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Preservation of reproductive behaviors during modest cooling: rapid cold-hardening fine-tunes organismal response

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Summary

The primary objectives of this study were to determine (1) whether rapid cold-hardening (RCH) preserves reproductive behaviors during modest cooling, (2) whether increased mating success at a lower temperature comes at the cost of decreased performance at a higher temperature and (3) whether RCH is associated with an elevated metabolic rate. *Drosophila melanogaster* (Diptera: Drosphilidae) were rapidly cold-hardened by a 2-h exposure to 16°C prior to experiments. A temperature decrease of only 7°C (23°C to 16°C) prevented half (11/22) of the control pairs of *D. melanogaster* from engaging in any courtship activity. By contrast, most RCH pairs courted (17/20). Additionally, the 7°C transfer prevented mating in every pair of control flies, whereas more than half (11/20) of the RCH pairs mated. There was no evidence of impaired courtship or mating performance when RCH pairs were tested at 23°C. Finally, RCH is apparently not an energy-demanding process because no increase in the metabolic rate was detected during its induction. Overall, these data demonstrate that RCH serves to constantly fine-tune an insect's physiological state to match slight changes in environmental temperature. Furthermore, the RCH response is not restricted to cryoprotection and survival in the cold but also preserves more subtle behaviors, such as courtship, at moderate to high temperatures throughout the year.

Key words: rapid cold-hardening, temperature, courtship, reproductive behavior, mating performance, metabolism, *Drosophila*.

Introduction

Traditionally, studies of insect cryobiology have focused on seasonal adaptations acquired over periods of weeks or months that promote winter survival. However, in the past 15 years a much more rapid process of cold hardening has come under increasing examination (Kelty and Lee, 1999). This process, known as rapid cold-hardening (RCH), is defined as the acquisition of enhanced tolerance to low temperatures over a short period of time, in the order of minutes to hours (Lee et al., 1987). Early studies of the response used severe chilling, such as direct transfers from 23° to 0°C, to induce and assess the RCH response (Lee et al., 1987; Coulson and Bale, 1990; Burks and Hagstrum, 1999).

Originally, Lee et al. (1987) hypothesized that RCH allows insects to 'instantaneously' enhance their cold tolerance in a thermally variable environment. Later investigations sought to test this hypothesis and elucidate the ecological relevance of RCH by employing more natural temperature regimes and moderate rates of cooling (Coulson and Bale, 1990). For example, Kelty and Lee (1999) found that cooling adult *Drosophila melanogaster* at low rates induced RCH, causing flies to enter a state of chill-coma at temperatures lower than those cooled more rapidly. Similar results were obtained using ecologically based thermoperiodic cycles (Kelty and Lee, 2001). In addition, Koveos (2001) induced RCH in the olive fruit fly, *Bactrocera oleae*, by maintaining them in outdoor field cages overnight. Flies tested at the coolest time of the day were more cold tolerant, based on survival rates after a 2-h exposure to –7°C, than flies tested at the warmest part. Recently, Bale (2002) re-stated the ecological relevance hypothesis, suggesting that the RCH response functions by 'resetting' the thermal thresholds for behavioral characteristics such as flight or critical thermal minimum (CT_{min}), the temperature at which insects enter a state of chill-coma.

Several investigators have argued that survival to reproduction or post-treatment fecundity, rather than immediate survival, provides more ecologically relevant measures of cold tolerance and RCH (Baust and Rojas, 1985; Coulson and Bale, 1992; Kelty and Lee, 1999). In addition to its long-term effects on female egg production (Coulson and Bale, 1992; Kelty and Lee, 1999) and on the rate of egg fertilization (Rinehart et al., 2000), RCH may also have a more immediate effect on reproductive success. Courtship and reproduction in *Drosophila*, as in other insects, require a complex series of reciprocal behaviors prior to successful mating (Spieth, 1974). RCH is known to preserve gross neuromuscular function at low temperature, as demonstrated

by its lowering of the chill-coma temperature (Kelty and Lee, 1999, 2001). Additionally, RCH prevents decreases in the resting membrane potential, reductions in neural conduction velocity and impairment of neuromuscular coordination that would otherwise occur as a result of chilling (Kelty et al., 1996). Therefore, it seems likely that RCH may also act to protect complex courtship behaviors, which require a fine degree of neuromuscular control.

