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Low temperature acclimated populations of the grain aphid *Sitobion avenae* retain ability to rapidly cold harden with enhanced fitness

S. J. Powell* and J. S. Bale

School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

*Author for correspondence (e-mail: sjp822@bham.ac.uk)

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Summary

In contrast to previous studies of rapid cold-hardening (RCH), which have investigated the responses of insects maintained under 'summer conditions' (20° to 25°C), this study focuses on the ability of low-temperature acclimated insects to undergo RCH. When the grain aphid *Sitobion avenae* Fabricus was low-temperature acclimated by rearing for three generations at 10°C, the discriminating temperatures (temperature that results in approximately 20% survival after direct transfer from the rearing temperature to a sub-zero temperature for a period of 3 h), of first instar nymphs and adult aphids were –11.5° and –12°C, respectively. Maximum rapid cold-hardening was induced by cooling aphids at 0°C for 2 h (nymphs) or 30 min (adults), resulting in survival at the respective discriminating temperatures increasing from 26% to 96% (nymphs) and 22% to 70% (adults). Cooling from 10° to 0°C at 1°, 0.1° and 0.05°C min^{-1} significantly increased survival of nymphs at the discriminating temperature, but not of adults. There were no 'ecological costs' associated with rapid cold-hardening at 0°C, or with exposure of rapidly cold-hardened aphids to the discriminating temperatures; fecundity and longevity, in both nymphs and adults were either similar to control aphids or significantly increased. The study demonstrates that rapid cold-hardening ability is retained in aphids that have already undergone cold-acclimation, as would be the case in overwintering aphids. Both rapid cold-hardening and subsequent exposure at previously lethal temperatures can enhance fitness in surviving individuals.

Key words: rapid cold-hardening, seasonal acclimation, aphid, *Sitobion avenae*, fitness.

Introduction

Rapid cold-hardening (RCH) in insects is detected as an increase in survival at a 'discriminating temperature' following a brief period of acclimation (1–3 h), at a low temperature (typically 0°C), compared with samples transferred directly from the rearing temperature to the discriminating temperature. The ability of insects to rapidly cold-harden was first reported by Lee et al. (1987). Many studies have since characterised the advantages and limitations of this response in a range of species groups (Broufas and Koveos, 2001; Coulson and Bale, 1990; Czajka and Lee, 1990; Larsen and Lee, 1994; Powell and Bale, 2004). Most of these studies have regarded rapid cold-hardening and seasonal (winter) acclimation as separate processes. For this reason, RCH experiments have usually been conducted on laboratory-reared insects maintained at temperatures typical of summer conditions, and in species in which the lethal temperature is markedly higher than the freezing temperature or supercooling point (SCP). In this respect, the recent study on RCH using shifts in the supercooling point in 'field-fresh' populations of Antarctic Collembola (Worland and Convey, 2001) and the study on the ability of the sub-Antarctic caterpillar *Pringleophaga marioni* to rapidly cold harden (Sinclair and Chown, 2003) are exceptions to this general trend.

Although summer-acclimated insects may have a greater potential to demonstrate a RCH response, in the Northern Hemisphere, the ecological importance of this ability is more associated with low-temperature survival, either in autumn and spring, when seasonal acclimation is incomplete or receding, or during periods with rapidly fluctuating temperatures (Lee et al., 1987; Coulson and Bale, 1990; Kelty and Lee, 1999). In other words, in natural environments, the conditions under which rapid cold-hardening might be induced are likely to be part of a trend of decreasing, or generally low, temperatures that would have already triggered the start of seasonal cold acclimation. As far as we are aware, this is the first time that the ability to rapidly cold-harden has been investigated in insects that have already undergone long-term, low temperature acclimation.

