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Supercooling point frequency distributions in Collembola are affected by moulting

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Summary

1. Many arthropods depress the freezing point of their body fluids (supercool) to avoid freezing at subzero temperatures. This is normally a seasonal response and is achieved by the production of specific biomolecules including cryoprotectants, a cessation in feeding, and the removal or masking of ice-nucleating material from their bodies.
2. In springtails, the mid-gut is shed during moulting which results in the complete evacuation of the gut and a concomitant reduction in the supercooling point (SCP). We determined whether this non-adaptive explanation could account for the variability observed in the SCP of summer-acclimatized springtails.
3. Moult preparation resulted in a highly significant reduction in the SCP. Feeding after moulting restored the SCP to previous high levels.
4. Significant differences in SCP between springtails sampled from vegetation and the soil surface, on different days, and at different sites on the same day were also documented, demonstrating that not all variation in SCP is environmentally induced.
5. Investigations of the responses of the SCP to environmental variation in springtails and other arthropods should take into account the effects of moulting before solely adaptive conclusions are drawn.

Key-words: Collembola, *Ceratophysella denticulata*, feeding, starvation, stratification, supercooling

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Introduction

Lower lethal temperature is a significant characteristic of all animals. In terrestrial arthropods this threshold varies substantially over both space and time at a variety of scales (Zachariassen 1985; Worland & Convey 2001; Sinclair, Addo-Bediako & Chown 2003a), with significant implications for the abundance and distribution of arthropods and how these characteristics will respond to a changing environment (Chown *et al*. 2004; Helmuth, Kingsolver & Carrington 2005). The array of responses to low temperatures employed by terrestrial arthropods is now reasonably well understood (Bale 1993; Worland, GruboLajsic & Montiel 1998; Sinclair 1999; Chown & Nicolson 2004). However, the relationships between these physiological responses, variation in the abiotic environment, and life-history strategies, in the context of fitness, are only beginning to be explored (Kelty & Lee 2001; Voituron *et al*. 2002; Brown, Bale & Walters 2004; Sinclair & Chown 2005a), although their importance has long been appreciated (Baust & Rojas 1985; Duman *et al*. 1991; Sømme 1999).

Arthropods employ one of three responses to survive subzero temperatures (Chown & Nicolson 2004), of which depression of the supercooling point (SCP) to well below zero is the most prevalent (Sinclair *et al*. 2003a). While some species in this freeze-intolerant group show substantial prefreeze mortality (Bale 1987, 1993), in others the lower lethal temperature is equivalent to the temperature at which an individual freezes – known as the crystallization temperature (T_c) or SCP (Cannon & Block 1988; Sømme 1999). The SCP has long been known to show substantial variation within a population, with a frequency distribution that is often clearly bimodal (Sømme & Block 1982; Klok & Chown 1998). Why the SCP should vary to such an extent has generated much debate, especially because this variation has been attributed directly to adaptive responses to changing environmental conditions (Block 1990). Typically, it is thought that in response to approaching winter

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conditions, individuals cease feeding, evacuate their gut contents and undergo a range of biochemical changes that include the production of cryoprotectants (Chown & Nicolson 2004). These changes depress SCPs in a variety of ways, including by reducing the likelihood of heterogeneous ice nucleation owing to the removal or masking of ice nucleators (Lee *et al*. 1991). In consequence, winter-acclimatized individuals tend to have unimodal SCP frequency distributions with low mean values.

