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Brief Communication

# High temperature pulses decrease indirect chilling injury and elevate ATP levels in the flesh fly, *Sarcophaga crassipalpis* ☆

Vanessa H. Dollo, Shu-Xia Yi, Richard E. Lee Jr. *

*Department of Zoology, Miami University, Oxford, OH 45056, USA*

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ABSTRACT

Indirect chilling injury commonly occurs during long-term exposure to low temperature in many organisms including insects. A previous study revealed increased rates of survival and reduced cold injury in flesh flies, *Sarcophaga crassipalpis*, that experienced an intermittent pulse of high temperature during a low-temperature regiment. We extended these studies by determining survival rates and ATP levels for flies that had undergone continuous long-term exposure at 0 °C versus those experiencing a 24-h warming pulse of either 15 or 20 °C. Survival among flies that had undergone a warming pulse was significantly greater than for flies that were maintained continuously at 0 °C. Furthermore, ATP levels of flies that had experienced a warming pulse were significantly higher than those of flies maintained at 0 °C. These data suggest that brief warming pulses during long-term cold storage allow regeneration of energy reserves that promote survival and reduce indirect chilling injury.



## Introduction

Indirect chilling injury is a widespread phenomenon that occurs in plants, insects and other organisms exposed to low temperature for extended periods on the order of days to weeks [5,10]. This type of cryoinjury is a major problem in the development of protocols for cryopreserving human tissues and organs in a chilled, but unfrozen state [6]. Commercially, indirect chilling injury is economically important because it determines the shelf life of fruits and vegetables. The development of protocols for storing biological agents, such as insects used in integrated pest management, also requires an understanding of indirect chilling injury [8].

Physiological problems associated with chilling injury stem from thermotropic damage to cell membranes resulting in metabolic imbalances and changes in membrane fluidity [5,7]. Cold-induced injuries are also associated with reduced rates of protein synthesis, production of free radicals, neuromuscular injuries, excessive thermoelastic stress, and changes in ion homeostasis and membrane potentials [4,5].

Many temperate insects experience long-term, exposure to temperatures in the range of 0–15 °C in winter or during periods of unseasonable cold [5]. In nondiapausing pharate adults of the flesh fly, *Sarcophaga crassipalpis*, continuous low-temperature exposure (0 °C) killed all flies within 25 days [1]. However, a brief warm-temperature pulse in the midst of an extended period of cold exposure increased survival to the adult stage. Chen and Denlinger [1] speculated that these brief, warm-temperature pulses allow insects to rebuild depleted energy reserves. We hypothesized that indirect chilling injury was linked to a shortage of ATP reserves and predicted that intermittent pulses of high temperature allow regeneration of energy supplies by initiating ATP synthesis. To test this hypothesis, we measured the effect of a brief warm-temperature pulse during extended low-temperature exposure on the emergence rate of adults of *S. crassipalpis* and their ATP levels.

## Warming pulse improved organismal survival

A flesh fly colony of *S. crassipalpis* (Diptera, Sarcophagidae) was reared on a liver and sugar diet at 25 °C and a long-day photoperiod (15 L:9 D), which produces nondiapausing pupae, after Chen and Denlinger [1]. The developmental status of pupae was determined by removing the anterior portion of the puparium and examining eye pigmentation. Flies that had reached the red-eye stage were considered pharate adults. Test tubes containing pharate adults were held at 0 °C for 20 d. Three groups of flies (20 replicates of 20 individuals per group) were used: a control, a 15 °C-pulse group, and a 20 °C-pulse group. The control remained at 0 °C for 20 d. The 15 °C- and 20 °C-pulse groups experienced a 24-h warming pulse on Day 10. On Day 20, all flies were transferred to an incubator at 25 °C and adult emergence was monitored during the next 4 days.

Percentage survival data were arcsine transformed prior to statistical analysis (Two-factor ANOVA, Bonferroni-Dunn test). Warm-

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* Corresponding author. Fax: +1 513 529 6900.
*E-mail address:* leere@muohio.edu (R.E. Lee).

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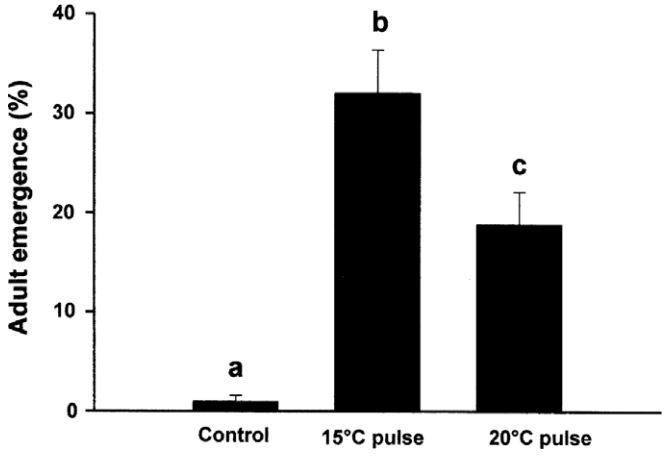


**Fig. 1.** Adult emergence following continuous exposure to 0 °C for 20 days compared to high temperature pulses for 24 h on Day 10 at either 15 or 20 °C before return to 0 °C. Each mean ± SEM is based on 20 replicates of 20 individuals per group. Different letters indicate a significant difference ($P < 0.05$) between treatment groups.

