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Brief communication

# Rapid cold-hardening increases membrane fluidity and cold tolerance of insect cells ☆

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**Abstract**

The rapid cold-hardening (RCH) response not only confers dramatic protection against cold-shock (non-freezing) injury, but also "instantaneously" enhances organismal performance. Since cold-shock injury is associated with damage to the cell membrane, we investigated the relationship between RCH and changes in cold tolerance and membrane fluidity at the cellular level. None of the adult flies (*Sarcophaga bullata*) in the cold-shocked treatment group survived direct transfer to −8 °C for 2 h; in contrast, 64.5% of flies in the RCH group survived exposure to −8 °C. Differences between the treatment groups also were reflected at the cellular level; only 21.3% of fat body cells in the cold-shocked group survived compared to 68.5% in the RCH group. Using $^{31}$P solid-state NMR spectroscopy, we determined that membrane fluidity increased concurrently with rapid cold-hardening of fat body cells. This result suggests that membrane characteristics may be modified very rapidly to protect cells against cold-shock injury.


*Keywords:* NMR; Membrane fluidity; Fat body; Rapid cold-hardening; Cold tolerance; *Sarcophaga bullata*

Many insects and other ectotherms in temperate regions increase their cold tolerance seasonally, allowing them to survive severe winter conditions. Although less studied, insects also have the capacity to acclimate rapidly to environmental cooling at other times of the year. The rapid cold-hardening (RCH) response is among the fastest, low-temperature responses ever reported [4]. The capacity for RCH is found in numerous arthropod orders [2].

Ecologically, the RCH response functions to allow insects to enhance "instantaneously" their cold tolerance in a thermally variable environment, as would occur during natural diurnal cycles. For example, Shreve et al. [8] found that RCH preserves normal reproductive behaviors in adult *Drosophila melanogaster* that are rapidly cooled; RCH pairs courted and mated more frequently than those from the control group. Hence, the significance of this rapid acclimatory response is not restricted simply to increasing cold survival, but also functions more generally to enhance organismal performance at multiple levels in response to environmental cooling [8].

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Despite the generality of this response, the nature of the underlying physiological mechanisms remains poorly understood. In many organisms, low-temperature acclimation over extended periods (weeks to months) frequently results in the modification of membrane lipids, often by the desaturation of fatty acids [5]. However, little is known about how rapidly these changes can occur. Could they happen within hours—the time course over which RCH occurs? Since cold-shock injury has been long associated with damage to the cell membrane, we decided to determine whether membrane properties changed during RCH in insect fat body. We selected fat body as our model because this tissue plays a central role in lipid, protein, and carbohydrate metabolism that is comparable to the function of mammalian liver [10]. In the present study, we investigated the relationship between RCH and changes in organismal and cellular cold tolerance and membrane fluidity in adults of the flesh fly, *Sarcophaga bullata*. Specifically, we tested the hypothesis that RCH increases membrane fluidity using solid-state NMR spectroscopy.

## Organismal cold tolerance

*Sarcophaga bullata* (Diptera, Tephritidae) were kindly provided by David Denlinger and reared on beef liver at 23 °C as previously described [4]. The species overwinters as a diapausing pupae. All experiments used 6- to 8-day-old adults. Flies were divided into three groups: the control (exposed to 0 °C for 2 h), the cold-shocked (directly transferred to −8 °C for 2 h), and the rapidly cold-hardened (RCH, exposing to 0 °C for 2 h prior to a 2-h exposure to −8 °C). For each treatment group, adults were placed in 15 ml glass test tubes (9 replicates of 10 flies per tube). After cold treatments, the percentage of surviving flies was determined after 2 h of recovery at room temperature; flies able to stand were judged to have survived. Flies transferred to −8 °C for 2 h do not freeze but remain supercooled as determined previously [4]. All flies survived 0 °C for 2 h (Fig. 1). When flies were directly transferred to −8 °C for 2 h, none survived, due to cold-shock injury; although some adults could slightly move their mouthparts or legs in an uncoordinated manner, none could stand normally. In contrast, the RCH group that was rapidly cold-hardened at 0 °C for 2 h had a significantly higher survival rate of 64.5% ($P < 0.01$, ANOVA Bonferroni–Dunn test) (Statview from SAS Institute) at −8 °C compared to the cold-shocked treatment group (Fig. 1). After 2 h of recovery at 23 °C, all surviving flies in the RCH group could right themselves and walk; furthermore, when these flies were released into a cage most flew upward and clung to the top of the cage.