By contrast, in several species low SCPs are also maintained in some individuals under summer conditions, despite the fact that the likelihood of subzero temperatures is low and that the remainder of the population has much higher SCPs (Block 1982; Block 1984; van der Woude 1987; Klok & Chown 1998). It has been suggested that these bimodal SCP distributions might represent a bet-hedging strategy that would allow animals to survive unexpected cold snaps (Klok & Chown 1998). However, recent work has shown that SCPs can be altered within hours in response to changing environmental conditions (Worland & Convey 2001; Sinclair *et al*. 2003b); that rapid cold hardening is a widely employed method to improve survival in the absence of freezing (Lee, Cheng & Denlinger 1987); and that, in environments where cold snaps are typical, freezing tolerance is a more commonly employed cold-hardiness strategy (Sinclair *et al*. 2003a; Sinclair & Chown 2005b). Therefore bet-hedging seems an unlikely strategy. Moreover, while several studies have demonstrated an effect of starvation on SCPs (Sømme & Block 1982; Leinaas & Sømme 1984; Leinaas & Fjellberg 1985; Zachariassen 1985; Cannon 1986), others have failed to find such an effect (Baust & Rojas 1985; Klok & Chown 1998; Worland & Block 1999), making the role of feeding and gut evacuation contentious as a mechanism influencing SCP frequency distributions. Thus both the proximate cause and ultimate evolutionary explanation for one of the most characteristic features of arthropod cold hardiness remain controversial at best and, at worst, obscure.

One reason why these variable and/or bimodal SCP distributions remain poorly understood might be that explanations for them, and for variation in cold hardiness strategies in general, have usually been sought within a framework that has an almost exclusive focus on adaptation (Lee & Denlinger 1991; Sømme 1995; Voituron *et al*. 2002; Sinclair *et al*. 2003a). However, it is widely appreciated that many characteristics of organisms are non-adaptive (Endler 1986; Baum & Larson 1991; Ketterson & Nolan 1999). In springtails, one of the groups in which variable SCP frequency distributions have commonly been documented (Cannon & Block 1988; Block 1990), the mid-gut is shed during moulting (Thibaud 1968). This process involves complete evacuation of the gut contents, which usually initiate ice nucleation at a relatively high temperature (Lee, Lee & Strong-Gunderson 1993). Moulting might therefore be expected to depress the SCP. Moreover, by contrast with the true insects, Collembola have indeterminate growth and continue to moult as long as they live (Rapoport & Aguirre 1973; Birkemoe & Leinaas 2000). Therefore moulting forms a significant part of juvenile and adult springtail life history, with a potentially strong, incidental effect on the supercooling ability of individuals. In other words, moulting might depress the freezing point of a springtail, not as a response to looming low temperature (an adaptive response), but rather simply as a consequence of gut clearance, so resulting in substantial variability in SCP frequency distributions.

Here we determine whether this non-adaptive explanation can account for variability in SCPs by investigating the effects of moulting on SCP frequency distributions of the springtail *Ceratophysella denticulata*, and the implications for the ecology and evolution of cold hardiness in arthropods.

Materials and methods

STUDY SITES AND ANIMAL

Ceratophysella denticulata is a pigmented, surface-dwelling hypogastrurid species in which preparation for moulting is easily observed by the white appearance of the last abdominal segment and the tips of the legs and antenna as the old cuticle starts to detach from the underlying pigmented epidermis (here defined as premoult animals). The species is a cosmopolitan inhabitant of damp areas, especially those rich in organic matter. It is easily spread by agricultural practices, and has been introduced to and become invasive on Marion Island in the Southern Ocean (Gabriel *et al*. 2001), where we carried out our study. The individuals used in this study were extracted from vegetation and litter, mainly *Cotula plumosa* (Asteraceae) collected from Trypot Beach (46°53·098′ S, 37°51·617′ E, April 2004), by returning plant samples to the laboratory and gently shaking them in a sieve over a funnel and collecting vial. Once extracted, springtails were placed in small plastic containers containing a base of moist plaster of Paris mixed with charcoal, and kept under controlled temperature conditions (15 ± 1 °C, 16 L : 8 D).

SUPERCOOLING POINT DETERMINATION

The temperature at which individual springtails froze (SCP) was determined using a Mettler Toledo differential scanning calorimeter DSC820 (Mettler Toledo Ltd, Leicester, UK) (Worland & Convey 2001). Up to 25 animals were placed in a hermetically sealed aluminium pan (40 µl) and cooled from +5 to –30 °C at 1 °C min^{-1}. This standard protocol was used in all experiments. The SCP of individual animals was taken as the start of the exotherm produced by the latent heat of freezing of the animal's body fluids. Some insects are killed by chilling injury before they freeze (Bale,

Harrington & Clough 1986; Bale 1993). Therefore, to test whether in this instance the SCP really reflected cold hardiness, we cooled (1 °C min^{-1}) a sample of 33 recently moulted animals, with presumably low SCPs, to –20 °C. After warming (1 °C min^{-1}) to room temperature (20 °C), survival was recorded and compared with the proportion of the sample from which exotherms (freezing) was recorded by the DSC.