Despite advances in understanding the ecological relevance of RCH, the physiological mechanism behind the process remains poorly understood (Kelty and Lee, 1999). Although Chen et al. (1987) documented modest levels of glycerol production during RCH in the flesh fly *Sarcophaga crassipalpis*, Kelty and Lee (1999) found no changes in glycerol, or any other sugar or polyol, levels in *D. melanogaster* during RCH. Thus, the elevation of sugars or polyols is not consistently associated with RCH, and so other factors must also play a role. Adjustments in metabolic rate may provide clues to the underlying mechanisms responsible for RCH. Coulson and Bale (1990) speculated that compensatory shifts in the metabolic rate might account, at least in part, for the observed effects of RCH. If so, the time required to reach a stable metabolic rate at a lower temperature after transfer from the rearing temperature should be less in RCH flies since the compensatory changes are already in place. In addition, if RCH is an energy-requiring process, then RCH flies should exhibit a higher metabolic rate at low temperatures than control flies.

In the present study, we determined whether the RCH response preserved reproductive behaviors and courtship success of *D. melanogaster* during brief periods of modest cooling that would be expected to occur frequently in nature. We also tested whether RCH came at the cost of reduced reproductive performance at a higher temperature. Lastly, we determined whether RCH was associated with an elevation in metabolic rate, a response that might provide clues as to the underlying mechanism of this response.

Materials and methods

Insect rearing

Drosophila melanogaster Oregon-R strain were reared on standard cornmeal–yeast–agar medium in ~0.5-liter bottles (approximately 200 adults per bottle) at 23°C and 15 h:9 h L:D (Kelty and Lee, 1999). Adults were allowed nine days to oviposit. Since both sexual activity (Spieth, 1974) and cold tolerance (Czajka and Lee, 1990) depend on the age of the adult, we used four-day-old flies for all experiments.

Mating trials

Since a mated female often exhibits decreased receptivity to subsequent matings (Spieth, 1974), we used virgin flies for the mating trials. We separated male and female adults within 5–6 h after pupal eclosion, before they reached sexual maturity (Spieth, 1974). The mating chambers for single pairs of flies were 60 mm×15 mm petri dishes with a layer of medium (~2 mm) that was sprinkled with a few grains of yeast. We used a video camera placed in an incubator to observe four chambers at a time.

In the first experiment, we compared the reproductive behavior of male and female pairs from the control and RCH groups. Control flies were transferred directly from 23°C to 16°C and their reproductive behaviors recorded for 1 h immediately after transfer. The RCH flies were given a 2-h acclimation period at 16°C, permitting them to rapidly cold-harden, before we recorded their reproductive activity for 1 h at 16°C. The males and females of the RCH group were kept segregated during this pre-treatment to be sure that they were still virgins during the mating trial.

We also performed a reciprocal study in order to determine whether the increased reproductive success exhibited by RCH flies at lower temperatures was gained at the cost of reduced mating performance and success at a higher temperature (23°C). Control flies were held continuously at 23°C. RCH flies were subjected to the cold-hardening treatment of 16°C for 2 h with the sexes separated before they were paired and returned to 23°C for a 1-h mating trial. For both experiments, we recorded whether the pair courted, the duration of each courtship event, whether the pair ultimately mated, and the courtship index, defined as the percentage of time a pair spent in courtship or mating behaviors.

Respirometry

We used carbon dioxide production as a measure of metabolic rate in *D. melanogaster* (Lee and Baust, 1982; Berrigan and Partridge, 1997). A Sable Systems (Las Vegas, NV, USA) flow-through respirometer with a Li-Cor CO_2/H_2O gas analyzer (LI-6262) was used to measure CO_2 production in the flies (Lighton, 1988; Berrigan and Partridge, 1997). The Datacan V software from Sable Systems was used to collect and analyze the data. We were unable to achieve the sensitivity needed to discern differences in metabolic rate at 23°C *versus* 16°C in individual flies. Therefore, 10 adult flies per replicate were placed in a small plastic chamber set inside a refrigerated bath (NESLAB RTE-8). Temperatures during all the respirometry experiments were recorded using a copper–constantan thermocouple inserted directly into the chamber.