The grain aphid *Sitobion avenae* Fabricus is a monoecious species overwintering on grasses and cereals as holocyclic (eggs) and anholocyclic (aphids) clones. The anholocyclic clones are chill susceptible (Bale, 1996), with high levels of pre-freeze mortality after brief exposures to relatively high sub-zero temperatures (Knight and Bale, 1986). A recent study (Powell and Bale, 2004) found that laboratory populations of an anholocyclic clone of *S. avenae* reared at 20°C are able to

rapidly cold-harden in response to 2–3 h acclimation at 0°C, or by slow cooling (0.1°C min^{-1}) from 10°C. It was hypothesised that rapid cold-hardening may be relatively more important in insects such as aphids (compared with longer-lived species) because their short generation times, even in winter, may prevent the 'full development' of seasonally induced cold-hardiness. Whilst it is known that aphids reared at 10°C for one or more generations are more cold hardy than those maintained continuously at 20°C (Clough et al., 1990), the ability of such low-temperature acclimated aphids to also rapidly cold-harden has never been investigated.

This paper describes a series of experiments that investigated the ability of populations of *S. avenae*, acclimated at 10°C, to further increase their cold tolerance *via* exposure to temperature regimes known to induce rapid cold-hardening in aphids reared at 20°C, and assesses the ecological costs (development, longevity and fecundity) associated with this response.

Materials and methods

A stock culture of an anholocyclic clone of *Sitobion avenae* Fabricius (DAV 95) was low-temperature acclimated by maintaining the population at 10°C for three generations prior to use in experiments. Aphids were reared singly on cut barley leaves (*Hordium vulgare* cv Heligan) inside Austin tubes (Austin et al., 1991), secured in Dufaylite (Dufaylite 25.9 mm×9.4 mm; Dufaylite Developments Ltd, Cromwell Road, St Neots, Hants PE19 1QW, UK) in trays of water at 10±1°C with a 18 h:6 h light:dark photocycle. All nymphs used in this study were first instars, produced between days 2 and 6 of the mother's birth sequence. First-born nymphs were discarded as previous studies have shown that the first born progeny from parent aphids of some species are physiologically atypical (Murdie, 1969; Clough et al., 1990). All adult aphids used in the study were newly moulted and pre-reproductive, ensuring consistency in the experimental material.

Determination of discriminating temperature

The discriminating temperature, defined as the exposure temperature resulting in approximately 20% survival, was determined by directly transferring aphids from the culture temperature (10°C), to progressively lower sub-zero temperatures for 3 h, before re-warming at 1°C min^{-1} to 10°C. Ten replicates of 10 aphids were placed inside plastic Eppendorf tubes, which were then placed inside glass boiling tubes stoppered with cotton wool and lowered into a Haake alcohol bath (F8-C50) set to the desired sub-zero temperature. A thermocouple placed inside an Eppendorf tube measured the temperature experienced by the aphids. Following the low-temperature treatment, aphids were placed on a recovery tray (strips of barley leaf on moist tissue under the lid of a small Petri dish) and returned to 10°C. Survival was assessed after 24 and 72 h. Surviving aphids were defined as those capable of co-ordinated movement.

Detection of a rapid cold-hardening response

To determine if a rapid cold-hardening response occurs in 10°C acclimated *S. avenae*, and subsequently, the optimum conditions for its induction, five replicates of 10 first instar nymphs and newly moulted pre-reproductive adults were transferred from the rearing temperature (10°C) to 0°C for between 10 min and 6 h, and then exposed to their respective discriminating temperatures for 3 h. In a separate experiment, aphids were cooled from 10° to 0°C at 1°, 0.1° or 0.05°C min^{-1}, prior to transfer to the discriminating temperature for 3 h. In both experiments, control groups of the same sample size were subjected to the same handling procedures as the treatment groups but maintained continuously at 10°C. On completion of the treatment exposures, aphids were re-warmed at 1°C min^{-1} and placed on recovery trays at 10°C. Survival was again assessed after 24 and 72 h.

Statistical analysis

All survival data was arcsine and square root transformed to ensure a normal distribution, then analysed using analysis of variance (ANOVA) and Tukey's multiple range test.