EFFECT OF MOULTING AND FEEDING ON COLD TOLERANCE

To estimate SCP frequency distributions in the natural population, we collected samples randomly from a composite sample: animals for the analyses were arbitrarily sampled from many hundred *C. denticulata* gently shaken from four to six samples (each ≈150–200 cm^2) taken from the selected sampling sites. From the same mass sample, we also selected animals in the early stages of ecdysis (see above) for additional analyses. The premoulting animals also provided a group of individuals, synchronized in their moulting cycle, for examination of the effects of moulting and subsequent feeding. Premoult animals were placed in separate containers without food, and examined twice daily. Animals that had moulted since the last inspection (having cast their old cuticle) were either selected for SCP analyses or placed in containers with soil and plant material from their natural habitat for 1 day at 10 ± 1 °C (18 L : 6 D) to examine how feeding after moulting affected the SCP.

To investigate the effect of starvation on SCP frequency distribution, ≈50 springtails were floated on the surface of distilled water for 2 days at 15 ± 1 °C (18 L : 6 D) and the SCPs of a subsample were determined. As animals may start moulting during this process, we also wanted to distinguish between the effects of starvation and moult preparation. It is difficult to observe the premoult stage in animals on water as they are densely packed and constantly moving, and in addition the water surface is a problematic background for observation of the detached part of the cuticle. We therefore measured the SCPs of a subsample obtained by selecting the first 20 animals collected on a fine brush. After this measurement, we inspected the animals under a stereomicroscope and selected all the (now dead) premoult animals. These were placed in a new sample pan and their SCPs measured a second time. As repeated freezing of the same springtail several times has been shown to produce very similar SCPs (Worland 2005), the distribution of the low group in the first measurement should correspond to the repeated SCPs of the selected premoult animals.

TEMPORAL AND SPATIAL VARIATION IN SCP

If moulting is to some degree synchronized within groups (Leinaas 1983), there could be rapid changes in SCP distribution associated with changes in the moulting status of the population (group), and also differences between sites on the same day. Moreover, as Collembola tend to seek moulting sites sheltered from drought exposure (Verhoef 1981; Leinaas & Fjellberg 1985), there could well be an exposure effect on the SCP distribution within a population. To test for these differences, we sampled animals from the same site at Trypot Beach with an interval of 3 days (7 and 10 April). Animals were extracted from samples consisting of both vegetation and the upper 2–3 cm of soil/litter from a 15 × 15-cm patch. Similarly, on 17 April we sampled animals from similar vegetation (*C. plumosa*) using the same method at two sites 1 km apart: Trypot Beach and Gentoo Lake (46°52·6′ S, 37°51·7′ E). Lastly, we tested whether there were differences between animals foraging up in the vegetation and animals found in the litter below (on algae and detritus). This was done on a rainy day when animal activity was high both in the vegetation and on the ground. Above-ground plant material of *C. plumosa* was collected by clipping vegetation from an area ≈15 × 15 cm, while litter and 1–2 cm of soil beneath the sampled area was removed. In all these tests the animals were extracted and their SCPs were recorded using the protocols described above.

ANALYSIS

In most of our data sets, SCP tended to show a form of bimodal distribution. This means that, while the range of SCPs remains similar, there is considerable variation in the distribution of SCPs within this range. With such data sets, statistical tests based on comparisons of mean values are less relevant than comparison of the distributional patterns. Comparison of the frequency distributions was also a major goal of the investigation. Therefore we used Kolmogorov–Smirnov two-sample tests (KS-stat) to compare treatments and samples.