The CO_2 production of the control flies was measured at room temperature (23±0.5°C) until a stable respiratory rate was observed, typically after ~30 min. To determine the effect of direct chilling to 16°C in the control group, we then placed the chamber directly in the cold bath, allowed CO_2 production to reach a new stable rate at 16°C, and recorded this rate and the time needed to reach a stable rate of CO_2 production. The air temperature within the chamber reached 16°C in less than 5 min after transfer. To determine the effect of RCH, flies from the RCH group were placed in the 16°C cold bath for 2 h. Next, we removed the chamber from the bath and held it at room temperature for 2–5 min, allowing it to increase to 23°C, and then returned it to 16°C. Because temperature transfer was achieved by simply immersing the chamber in a cold bath, the

respirometry system maintained a stable baseline throughout this experiment. The flies remained rapidly cold-hardened despite the brief exposure to 23°C (Kelty and Lee, 2001). We allowed CO_2 production to stabilize and then measured the metabolic rate and the time required to attain the stable rate.

During preliminary runs, flies often lost 15% or more of their body mass due to water loss over a span of 3 h in the dry airstream. In order to avoid excessive dehydration in all experimental trials, we rehydrated the air by bubbling it through a solution of 15% potassium hydroxide to humidify the airstream while keeping the CO_2 dissolved in the water in solution. The air then passed through a condensing chamber on a thermoelectric cold plate (TCP-2) to remove excess moisture that would form droplets on the inside of the tubing. This also had the effect of pre-cooling the airstream during the RCH trials. However, the thermocouple inside the chamber showed that this did not affect the temperature during the control trials. Despite these measures, male *D. melanogaster* still lost ~10% of their mass during the experiments. Therefore, female flies, which only lost about 3% of their mass, were used in the respirometry experiments.

Statistics

All values are given as means ± S.E.M. When comparing courtship indices, the percentage data was first transformed by taking the arcsine of the square root of the observed index. The numbers of control and RCH pairs that courted or mated were compared using a chi-squared analysis. Comparison of the acclimation time in the respiration experiments was performed using an unpaired *t*-test. We used analysis of variance (ANOVA) with Bonferroni *post-hoc* tests to compare all other parametric data in both the mating trials and respiration experiments. Non-parametric data were compared using Kruskal–Wallis with multiple comparisons (Gibbons, 1997). Significance for all tests was determined at $P<0.05$. All statistical analyses were performed using StatView 5.0.

Results

Courtship success

RCH flies were more successful than control flies in courtship and mating ability at temperatures that would otherwise prohibit mating. After direct transfer from 23°C to 16°C, only 11 of the 22 pairs of flies in the control group courted, and none of these pairs mated (Fig. 1). By contrast, RCH allowed the majority of pairs to court (17/20) during the 1-h trial, and more than half (11/20) to mate (Fig. 1). The number of control and RCH pairs that courted ($\chi^2=5.775$, $P=0.016$) and the number of pairs that mated ($\chi^2=16.394$, $P<0.0001$) were significantly different.

In addition, RCH pairs engaged in courtship for longer continuous periods and spent more time overall in reproductive activities at 16°C. The mean duration of individual courtship events of the RCH flies was significantly longer than that of flies in the control group (Table 1), suggesting that the RCH pairs had greater endurance during courtship. They also had a significantly greater courtship index than control pairs. Flies in the RCH group spent more than half of the 1-h trial period (53.3±8.4%) either courting or mating, whereas control flies exhibited reproductive behaviors for only ~12 min (20.6±5.7%) (Table 1).

We hypothesized that enhancement of courtship success at 16°C by RCH would diminish the rate of success at 23°C. To discern if RCH diminished the flies' ability to mate at a higher temperature, we tested control and RCH groups at 23°C. Pairs

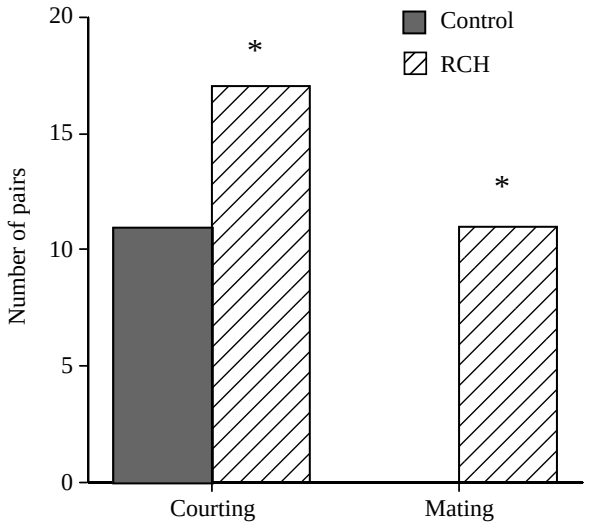

Fig. 1. Comparison of the reproductive activity of control *versus* rapidly cold-hardened (RCH) *D. melanogaster* tested at 16°C. Immediately prior to the mating trial, RCH pairs ($N=20$) were acclimated to 16°C for 2 h, while control pairs ($N=22$) were transferred from 23°C to 16°C immediately prior to the 1-h observation period. Asterisks denote significant differences from control (χ^2, $P<0.05$).