Ecological costs of rapid cold-hardening

The occurrence of possible deleterious effects associated with rapid cold-hardening on the development, longevity and fecundity of first instar and adult *S. avenae* was investigated by establishing three treatment groups: (i) control: aphids maintained continuously at 10°C; (ii) rapidly cold-hardened: aphids acclimated at 0°C for the periods of time producing the highest survival at the discriminating temperature (2 h for nymphs and 30 min for adults) and then returned to 10°C; (iii) exposed: aphids rapidly cold-hardened at 0°C for 2 h (nymphs) or 30 min (adults), and then exposed to the appropriate discriminating temperature for 3 h before being returned to 10°C. For each treatment five groups of 10 aphids were placed inside plastic Eppendorf tubes, which were then placed inside glass boiling tubes stoppered with cotton wool and lowered into a Haake alcohol bath (F8-C50) set to the desired temperature regime. Following treatment, aphids were placed singly onto cut barley inside an Austin tube. Development and reproduction were monitored daily by counting and removing moulted cuticles or new-born nymphs respectively.

Results

Determination of the discriminating temperature

Mean survival of first instar nymphs and newly moulted pre-reproductive adults exposed directly from their rearing temperature (10°C) to a range of sub-zero temperatures is shown in Fig. 1. The survival of both nymphs and adults declined with each 1°C reduction in temperature. No aphid (nymph or adult) survived exposure to temperatures below –12.5°C. The discriminating temperatures (approximately 20% survival in a 3 h exposure) were determined as –11.5° and

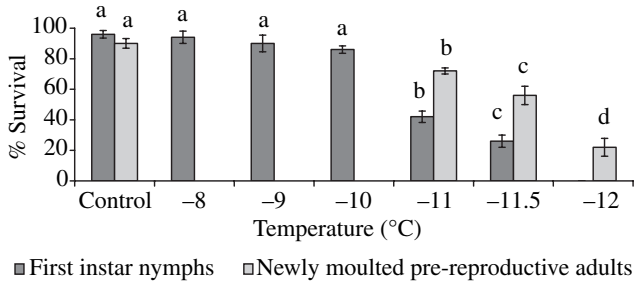

Fig. 1. Percentage survival (mean ± S.E.M.; N=100) of first instar and newly moulted pre-reproductive *S. avenae* reared at 10°C, 72 h after direct exposure to a range of sub-zero temperatures. Discriminating temperatures for first instar nymphs and newly moulted adult aphids were determined as –11.5°C and –12.0°C, respectively. Means with the same letter are not significantly different at P<0.05 (Tukey multiple comparison test).

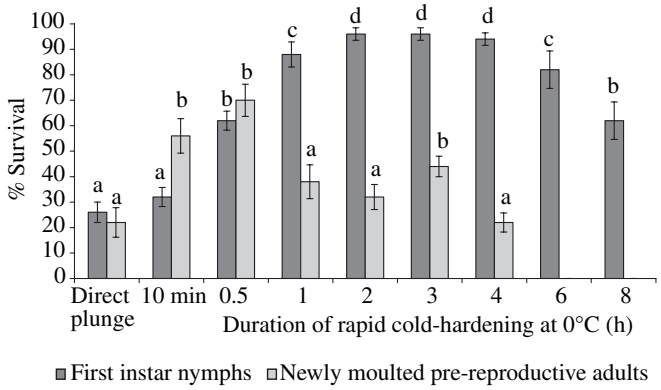

Fig. 2. Percentage survival (mean ± S.E.M.; N=50) of first instar and newly moulted pre-reproductive *S. avenae* reared at 10°C, 72 h after rapid cold-hardening (RCH) treatment. Aphids were rapidly cold-hardened at 0°C for increasing periods of time, prior to exposure to the discriminating temperature (–11.5°C for first instar nymphs and –12°C for newly moulted adults). Means with the same letter are not significantly different at P<0.05 (Tukey multiple comparison test).

–12°C for first instar and newly moulted pre-reproductive adults, respectively.

Detection of a rapid cold-hardening response: varying the acclimation period at 0°C

When aphids were transferred directly from the culture temperature (10°C) to their respective discriminating temperature, 26% of first instar nymphs and 22% of adults survived (Fig. 2). First instar nymphs required a minimum acclimation period of 30 min at 0°C to significantly increase survival from 26% to 62%, compared with the 'direct plunge' exposure at –11.5°C ($F_{8,32}$=19.1, P<0.01). Nymphal cold hardiness reached its maximum following a 2 h acclimation period at 0°C with 96% surviving 3 h at the discriminating temperature; acclimation for longer than 4 h resulted in a trend of steadily decreasing survival that became significantly different from maximum survival after 8 h of acclimation (Fig. 2).