Results

Of the 33 springtails cooled to –20 °C, 18 froze with a mean SCP of –15 ± 3 °C (mean ± SD). Post-treatment assessment of all the animals revealed that 18 had died during the treatment (corresponding exactly to the 18 freezing points) but the remaining 15 had survived. Of these 15 animals, five moulted during the following 24 h. As no springtails have been shown to be freeze-tolerant so far, this result is clear evidence that the freezing point represents the lower lethal temperature for this species.

Moult preparation had dramatic consequences for the SCP frequency distributions. The random sample of freshly caught animals (Fig. 1a) differed significantly and substantially from a subsample of premoult animals (Fig. 1b) (KS-stat = 0·6032; $P < 0·0001$; random sample mean ± SD –12·5 °C ± 5·0, $n = 31$;

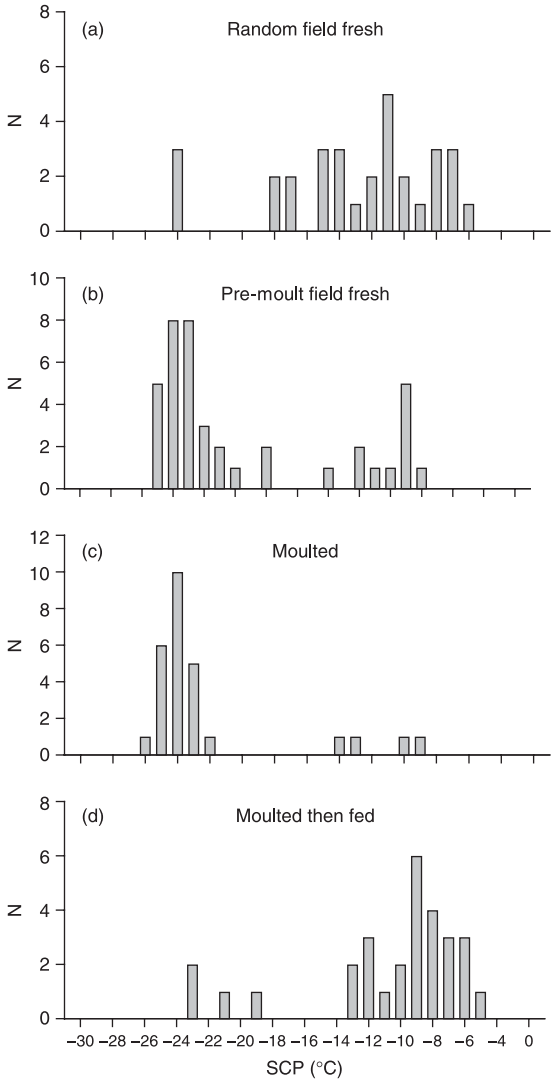

Fig. 1. Supercooling point (SCP) distribution in the Collembola *Ceratophysella denticulata* from (a) an arbitrary field sample; (b) premoulting animals from the same main sample; (c) recently moulted animals; (d) recently moulted animals that had been fed for 1 day (10 °C).

premoult animals: −18·9 °C ± 6·1, $n = 40$). Both samples included a wide range of SCP classes, but the random sample consisted mainly of animals with SCPs ranging from −5 to −17 °C, with only a few as low as −23 °C. By contrast, premoulting animals showed a distinct peak of low SCPs (−19 to −24 °C). As premoult animals were not excluded from the random sample, the few animals with low SCPs suggest that only a small fraction of the animals sampled from that date were preparing to moult. The SCP distribution of recently moulted animals (Fig. 1c) did not differ significantly from the premoult sample (KS-stat = 0·3046; $P = 0·078$) (−21·6 °C ± 4·6, $n = 27$). However, the recently moulted group tended to have a more concentrated, unimodal distribution between −21 and −25 °C, with only individuals showing high SCPs.