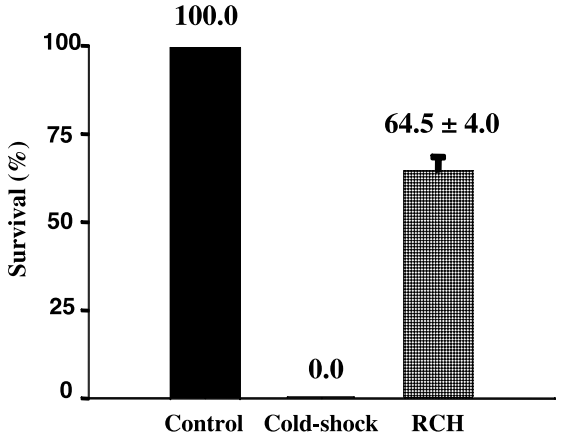


Fig. 1. The survival rate (%) of adult flies in each of the three treatment groups: control (black bar), cold-shocked (white bar), and rapidly cold-hardened (checked bar) groups. Data are expressed as means ± SEM.

## Viability of fat body cells

In a separate experiment, adults were divided into four groups (the three described above plus an additional untreated control group held at 25 °C). Following each treatment, fat bodies were dissected in Coast's solution immediately before cell viability was assessed using fluorescent vital dyes provided in a LIVE/DEAD sperm viability kit (Molecular Probes, Inc., Eugene, OR) as modified by Yi and Lee [10]. For each replicate, 10 microscope fields of 100 cells were counted. Means were based on the average value for each of three replicates and compared using ANOVA Bonferroni–Dunn test (Statview from SAS Institute) with statistical significance set at $P < 0.01$. Cell viability did not differ between the 25 °C control group or the 0 °C—2-h control group (Figs. 2 and 3). However, 78.7% of fat body cells from cold-shocked flies (directly transferred to −8 °C) were killed (Figs. 2B and 3). In contrast, the RCH treatment significantly ($P < 0.001$) improved the rate of cell survival by 47.2% compared to the cold-shocked group.

## Membrane analysis of fat body cells using solid-state NMR spectroscopy

Fat bodies were dissected from untreated control and RCH (2 h at 0 °C) acclimated flies in insect

