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Overwintering Red Velvet Mites Are Freeze Tolerant

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Freeze-tolerant mite

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What is already known:

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

What this study adds:

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

Abstract

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

Keywords

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

Introduction

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

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

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

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

Methods

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

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

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

We estimated LLT_{50} in March and November 2016, and January 2017 in groups of four mites exposed for 1h to a range of temperatures below the SCP. We began cooling at 21°C (for March measurements), 15°C (November), or 4°C (January; we based these temperatures on approximate maximum air temperatures for each month), and cooled and rewarmed at 0.25°C min^{-1} (March measurements), or to 0°C at 1°C·min^{-1}, and 0.5°C·min^{-1} thereafter, and rewarmed at 0.5°C·min^{-1}. We assessed survival after 24h at 20°C (March, November) or 4°C (January).

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

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 0.25°C·min^{-1} and assessed survival (as above). In March 2017, we cooled three groups of four 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 week, before rewarming at 0.5°C·min^{-1}.

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

We compared SCP, osmolality, water content, and glycerol content using ANOVA in R (version 3.2.2). We calculated LLT_{50} from a generalized linear model in R and used non-overlapping 95% confidence intervals to compare months.

Results

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

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

Discussion

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

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

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

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

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

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

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

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Tables

Table 1: Freeze tolerance in *Allothrombium* sp. We cooled mites until c. 50% froze, then removed them from the cold for recovery.

	Frozen		Unfrozen	
Date collected	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

Figure Legends

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

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