Supercooling points also responded strongly to feeding, showing an increase after a single day of postmoult feeding, resulting in a significant difference in the SCP frequency distribution (Fig. 1d, mean ± SD −10·5 °C ± 4·7, $n = 29$) from that found in postmoult animals (KS-stat = 0·7778; $P < 0·0001$). Although this SCP distribution was not significantly different from that of the original, randomly sampled field fresh animals (KS-stat = 0·3345; $P = 0·060$), it tended to be more unimodal, with individual values clustering around −5 to −13 °C. The virtually unimodal, although quite different frequency distributions of the postmoult animals before and after feeding indicated a rapid shift between two well defined states. In both instances only a few animals with SCPs outside the main distribution peaks were found. These may have been animals that either had not commenced feeding, on the one hand, or had eaten all or part of the exuvium, on the other.

The SCP frequency distribution of animals that had been feeding for 1 day after moulting (Fig. 1d) and subsequently starved on distilled water for 2 days at 15 °C (Fig. 2a) differed significantly from those that had been feeding (KS-stat = 0·7269; $P < 0·0001$, mean value declined to −19·2 ± 5·0 °C, $n = 16$). Of this sample, six individuals were preparing to moult, and a second analysis showed that (except for one individual) these premoulting animals represented the group with the lowest SCP (−23 to −26 °C) (Fig. 2b). When removing the values for premoult animals from the result of the first analysis, it become evident that the remaining starved intermoult animals had intermediate SCPs. However, comparison with the previously feeding animals showed that starvation, in itself, also had a significant lowering effect on the SCP (KS-stat = 0·7519; $P = 0·0001$).

Samples of field-fresh animals taken at Trypot Beach at only a 3-day interval differed significantly in their SCP distributions (KS = 0·4575; $P = 0·0003$) (Fig. 3a,b). An increase with time in the proportion of individuals with low SCPs (Fig. 3b) suggested that

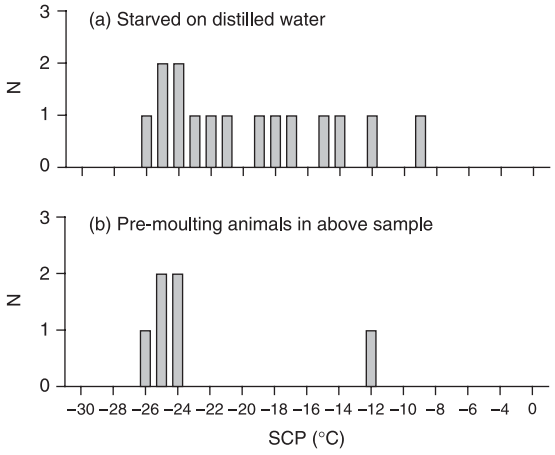

Fig. 2. Supercooling point distribution of *Ceratophysella denticulata* (a) fed for 1 day after moulting (cf. Fig. 1d) then starved on distilled water for 2 days at 15 °C; (b) repeat measurement of selected individuals in the premoult stage.

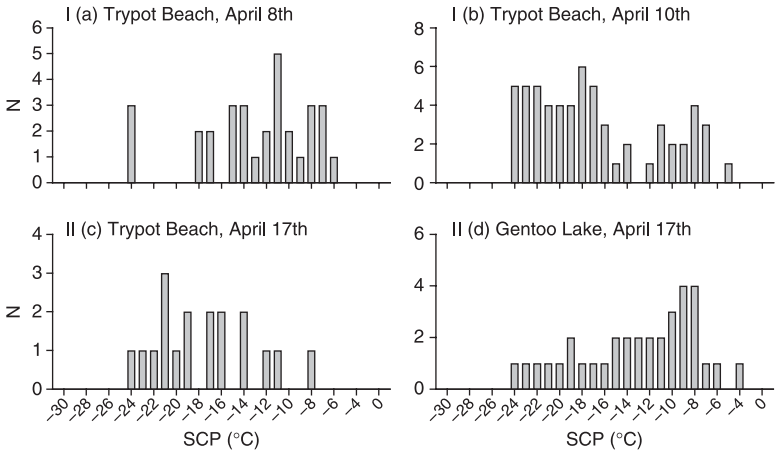

Fig. 3. Variations in supercooling point distribution (I) in time (within site); (II) between sites on the same day.