Table 1. *Effects of temperature and rapid cold-hardening on courtship parameters in* D. melanogaster

Mating temperature	Treatment group (*N*)	Number of courtship events	Duration of individual courtship events (min)	Courtship index (%)
16°C	Control (22)	0.9±0.2^{a}	9.6±0.8^{a}	20.6±5.7^{a}
16°C	RCH (20)	1.6±0.3^{a,b}	19.1±3.4^{b}	53.3±8.4^{b}
23°C	Control (20)	3.0±0.8^{b,c}	7.9±1.2^{a}	40.8±6.3^{a,b}
23°C	RCH (19)	6.6±2.1^{c}	4.9±0.7^{a}	52.3±3.9^{b}

Flies in the rapid cold-hardening (RCH) group were subjected to a 2-h 16ºC pre-treatment prior to mating trials. Control flies were held at 23ºC until immediately before transfer to the mating temperature. Courtship index is defined as the percentage of the 1-h mating trial that a pair spends in reproductive behavior. Values are given as means ± S.E.M.; *N*=number of pairs. Values within columns are statistically different if they do not share a common letter (Kruskal–Wallis, multiple comparisons *post-hoc* tests, $P<0.05$).

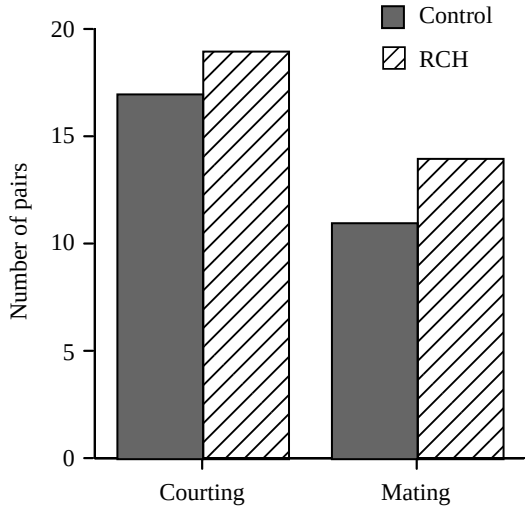

Fig. 2. Reciprocal test to determine whether rapid cold-hardening (RCH) at 16°C reduced mating performance at the original rearing temperature of 23°C. RCH pairs (N=19) were acclimated at 16°C for 2 h immediately prior to a 1-h mating trial. Control pairs (N=20) were held continuously at 23°C prior to the mating trial.

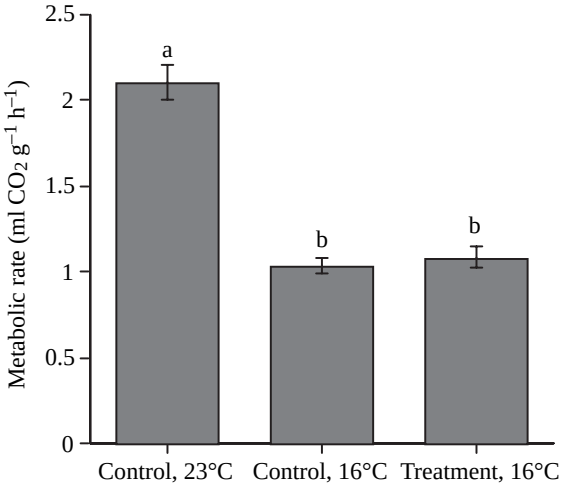

Fig. 3. Comparison of metabolic rate of *D. melanogaster*, as measured by CO_2 production, for control flies at 23°C and 16°C, and rapidly cold-hardened (RCH) flies at 16°C after a brief exposure (~5 min) to 23°C. Error bars represent ± S.E.M. Bars with different letters are significantly different (Bonferroni multiple comparisons test, P<0.0001).

from the RCH group did not appear to exhibit impaired courtship abilities at 23°C, as there was no difference in the number of pairs courting or mating between the two groups (Fig. 2). Nor were there differences in event duration and courtship index (Table 1).