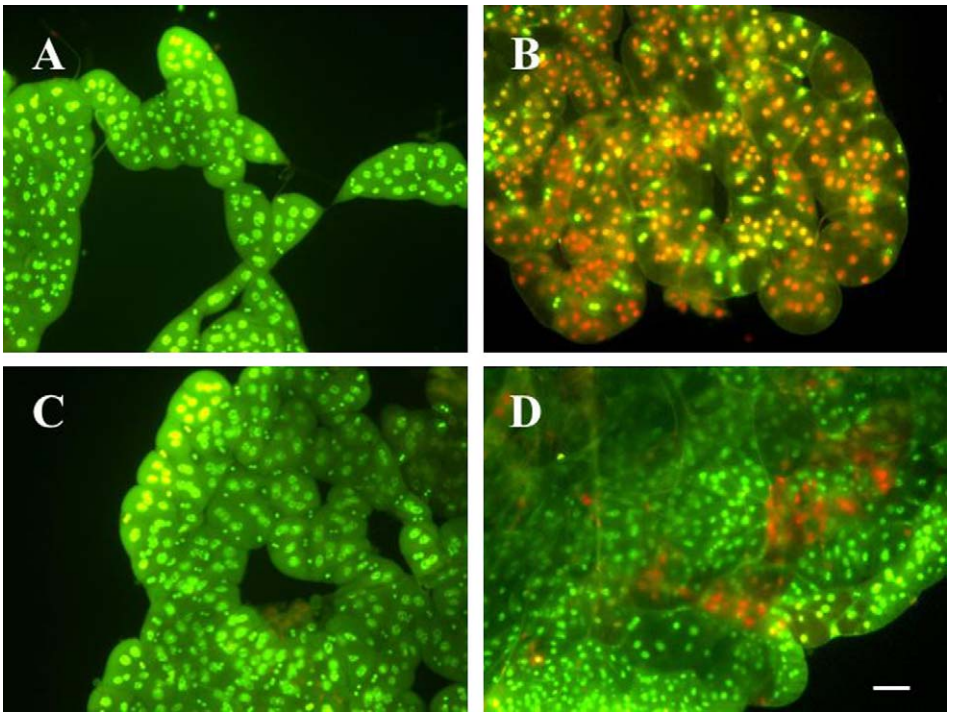


Fig. 2. Representative fluorescent images showing the effects on fat body cell viability from flies that had been control, cold-shock or rapidly cold-hardening. (A) Untreated control at 25 °C; (B) cold-shocked group at −8 °C, 2 h; (C) 0 °C—2-h control; (D) rapidly cold-hardened group at 0 °C, 2 h followed by −8 °C, 2 h. Green, live cells; red-orange, dead or injured cells. Scale bar = 25 μm.

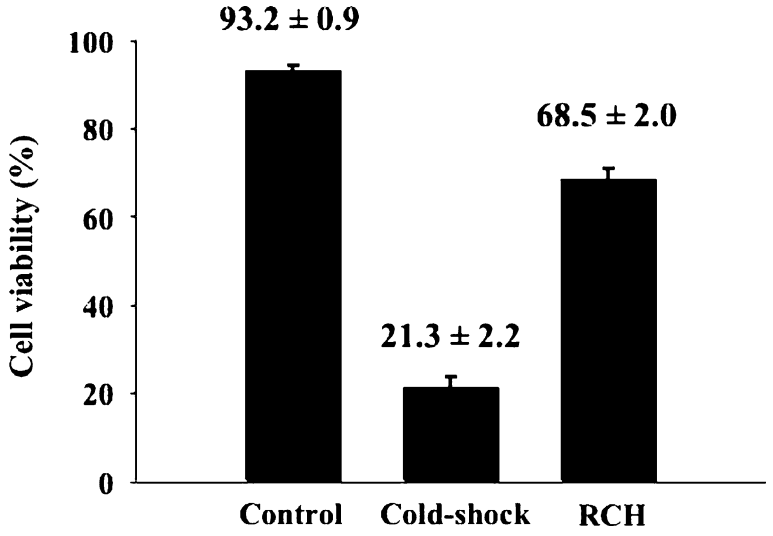


Fig. 3. Fat body cell viability (%) from flies in the control, cold-shock or rapidly cold-hardened treatment groups as described in Fig. 2. Data are expressed as means ± SEM. Data for the 25 °C untreated control group and the 0 °C—2-h control group were combined because the survival rates were not significantly different between these treatments.

Ringer's solution containing 187 mM NaCl, 21 mM KCl, 7 mM $CaCl_2$, and 1 mM $MgCl_2$. After dissection, the fat body cells were pooled and placed into a 4 mm rotor for immediate NMR analysis (Fig. 4). The $^{31}P$ solid-state NMR spectra were recorded on a Bruker AVANCE 500 MHz WB solid-state NMR spectrometer utilizing a triple resonance 4 mm CP-MAS probe. $^{31}P$ NMR data were collected at 202.4 MHz with high power $^{1}H$ decoupling using a 4 ms p/2 pulse for $^{31}P$ and a 5 s recycle delay. The