more animals had started to prepare for moulting, or had moulted very recently. Similarly, animals collected on the same day, in similar vegetation and using an identical method (size and depth of samples), but at two sites 1 km apart, differed significantly in their SCP distributions (KS-stat = 0·4281; $P = 0{\cdot}018$). (Fig. 3c,d). There were also significant differences in SCP frequency distributions between samples taken in the lower vegetation and from those taken in the upper litter/soil layer. On the wet day when this sampling was undertaken numerous animals were observed in the vegetation, and they showed significantly higher SCPs (Fig. 4a) than animals collected from the soil fraction of the same area (Fig. 4b) (KS = 0·4720; $P = 0{\cdot}0004$; $-13{\cdot}3 \pm 5{\cdot}3$ °C, $n = 33$ *vs* $-17{\cdot}4 \pm 4{\cdot}9$ °C, $n = 40$). Both samples tended to have a bimodal distribution, but the dominance changed from animals

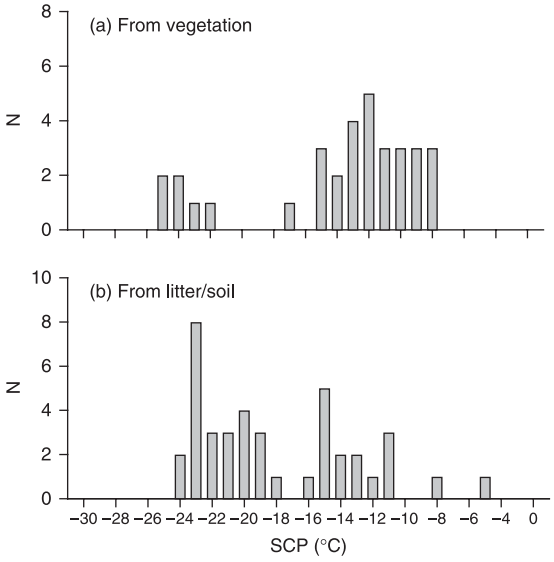

Fig. 4. Stratified variations in supercooling point distribution between animals (a) in vegetation; (b) in litter/upper soil.

with high SCP in the vegetation to those with low SCP in the litter and soil.

Discussion

Our data show that ecdysis in *C. denticulata* significantly alters SCP frequency distributions, causing a substantial decline in individual SCP. Similar effects of moulting on SCP frequency distributions have been observed in two other springtail species: *Tullbergia antarctica* (Worland 2005) and *Cryptopygus antarcticus* (M.R.W. and H.P.L., unpublished data). These species represent three different springtail families, suggesting that the effect of moulting on SCP might be a general phenomenon in the Collembola. Unlike insects, springtails evacuate their gut and shed the mid-gut cells into the lumen during moulting (Humbert 1978), and it seems likely that this process removes ice-nucleating agents from the gut. Moreover, moulting forms a significant proportion of the lifespan of an individual springtail. Collembola continue to moult as long as they live (Hopkin 1997), even after they attain maximum size (Birkemoe & Leinaas 2000). Individuals have been observed to moult up to 40 times (Mari Mutt & Soto-Adams 1987), with up to 40% of a population being in the moulting cycle at any time. (de With & Joosse 1971; Leinaas 1983). Thus, in springtails, a considerable part of the growth season may be spent in a moulting, non-feeding state characterized by a low SCP, which is likely to be independent of any response to the environment, at least in the summer months. As soon as moulting is completed and feeding resumes, the SCP frequency distribution changes to a reasonably well defined group with a high SCP. In consequence, the substantial variation found in springtail SCP distributions (Sinclair *et al*. 2003b; Worland & Convey 2001), especially in summer-acclimatized individuals, is unlikely to be solely a result of an adaptive response, but rather is also a consequence of an integral part of the life history of the group.