Respirometry

There was no difference in the metabolic rate at 16°C between control *D. melanogaster* that did not have any prior exposure to 16°C and flies that had rapidly cold-hardened to 16°C. In the control group (N=5 groups of 10 flies), the rate of CO_2 production was greater at 23°C than at 16°C (P<0.0001). The respiratory rate of the RCH group (N=6 groups of 10 flies) at 16°C after the brief exposure (~5 min) to 23°C was also lower than that of the control flies at 23°C (P<0.0001) but was not significantly different from the control flies at 16°C (P=0.64) (Fig. 3). We hypothesized that the time to reach a stable metabolic rate after the temperature transfer was longer for control flies (51.8±11.3 min) with no prior low temperature exposure than for flies rapidly cold-hardened to 16°C (37.6±6.0 min). However, the difference was not statistically significant (t=1.167, P=0.27).

Discussion

Rapid cold-hardening preserved reproductive behaviors that would otherwise be severely impaired by transfer to a temperature just 7°C lower than the rearing temperature. As such, this reproductive effect of RCH is evidence for its ecological relevance in terms of resetting thermal thresholds for various behaviors (Bale, 2002). RCH allows insects to court and mate at temperatures that would otherwise prohibit reproductive activities, thus increasing the range of temperatures over which mating is possible and augmenting evolutionary fitness. For insects such as *D. melanogaster* that have short adult life spans, the capacity to rapidly adjust to changes in environmental temperature may be critical for mating success as they may not have time to wait until conditions are ideal. In addition, RCH protected the fine motor function necessary for successful courtship in *D. melanogaster*. This protection probably results from the preservation of required neuronal and muscular resting potentials, neural conduction velocities and neuromuscular coordination (Kelty et al., 1996).

The adaptive significance of compensatory responses is an important issue in the evolution of physiological acclimation (Woods and Harrison, 2002). Leroi et al. (1994) formulated the beneficial acclimation hypothesis (BAH), which states "*acclimation to a particular environment gives an organism a performance advantage in that environment over another organism that has not had the opportunity to acclimate to that particular environment.*" Our results support the BAH; flies that were acclimated to 16°C had a reproductive advantage over flies that were not given the chance to acclimate to the lower temperature. Many other researchers, however, have found that the BAH is not well-supported (see references in Woods and Harrison, 2002). These studies have tended to subject organisms to chronic, relatively severe stresses, whereas the flies in our experiments had a single comparatively brief exposure to a mild temperature. The differing results of the tests of the BAH illustrate the differences between acclimation and hardening. Acclimation is commonly referred to as a longer-term process that prepares the organism for more severe conditions (Menke and Claussen, 1982; Hoffmann et al., 2003). Acclimatory processes tend not to follow the BAH since they function not to increase fitness at the acclimation

temperature but rather to increase fitness at an even lower (or higher) temperature. Hardening, on the other hand, supports the BAH because it is a shorter-term phenomenon that increases fitness during a usually less severe stress (Hoffmann et al., 2003).

When the RCH response resets the lower thermal limit for a behavioral or physiological parameter (Bale, 2002), it may come at the cost of a corresponding decrease in the upper thermal limit. As the sheepshead minnow, *Cyprinodon variegatus*, is acclimated to temperatures from 5°C to 40°C, its CT_{max} increases; concomitantly, its CT_{min} increases, so that the overall thermal range of activity remains more or less constant (Beitinger et al., 2000). In addition, Layne et al. (1987) found that crayfish acclimated to 5°C had lower CT_{min} and CT_{max} than crayfish acclimated to 25°C. However, the data of Klok and Chown (2003) indicate that CT_{min} and CT_{max} are decoupled in several species of weevils. The protective effects of RCH have been demonstrated in numerous insect species; however, few studies have investigated its potential costs. Kelty and Lee (1999) found that RCH *D. melanogaster* females possessed the same early fecundity as control flies. However, RCH resulted in shorter adult life spans in the housefly, *Musca domestica* (Coulson and Bale, 1992). We hypothesized that although RCH enabled courtship and mating performance at a lower temperature, it would come at the cost of decreased performance at higher temperatures. Although we did not observe any evidence of impaired reproductive activity at 23°C in flies that had been rapidly cold-hardened to 16°C, it is possible that the temperature range used in our experiments was too small to detect such trade-offs. Perhaps if flies had been tested at a higher temperature, e.g. 27°C, then we might have observed such costs.