The cold hardiness of adult aphids increased more rapidly than that of nymphs. Acclimation at 0°C for only 10 min significantly increased survival at the discriminating temperature, from 22% to 56% ($F_{5,20}$=9.2, P<0.01). Maximum cold tolerance (70% survival) was induced by 30 min at 0°C. However, acclimation at 0°C for longer than 30 min significantly reduced survival of adult aphids when subsequently exposed at –12°C; thus, survival at –12°C after 4 h acclimation at 0°C was similar to that after a 'direct plunge' to the same temperature (Fig. 2).

Detection of a rapid cold-hardening response: varying the rate of cooling

Fig. 3 shows mean percentage survival of 10°C-reared first instar nymphs and newly moulted pre-reproductive adults, cooled from 10° to 0°C at 1°, 0.1° and 0.05°C min^{-1}, prior to a 3 h exposure at the respective discriminating temperatures. At all three rates of cooling, survival of first instar nymphs after 3 h at the discriminating temperature, was significantly increased compared with a direct plunge to –11.5°C for the same period ($F_{3,12}$=25.1, P<0.01). The fastest cooling rate of 1°C min^{-1} increased survival from 25% to 78%; maximum cold tolerance was induced by cooling at 0.1°C min^{-1}, resulting in 86% of nymphs surviving exposure at the discriminating temperature. In contrast, the cold hardiness of adult *S. avenae* was not significantly increased by any of the cooling rates investigated.

Ecological cost of rapid-cold hardening: aphids treated at the first instar nymph stage

Data on the development, fecundity and longevity of the three treatment groups (control, rapidly cold-hardened and exposed) are shown in Table 1. Development time was not significantly affected by the rapid cold-hardening or exposure treatments compared with the control. Daily fecundity increased from the control through rapid cold-hardening to the exposed treatments, suggesting that increasing the level of cold stress increases fecundity in surviving aphids. Compared with the control, rapid cold-hardening did not affect daily reproduction; however, the exposed treatment resulted in a significant increase (1.6±0.1 nymphs per day) compared with both the control (1.1±0.1 nymphs per day) and RCH treatments (1.3±0.1 nymphs per day) ($F_{2,73}$=13.3, P<0.01), fecundity (recorded over the entire life span) followed a similar pattern with the exposed treatment producing a significantly higher number of nymphs (50.7±2.6) ($F_{2,73}$=5.4, P<0.01) compared with both the RCH and control treatments, which in turn were not significantly different. Mean length of reproductive life and mean longevity were similar in all three treatments.

Ecological cost of rapid cold-hardening: aphids treated at the newly moulted pre-reproductive adult stage

Neither the RCH nor exposure treatments affected the daily fecundity of aphids treated at the newly moulted pre-reproductive adult stage (Table 1). However, as with first instar

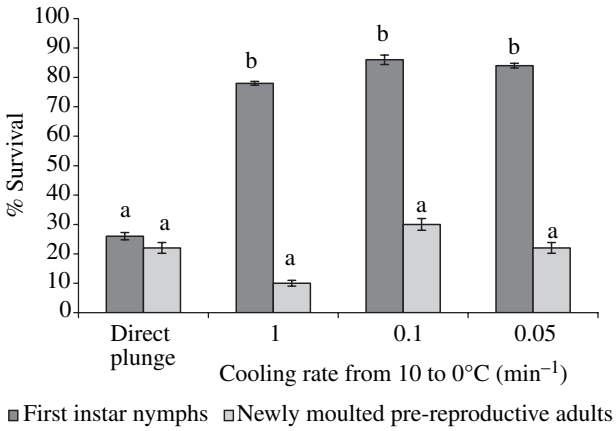

Fig. 3. Percentage survival (mean ± S.E.M.; N=50) of 10°C-reared first instar and newly moulted pre-reproductive *S. avenae*, 72 h after cooling from 10° to 0°C at various rates, prior to exposure to the discriminating temperature (–11.5°C for first instar nymphs and –12°C for newly moulted adults). Means with the same letter are not significantly different at P<0.05 (Tukey multiple comparison test).

nymphs, mean total fecundity increased significantly with increasing levels of cold stress ($F_{2,73}$=22.9, P<0.01); RCH treatment increased mean total fecundity from 16.8±1.5 (control) to 33.1±1.7 nymphs, with a further increase to 34.6±2.7 nymphs in the exposed treatment. The RCH and exposed treatments significantly increased the length of reproductive life and longevity compared with the control.