If this is the case, then the question arises as to why several springtail species show no evidence of bimodality or substantial variation in SCP in summer populations, and have a persistently high SCP (Sømme & Conradi-Larsen 1977; Sømme & Block 1982; Leinaas & Sømme 1984; Leinaas & Fjellberg 1985). Where moulting is completely synchronized within populations, as in the group-living *Hypogastrura lapponica* and *Hypogastrura socialis* (Leinaas 1983), whole colonies can switch from low to high SCP within a very short time (H.P.L., unpublished data). In this case, some of the summer collections of these species would be dominated by low SCPs, contrasting with the unimodal distribution found in the studies referred to above. A more likely explanation is the tendency of premoulting animals to seek shelter. For instance, *Tetracanthella wahlgreni*, *Vertagopus westerlundi* and *Vertagopus sarekensis* inhabit thin moss or lichen cover on alpine rocks and windswept ridges, but moult under small

stones, loose rock flakes and in crevices. These moulting sites can be identified easily by accumulations of often large amounts of old exuviae. If these animals are sampled from their main vegetation habitat, only actively feeding animals in the intermoult stage will be obtained, which may explain the distinctly unimodal distribution of high SCPs in summer described by Sømme & Conradi-Larsen (1977); Leinaas & Fjellberg (1985). A similar, but less clear tendency was seen in our data comparing *C. denticulata* sampled from the vegetation and from the litter layer. Thus a representative picture of the SCP distribution of a population requires a stratified sampling of the habitat, including both foraging and moulting sites. In this context, a description of the supercooling state of a population is less of a problem in species with distinct foraging and moulting microhabitats (e.g. *T. wahlgreni* and *Vertagopus* spp.). However, in species such as *C. denticulata*, feeding and moulting occur in close vicinity, requiring a set of randomized samples for each estimate.

Such variation of SCPs in time and space associated with moulting has important implications for the interpretation of SCP observations made during the growth season. To distinguish between environmental responses (adaptive or plastic, at any spatial or temporal scale) and changes in SCP related to random or synchronized moulting independent of survival strategies in the life history of a species, SCP distributions need to be assessed repeatedly, preferably at a scale that is appropriate for the question being asked (Worland & Convey 2001; Sinclair *et al*. 2003b).

This raises the question of the relative effects on SCPs of starvation and moulting, given their asymmetrical association (moult does not necessarily follow cessation of feeding or starvation). Several studies have previously concluded that starvation may strongly reduce the freezing point in Collembola (Sømme & Block 1982; Leinaas & Sømme 1984; Leinaas & Fjellberg 1985; Cannon 1986). A complicating factor not accounted for in these studies is that animals may start preparing and even moult during the process, thus confounding the effects of the two processes. By discriminating here between starved, premoult and non-moulting animals, we have not only shown that the results were affected by moult preparation, but also that starvation in itself reduced SCPs significantly, although not as much as moulting.

Even if the lowering effect on SCPs is a non-adaptive consequence of moulting, it could nevertheless be utilized in a cold-hardiness strategy. A phenological timing of moult synchronization might increase population cold hardiness at the end of the season. Possible effects of temperature on moult synchronization are less well known. Burn (1981) noted that the non-feeding premoult period is disproportionately extended at reduced temperatures compared with the actively foraging intermoult stage, which might lead to an accumulation of premoult animals and thus lowered SCPs before winter. However, although such a wintering strategy is possible, no evidence has been found. Moreover, it appears that Collembola in cool boreal forests do not moult in winter (Leinaas 1978), and that the most cold-exposed of these species, the snow surface-active *H. socialis*, undergoes a last autumn moult ≈2 months before the onset of winter (Leinaas 1981). Nevertheless, we believe this question is worth further investigation. In habitats where environmental conditions are unpredictable and cold snaps common, the incidental occurrence of a proportion of individuals being in a moulting state could be seen as a co-opted survival strategy. In this case one would not expect to see evidence of synchronized moulting. This possibility has yet to be investigated.

In conclusion, we have shown that SCP variation within a springtail species is not simply induced as a response to changing environmental temperatures, but is also a consequence of endogenous physiological variation associated with a separate process: moulting. This finding has considerable consequences for investigations of the environmental physiology of springtails and other arthropods.

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