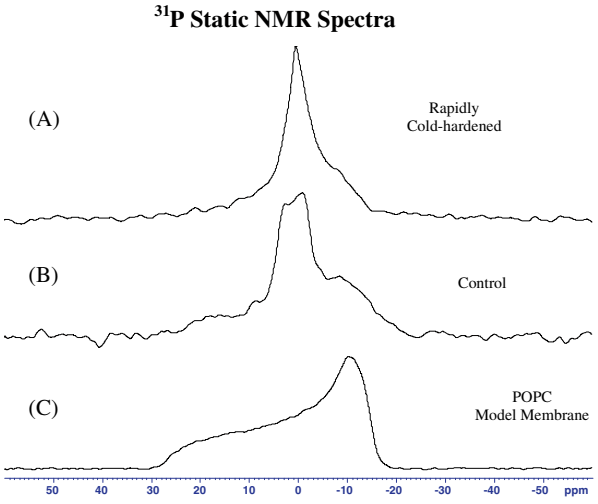


Fig. 4. Comparison of $^{31}$P static NMR spectra for fat body cells from flesh fly adults, (A) rapidly cold-hardened and (B) control. For reference, a $^{31}$P NMR spectrum from a POPC model membrane is included (C).

spectral width was set at 300 ppm. The free induction decay was processed using 100 Hz of line broadening.

The $^{31}$P solid-state NMR spectrum of a model membrane system containing 1-palmitoyl-2-oleoyl-*sn*-glycero-phosphocholine (POPC) in the liquid-crystalline phase at 25 °C is shown in Fig. 4C [1,9], which served as an internal control for the analysis. The chemical shift anisotropy (CSA) width is 44 ppm. The CSA is measured by $\sigma_{ll} - \sigma_{\perp}$. The spectrum indicates that the POPC multi-lamellar vesicles (MLVs) form lipid bilayers in the liquid-crystalline phase ($L_{\alpha}$) and are expected for POPC at a temperature well above its chain melting transition temperature of −3 °C. The $^{31}$P NMR spectrum exhibits axially symmetry ($\eta = 0$) and indicates that the phospholipids do not form isotropic or inverse hexagonal phases with high curvatures. The $^{31}$P NMR spectra shown in Figs. 4A and B indicate that the membrane compositions are also in the $L_{\alpha}$ phase, but are more motionally averaged (e.g., more fluid), when compared to the model NMR spectrum displayed in Fig. 4C. The control fat body spectrum (Fig. 4B) consists of two different membrane components. One has a small $^{31}$P CSA width of approximately 35 ppm indicative of more motionally averaged membrane bilayers, when compared to the model in Fig. 4C. The second component is isotropic and is observed as a very fast motionally averaged component (peak centered at 0 ppm). The $^{31}$P NMR peaks for the RCH fat body spectrum (Fig. 4A) also exhibits two membrane components. The first consists of an even smaller CSA width of approximately 24 ppm, while the same isotropic component (peak centered at 0 ppm) is observed as well; taken together, these peaks indicate even greater membrane fluidity than the data shown in Fig. 4B.

The cell membrane is a primary site of chilling or cold-shock injury, which is manifested by damage to intracellular organelles and the leakage of ions and other solutes across cell membranes [2]. Injury is believed to be caused by thermotropic phase transitions in constituent lipids during rapid cooling [3]. Abrupt chilling can cause a membrane transition from the liquid-crystalline phase to the lethal gel phase.

Laboratory acclimation or field acclimatization to cold is often associated with an increase in membrane fluidity in microorganisms, plants, and ectothermic animals [5]. Furthermore, the increase in membrane fluidity is directly associated with increased cell survival at low temperature. Low-temperature acclimation commonly remodels membrane lipids by increasing proportions of *cis* unsaturated and long-chain polyunsaturated fatty acids and by increasing the ratio of phosphatidylethanolamine to phosphatidylcholine [5].

Evidence from the literature on the cryopreservation of mammalian sperm and other cells also indicates that membrane fluidity is a key factor in susceptibility to cold-shock injury [3]. The membrane phase transition temperature in shrimp sperm, which are highly resistant to cold shock, is lower than that of chilling-intolerant pig sperm [3]. Interestingly, human sperm were most resistant to cold shock and did not exhibit a marked lipid phase transition as did the shrimp and pig sperm [3].

In the present study, we hypothesized that RCH increased membrane fluidity in fat body cells. Flies in the RCH group were more tolerant of subzero temperature than unhardened flies in the control group (Fig. 1). Correspondingly, fat body cells from RCH flies were significantly more cold-tolerant than ones from control flies (Figs. 2 and 3).