Discussion

The relationship between seasonal low-temperature acclimation and rapid cold-hardening remains unclear, though it does appear that the key biochemical components involved in seasonal increases in cold-hardiness (ice nucleating agents, antifreeze proteins and polyols) have little or no role in rapid cold-hardening (Kelty and Lee, 1999). It has been hypothesised that RCH may act to preserve neuronal and muscular resting potentials, neural conduction velocities, neuromuscular co-ordination and the fluidity of membranes (Kelty et al., 1996). Also, it is known that stress other than low temperature (such as anoxia and high temperature) can induce the rapid cold-hardening response (Coulson and Bale, 1991; Rinehart et al., 2000). The majority of reports of rapid cold hardening have involved laboratory populations of insects reared at 20°C or higher, in species that were known to die at temperatures considerably above the freezing temperature (SCP).

As far as we are aware, there have been no previous studies comparing rapid cold-hardening between low-temperature acclimated and non-acclimated populations of the same species. The first report of rapid cold-hardening in an aphid was with an anholocyclic clone of *S. avenae* reared at 20°C (Powell and Bale, 2004). The data for populations of *S. avenae* reared at 20°C provide a comparative baseline by which to assess the occurrence and extent of RCH in acclimated aphids. Thus at 20°C, survival of nymphs and adults increased from 18% and 16% at their respective discriminating temperatures (–8°C and –8.5°C) to 83% and 68%. There were no ecological costs (effects on development, longevity or reproduction) associated with rapid cold-hardening of nymphs or adults, compared with aphids maintained continuously at 20°C, but exposure of rapidly cold-hardened aphids at their respective discriminating temperatures significantly reduced fecundity and longevity of both age groups (Powell and Bale, 2004). Recent research has found that aphids acclimated at 10°C for three generations are more cold-hardy than populations maintained at 20°C, as revealed by a lowering of the $LTemp_{50}$ (temperature that results in 50% mortality of the experimental population) of nymphs from –8.0°C to –16.0°C, and of adults from –9.3°C to –13.5°C (S.J.P. and J.S.B., unpublished data). This study demonstrated that a greater level of cold tolerance was also reflected in the rapid cold-hardening response, in which the discriminating temperature was lowered from –8° to

Table 1. *Development time, daily reproduction, fecundity and longevity of first instar nymph and newly moulted pre-reproductive adults*

	Development time to adult (days)	Mean daily fecundity (nymphs per day)	Mean total fecundity (nymphs per adult)	Mean length of reproductive life (days)	Longevity (days)
Control nymphs	18.8±0.2^{a}	1.1±0.1^{a}	37.3±2.6^{a}	37.7±2.6^{a}	58.5±2.6^{a}
RCH nymphs	18.7±0.5^{a}	1.3±0.1^{a}	46.1±2.6^{a}	38.9±2.5^{a}	59.5±2.4^{a}
Exposed nymphs	18.1±0.4^{a}	1.6±0.1^{b}	50.7±2.6^{b}	35.7±2.5^{a}	56.3±2.5^{a}
Control adults	n/a	0.9±0.1^{y}	16.8±1.5^{y}	21.7±2.2^{y}	42.7±2.2^{y}
RCH adults	n/a	1.1±0.1^{y}	33.1±1.7^{z}	32.3±2.1^{z}	53.3±2.1^{z}
Exposed adults	n/a	1.1±0.1^{y}	34.6±2.7^{z}	33.2±2.7^{z}	54.2±2.7^{z}

Control: aphids were maintained continuously at 10°C; RCH: aphids acclimated at 0°C for periods of time producing the highest survival at the discriminating temperature (2 h for nymphs and 30 min for adults) and then returned to 10°C. Exposed: aphids rapidly cold-hardened at 0°C for 2 h (nymphs) or 30 min (adults), prior to a 3 h exposure at the discriminating temperature (–11.5°C for nymphs and –12°C for adults).

n/a, not applicable.

