwinter. Decreased water content accounts for
176 c.215 mOsm of that increase, with another 15 mOsm from increased [glycerol]. Glycerol has
177 been reported as a cryoprotectant in other arachnids (Aitchison and Hegdekar 1982; Kirchner
178 and Kestler 1969; Young and Block 1980) and is thought to enhance freeze tolerance by
179 stabilising macromolecules and reducing ice content and minimum cell volume (Lee 2010).
180 Although our small sample sizes (and consequently high variance) mean that we probably lack
181 statistical power to detect small differences in [glycerol], the magnitude of the change from
182 summer to winter (~15 mM) is substantially lower than the large changes observed in other
183 mites; for example, the freeze-avoidant *A. antarcticus* accumulates c. 0.5 M glycerol (Young and
184 Block 1980). Approximately 530 mOsm remain unaccounted for; we hypothesize that other
185 osmotically-active agents (such as other polyols or amino acids, see Sinclair and Toxopeus, in

186 press) contribute to the increase of hemolymph osmolality could also act as cryoprotectants.
187 Possibly, the small decrease in SCP in midwinter results from this increased osmolality.

188

189 In arachnids, freeze tolerance has evolved in two desert scorpions (Crawford and Riddle
190 1975; Whitmore et al. 1985), but not in any other mites as far as we are aware (Cannon and
191 Block 1988). Winter temperatures in southwestern Ontario can be highly variable (see, e.g.,
192 Marshall and Sinclair 2012), and we suggest that this thermal variability coupled with winter
193 activity (which likely precludes the accumulation of very high cryoprotectant concentrations),
194 and extensive environmental moisture (promoting inoculative freezing) has favored freeze
195 tolerance in this species. Thus, we show that neither small size nor a phylogenetic tendency
196 towards freeze avoidance in mites prevents them from evolving freeze tolerance, and we
197 speculate that other mites in similar circumstances may also be freeze tolerant.

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206

207

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251

252

253 **Tables**

254 Table 1: Freeze tolerance in *Allothrombium* sp. We cooled mites until c. 50% froze, then

255 removed them from the cold for recovery.

Date collected	Frozen		Unfrozen	
	No. alive	No. dead	No. alive	No. dead
March 2016	9	0	8	1
September 2016	5	3	8	0
October 2016	2	2	4	0
November 2016	8	0	8	0
January 2017	7	0	3	0

256

257

258 **Figure Legends**

259 Figure 1: Cold tolerance of field-collected red velvet mites, *Allothrombium* sp. (a) Mean \pm SEM
260 supercooling point (SCP; numbers indicate sample size; different letters indicate points that are
261 significantly different, $F_{7,147} = 22.07$, $p < 0.01$). (b) Survival after 1h cold exposure; curves are the
262 result of a generalized linear model with 95% confidence intervals shown in grey. Note that
263 cooling rates in the 2015-2016 winter were $0.25^{\circ}\text{C}\cdot\text{min}^{-1}$, but $0.5^{\circ}\text{C}\cdot\text{min}^{-1}$ in the 2016-17 winter
264 for both datasets.

265

266 Figure 2: Hemolymph composition of overwintering red velvet mites, *Allothrombium* sp. (a)
267 Water content, osmolality; (b) glycerol concentration. Different letters indicate points that are
268 significantly different (Water content: $F_{4,48} = 6.33$, $p < 0.01$; Osmolality: $F_{3,24} = 21.88$, $p < 0.001$;
269 [glycerol]: $F_{4,21} = 3.52$, $P = 0.03$), numbers indicate sample sizes; mean \pm SEM shown throughout.

270