Changes in the metabolic rate of ectotherms that are observed during cooling are two-fold: (1) immediate, direct Q_{10} effects of temperature on chemical reactions and (2) biological, compensatory adjustments that appear over time after the change in temperature (Bullock, 1955; Keister and Buck, 1974; Clarke, 1980). Like the compensatory adjustments of metabolic rate that occur during temperature acclimation, the protective effects of RCH, and therefore the underlying physiological mechanisms, also require time to develop (Lee et al., 1987; Coulson and Bale, 1990). We hypothesized that the metabolic rate would be elevated during RCH if energy were required for this response. We compared the metabolic rate at 16°C of flies with and without a 2-h pre-treatment at 16°C that would induce RCH and the hypothesized elevation of metabolic rate. However, no differences that would suggest biological, compensatory adjustments were found (Fig. 3), and the times required to reach a stable metabolic rate did not differ between the control and RCH groups. These results suggest that RCH is not associated with a notable increase in the metabolic rate. Our results are consistent with the findings of Misener et al. (2001), which suggest that the RCH response does not require the synthesis of a new suite of proteins. They found that the inhibition of protein synthesis by cycloheximide did not inhibit RCH. The precise physiological mechanism of RCH remains unknown, although these results suggest that it does not require a substantial energy input for its induction or maintenance.

It is becoming increasingly evident that the RCH response represents a constant fine-tuning of the physiological function of an insect to match environmental conditions. Previously, investigators hypothesized that RCH allows an insect's overall cold tolerance to track rapid environmental temperature shifts, especially during the spring and autumn months when diurnal temperature extremes are most dramatic (Lee et al., 1987; Coulson and Bale, 1990; Kelty and Lee, 1999). However, Kelty and Lee (2001) showed that cooling *D. melanogaster* from 23°C to 16°C induces an RCH response that increases sub-zero survival. Finally, in the present study we demonstrated that RCH preserved reproductive behaviors during a decrease of only 7°C to 16°C, which represents the highest reported temperature at which the protective effects of RCH are evident. As such, this strongly suggests that the RCH response is much more pervasive and subtle than previously thought. In addition to operating over diurnal patterns of warming and cooling (Kelty and Lee, 2001), RCH may also be induced as an insect experiences slight variations in temperature that occur during a single afternoon or while moving from sunlight to shade.

Minute organisms, including small insects, experience the external environment on a very fine scale, spatially and temporally. Because of their small size and correspondingly large surface area to volume ratio, the internal body temperature closely tracks environmental temperature. Since they lack the thermal inertia of large animals, their body temperature changes rapidly in response to small, momentary variations in the environmental temperature. For example, Willmer (1986) found that the temperature on the upper surface of a leaf can decrease from 23°C to 16°C in 2 h during a summer afternoon. In addition, temperatures just 3 cm above a leaf may be as much as 7°C higher than those at the leaf surface (Willmer, 1982). Thus, insects in nature routinely experience the temperature change used in our experiments during a summer afternoon, or even while landing on a leaf.

The RCH response probably permits small insects like *D. melanogaster* to quickly improve their behavioral performance in response to even slight changes in environmental temperature. This distinguishes it from the acclimation responses that do not necessarily benefit the organism at the acclimation temperature but instead prepare it for more severe temperatures (Hoffmann et al., 2003). Consequently, the term rapid cold-hardening is, in a sense, a misnomer because it is too restrictive to encompass the wide range of temperature changes that elicit this rapid acclimation response. Insects probably rapidly cold-harden in response to thermal variations in the mid-summer that can hardly be considered 'cold' in the same way that –5°C is 'cold'. The RCH response is not a mechanism that is used only on an occasional basis, during a sudden cold snap, but rather a much more common process that occurs at relatively high temperatures and in response to slight thermal changes in active insects throughout the year. The term

rapid cold-hardening places the conceptual emphasis on the prevention of cold injury rather than on a continual fine-tuning of physiological function to match small changes in environmental temperature. Our data further support the idea that insects continuously make rapid, subtle acclimatory adjustments to very minor thermal changes (Lee et al., 1987; Kelty and Lee, 2001).

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