Values are mean ± S.E.M. The letters in brackets indicate statistical significance; those with the same letter are not significantly different at P<0.05 (Tukey multiple comparison test). N=50.

–11.5°C for nymphs and –8.5° to –12°C for adults. Importantly, these low-temperature acclimated aphids still retained the ability to rapidly cold-harden, increasing survival at the discriminating temperature from 26% to 96% and 22% to 70% for nymphs and adults, respectively. In contrast to aphids reared at 20°C, which demonstrated no ecological costs or benefits as a result of rapid cold-hardening (Powell and Bale, 2004), the fecundity of low-temperature acclimated aphids treated at the adult stage, increased significantly after rapid cold-hardening at 0°C, and was further significantly increased (compared to the control) after exposure at the discriminating temperature. Supporting the hypothesis that RCH can re-set the thermal thresholds for certain behaviours such as reproduction (Bale, 2002). The longevity of adults was also significantly increased after 30 min of acclimation at 0°C. However, the fecundity and longevity of aphids treated at the first instar nymph stage was unaffected by rapid cold-hardening at 0°C for 2 h. The absence of any deleterious effects of RCH on the fecundity and longevity of *S. avenae* contrasts with the results of Coulson and Bale (1992) who found that in *Musca domestica,* both of these performance parameters were significantly decreased by RCH. However, Broufas and Koveos (2001) reported that the cost of RCH on the post diapause reproductive output of *Euseius (Amblyseius) finandicus,* was negligible, and a recent study by Shreve, Kelty and Lee (Shreve et al., 2004) reported an increase in successful mating of *Drosophila melanogaster* following RCH, compared with flies exposed directly to low temperature. It is therefore clear that the ecological cost of RCH varies greatly between different species. When the ability of low-temperature acclimated nymphs and adults to rapidly cold-harden are compared, the main difference between the two life stages is the increase in nymph survival at their discriminating temperature after slow cooling from 10° to 0°C, but the absence of a similar response in adults.

In a wider context, RCH may influence the overwintering success of anholocyclic aphids, and hence their economic importance. Winter exerts a major influence on the annual population dynamics of aphid species with anholocyclic clones, particularly the timing of the spring migration and the number of migrating aphids (Harrington et al., 1989; Werker et al., 1998). It is known that aphids have low supercooling points (<–20°C) and die before they freeze ($LTemp_{50}$ of –8.1°C for *Myzus persicae* reared at 20°C), but can acclimate when reared at lower temperatures ($LTemp_{50}$ of *M. persicae* is lowered to –11.5°C after two generations at 10°C) (Clough et al., 1990). However, the overwintering biology of anholocyclic aphids differs from that of most other insects in one key area: aphids continue to develop and reproduce throughout winter. As a consequence, an aphid found in the field in early spring is likely to be the second, third or fourth generation descendant of the individual that entered the winter 4–5 months earlier. Owing to these short generation times, it has been suggested that aphids may have a comparatively limited ability to seasonally acclimate, and that rapid cold-hardening may be relatively more important in these insects (Powell and Bale, 2004). It is certainly the case that aphid mortality increases rapidly over a narrow range of temperatures (–5°C to –15°C; Knight and Bale, 1986), hence even modest decreases in the lethal temperature may be important for the winter survival of anholocyclic clones.

In summary, this study demonstrates that low-temperature acclimated *S. avenae* retain the ability to rapidly cold-harden as a result of a short acclimation period at 0°C (nymphs and adults) or by slow cooling at rates between 1 and 0.05 min^{-1} (nymphs only). This suggests that rapid cold-hardening may be an important component in the overwintering survival of *S. avenae* and other aphids with anholocyclic clones, with the additional benefit of increasing longevity and fecundity.

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