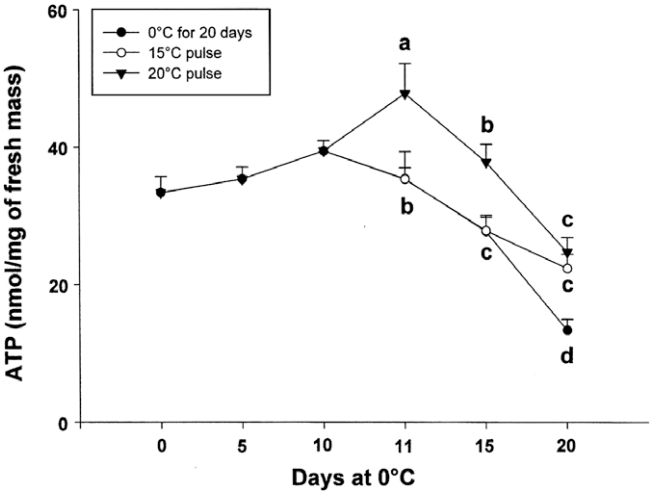


**Fig. 2.** Effect of high temperatures pulses (15 or 20 °C) on ATP levels in pharate adults. Each mean ± SEM is based on three replicates of five individuals. Different letters indicate a significant difference ($P < 0.05$) between groups.

ing pulses markedly increased the rate of fly survival (Fig. 1). Flies that underwent a 15 °C pulse experienced a higher rate of adult emergence (32 ± 4%) than those that did not (1 ± 0.6%) ($P < 0.001$). Insects that underwent a 20 °C pulse also experienced greater emergence (18 ± 3%) than those that did not ($P < 0.001$). Furthermore, flies that experienced the 15 °C pulse survived better than ones receiving a 20 °C-pulse ($P < 0.05$), which matches with a previous report for this species [1]. Our results were consistent with those obtained for other insects in which survival is improved by a brief transfer to higher temperatures during long-term, low-temperature exposure [2,5,8].

## Warming pulse elevated ATP levels

ATP was extracted according to Ebina and Ohto [3] from each treatment group on Day 0, 5, 10 (pre-24 h pulse), 11 (post-24 h pulse), 15, and 20 ($n = 3$ replicates of five individuals per sample day per group). Flies were weighed and homogenized in 5% (w/v) trichloroacetic acid in a 2-ml glass homogenizer. After a 10-min incubation period at room temperature, the homogenate was centrifuged at 14,000*g* for 20 min. Supernatants were aliquoted and stored at −20 °C. Before the ATP assay, fly ATP extracts were diluted 40-fold with 40 mM Tris–acetate buffer containing 1 mM EDTA (pH 7.8). Reagents and standard reaction solutions were prepared according to instructions provided in an ATP Determination Kit (Molecular Probes Inc., Eugene, OR). The assay reaction, ATP with firefly luciferase and its substrate luciferin, was recorded and quantified using a chemiluminometer (light emission ~560 nm at pH 7.8, Beckman-Coulter DTX 880 MultiMode Plate Reader). A standard curve was generated using a series of standard ATP concentrations. ATP values of fly samples were calculated using the standard curve as a reference. Mean ATP levels and emergence were statistically analyzed using the SAS System and SigmaPlot 9.0.

ATP levels of treatment groups differed significantly (Two-factor ANOVA, Bonferroni-Dunn test, $P < 0.05$) on Day 20 (Fig. 2). Flies that experienced a warming pulse had higher levels of ATP. Furthermore, on Days 11 and 15, 20 °C-pulse flies demonstrated significantly higher levels of ATP than insects that had undergone a lower 15 °C pulse. Similar results were obtained using 3-day-old pupae held at 0 °C for 10 days and then exposed to a 24 h, 15 °C pulse. The mean ATP level for these pupae was 27.8 ± 4.2 nmol/mg before the pulse. Following the warming pulse, significantly higher levels of ATP were observed (40.9 ± 4.0 nmol/mg). Consequently, a warming pulse significantly elevated ATP levels in both pupae and pharate adults.

Fluctuations in environmental temperature affect a wide range of physiological processes in insects, including metabolic activities, developmental rates and growth [10]. However, few studies have investigated the physiological basis for increased survival during thermal fluctuations, especially with respect to indirect chilling injury. Our results provide the first direct evidence that warming pulses reduce indirect chilling injury by allowing the restoration of ATP levels. These results are consistent with Chen and Denlinger's [1] speculation that brief exposure to elevated temperatures allow insects to re-establish energy reserves. In other mesophilic species from temperate regions low temperature inhibits ATP synthesis while warming stimulates their capacity for ATP production [9]. Transferring insects from low to higher temperatures, allows them to re-establish ion gradients [5] and up-regulate production of a variety of cellular components, including proteins involved in energy metabolism, cytoskeletal components, and protein chaperones [2].

There are several reasons why elevated ATP levels might diminish the deleterious effects of indirect chilling injury. Insects need energy reserves to maintain their metabolic processes [2]. One of the greatest energy-consuming processes is active transport of ions across cellular membranes, which controls many critical processes including cell volume regulation, nutrient uptake and membrane fluidity. Napolitano and Shain [9] showed that obligate cold-adapted species, ranging from ice worms to fungi to bacteria, maintain higher ATP levels than mesophilic species at low temperature. They conclude that elevated ATP levels are a compensatory mechanism that is critical for maintaining biochemical processes and active growth at low temperature. Consequently, by increasing ATP levels during brief warming pulses, insects may be better able to maintain critical biochemical reactions at viable rates and reduce indirect chilling injury during future low-temperature exposure.

In nature insects are exposed cycling thermal regimes rather than constant low temperatures. Protection against indirect chilling injury is likely an intrinsic process that has evolved in response to the alternating low and high temperatures experienced in the field. The cryoprotective response that we observed to a single warming pulse likely mimics similar fluctuations that occur under the field conditions [1]. By studying insects that are naturally able to protect themselves against indirect chilling injury, we can gain insight, and perhaps cues for avoiding or repairing, this type of cryoinjury in other organisms.

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