The $^{31}$P CSA of POPC MLVs was 44 ppm at room temperature in the in $L_{\alpha}$ phase (Fig. 4C). For fat body from control flies, the $^{31}$P CSA was equal to 35 ppm along with the appearance of a mobile isotropic component. However, for RCH sample the $^{31}$P CSA was reduced to 24 ppm and the isotropic component was larger. The $^{31}$P NMR lineshape and CSA width can provide unique information about the formation and type of phospholipid bilayers and their state of membrane fluidity. For example, both solid-state NMR

and EPR spectroscopic techniques have indicated that the addition of cholesterol to lipid bilayers decreases membrane fluidity [6,9]. Clearly, the shorter $^{31}P$ CSA and increased isotropic component in the solid-state NMR data in Fig. 4 indicate that the membrane composition of flies in the RCH group is more fluid than that of the control group. Also, we were able to identify the chemical composition of the lipids, including chemical shifts in the carbonyl and acyl C=C groups of the membrane phospholipids, using CPMAS solid-state NMR spectroscopy (data not shown).

To our knowledge our study is the first to measure directly an increase in membrane fluidity associated with the RCH response. Our data are consistent with a recent report by Overgaard et al. [7] who found slight, but statistically significant, increases in unsaturated phospholipid fatty acids of cell membranes in *Drosophila melanogaster* during RCH. Since cold-shock injury is commonly believed to be associated with cell membrane damage, this result suggests that membrane modification may play a vital role in protecting against cold-shock injury.

## References

[1] P.C. Dave, E.K. Tiburu, N.A. Nusair, G.A. Lorigan, Solid State, Nucl. Magn. Reson. 24 (2003) 137–149.

[2] D.L. Denlinger, R.E. Lee, Physiology of cold sensitivity, in: G.J. Hallman, D.L. Denlinger (Eds.), Temperature Sensitivity in Insects and Application in Integrated Pest Management, Westview Press, Boulder, 1998, pp. 55–95.

[3] E.Z. Drobnis, L.M. Crowe, T. Berger, T.J. Anchordoquy, J.W. Overstreet, J.H. Crowe, Cold shock damage is due to lipid phase transitions in cell membranes: a demonstration using sperm as a model, J. Exp. Zool. 265 (1993) 432–437.

[4] R.E. Lee, C.-P. Chen, D.L. Denlinger, A rapid cold-hardening process in insects, Science 238 (1987) 1415–1417.

[5] D.A. Los, N. Murata, Membrane fluidity and its roles in the perception of environmental signals, Biochim. Biophys. Acta 1666 (2004) 142–157.

[6] J.X. Lu, M.A. Caporini, G.A. Lorigan, The effects of cholesterol on magnetically aligned phospholipid bilayers: a solid-state NMR and EPR spectroscopy study, J. Magn. Reson. 168 (2004) 18–30.

[7] J. Overgaard, J.G. Sørensen, S.O. Petersen, V. Loeschcke, M. Holmstrup, Changes in membrane lipid composition following rapid cold hardening in *Drosophila melanogaster*, J. Insect Physiol. 51 (2005) 1173–1182.

[8] S.M. Shreve, J.D. Kelty, R.E. Lee, Preservation of reproductive behaviors during modest cooling: rapid cold-hardening fine-tunes organismal response, J. Exp. Biol. 207 (2004) 1797–1802.

[9] E.K. Tiburu, P.C. Dave, G.A. Lorigan, Solid-state $^{2}H$ NMR studies of the effects of cholesterol on the acyl chain dynamics of magnetically aligned phospholipid bilayers, Magn. Reson. Chem. 42 (2004) 132–138.

[10] S.-X. Yi, R.E. Lee, Detecting freeze injury and seasonal cold-hardening of cells and tissues in the gall fly larvae, *Eurosta solidaginis* (Diptera: Tephritidae) using fluorescent vital dyes, J. Insect Physiol. 49 (2003) 999–1004.