Cold shock and rapid cold-hardening of pharate adult flesh flies (Sarcophaga crassipalpis): effects on behaviour and neuromuscular function following eclosion

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Abstract. Little is known about the nature of injury due to cold shock, or its prevention by rapid cold-hardening, in insects. To understand these phenomena better at the system level, physiological and behavioural comparisons were made between control, cold shock-injured, and rapidly cold-hardened flesh flies, Sarcophaga crassipalpis Macquart (Diptera, Sarcophagidae). Cold shock impaired the proboscis extension reflex in response to 0.125, 0.5 and 1.0 M sucrose solutions. Cold shock-injured flies were unable to groom effectively and spent only 12.5% of the first 5 min following dust application producing ineffectual leg movements. In contrast, control and rapidly cold-hardened flies exhibited normal grooming behaviour spending 92.4% and 94.1% of the first 5 min following generalized dust application grooming. Cold shock also decreased the mean resting membrane potential of tergotrochanteral muscle fibres from −65.9 mV in control flies to −41.6 mV. Conduction velocities of the three motor neurone populations innervating the tergotrochanteral muscle were all significantly lower in cold-shocked flies than in control or rapidly cold-hardened flies. Finally, cold shock impaired neuromuscular transmission as evidenced by a lack of evoked end plate potentials.

Key words. Sarcophaga crassipalpis, cold shock, rapid cold-hardening, nervous system, muscle.

Introduction

Insects are susceptible to three forms of low temperature injury. Freezing injury, of particular importance to overwintering insects, results from ice formation within the body that can result in mechanical injury and osmotic stress (Meryman, 1974; Mazur, 1984; Taylor, 1987). For many insects, the ability to supercool (i.e. remain unfrozen) to temperatures well below the freezing point of their body fluids facilitates avoidance of this form of injury (Lee, 1991). For this reason, the supercooling point, the temperature at which ice crystals spontaneously begin to form, was previously thought to represent the lower lethal limit of temperature tolerance for freeze-susceptible organisms. However, more recent data indicate that some species of insects are susceptible to two forms of non-freezing low temperature injury (i.e. injury above the supercooling point) (Lee & Denlinger, 1985; Bale, 1987). One of these, termed indirect chilling injury, is caused by prolonged exposure to moderately low temperatures, near or slightly above 0°C (Lee, 1991). The other, termed cold shock, is caused by brief (as little as 30 min) exposures to more extreme, generally sub-zero temperatures (Morris & Watson, 1984; Lee, 1991; Chen & Walker, 1994).

Although observed in a variety of cell types, tissues and organisms (Morris et al., 1983; Morris & Watson, 1984; Watson & Morris, 1987), little is known about the nature of cold shock injury. At the cellular level, it is hypothesized that exposure to injurious low temperatures leads to membrane phase changes (Quinn, 1985; Drobnić et al., 1993), thermoelastic stress (McGrath, 1987), or damage to critical proteins (Zachariassen, 1985; Lee et al., 1987). At the whole organism level, cold shock may lead to the immediate death of the affected animal, or its effects may be exhibited at some later time, as for pharate adult flesh flies (Sarcophaga crassipalpis) (Lee & Denlinger, 1985; Denlinger et al., 1991; Yocum et al., 1994). For pharate adults of this fly, cold shock injury is exhibited 3 days after low temperature exposure when the adult attempts to emerge from the puparium. Recordings of the mechanical activity associated...
with the behavioural programme for forward movement and obstacle removal show that these eclosion-associated activities are impaired (Yocum et al., 1994). Because successful emergence requires the coordinated activity of the nervous and muscle systems, it was hypothesized that one or both of these systems are damaged by exposure to low temperature.

Although low temperature may prove harmful to the unprepared insect, a number of species from temperate regions are capable of avoiding cold injury by physiologically preparing for low-temperature exposure through one of two mechanisms. Cold-hardening associated with overwintering occurs over days or weeks as the organism physiologically prepares to undergo prolonged exposure to low temperature (Lee, 1991). In contrast, rapid cold-hardening is acquired during moderate low temperature exposures (0°C to 6°C) for as little as 30 min. This type of low temperature protection, described in a number of species, is a physiological response defending against cold shock injury (Chen et al., 1987; Lee et al., 1987; Czajka & Lee, 1990). Much like the injury against which it protects, little is known about the nature of rapid cold-hardening.

This study examined the effects of cold shock injury on behavioal and physiological parameters in S. crassipalpis, and the ability of rapid cold-hardening to prevent this injury. The proboscis extension reflex is a stereotyped dipteran response to the activation of chemoreceptors on the tarsi or labellum whose anatomical and physiological components are well characterized (Dethier, 1976). In the adult fly, ascending axons of labellar and tarsal chemoreceptor cells synapse with interneurones in the subesophageal ganglion which in turn control motor neurones activating muscles in the proboscis (Dethier, 1959, 1971, 1976; Sturckow, 1967; Mitchell & Itagaki, 1992). The effects of cold shock injury and rapid cold-hardening on grooming behaviour were also studied. This behaviour involves a stereotyped sequence of leg and body movements activated by cuticular mechanoreceptor excitation (Dawkins & Dawkins, 1976).

In this study, physiological tests compared parameters from a component of the flight initiation system, the tergotrochanteral muscle (TTM) and its innervation. The cell bodies of the three motor neurones innervating this muscle lie in the thoracic ganglion where they receive synaptic input from, among others, the giant fibres of the cervical connective (Trimarchi & Schneiderman, 1993). The axons of the motor neurones leave the ganglion through the dorsal medial nerve (DMN) from which they bifurcate to form the tergotrochanteral muscle nerve (TTMn) supplying the TTM (Schouest et al., 1986).

Materials and Methods

Experimental Design. All experiments (behavioural and physiological) were carried out on male and female Sarcophaga crassipalpis Macquart (Diptera, Sarcophagidae) within 2 days following adult emergence. The effects of cold shock injury and rapid cold-hardening were determined by dividing the animals into three treatment groups for each experiment. These treatment groups were: (1) control (untreated flies), (2) cold shock injured (exposed to −10°C for 1 h with no prior low temperature exposure), and (3) rapidly cold-hardened (exposed to −10°C for 1 h following a 2 h exposure to 0°C). All low temperature exposures were performed 3–4 days prior to the expected date of eclosion from the puparium.

Insect rearing. Non-diapausing S. crassipalpis were reared according to the methods outlined by Lee & Denlinger (1985). To avoid diapause induction and associated cold-hardening, animals were maintained at 25°C under a long-day photoperiod (LD 15:9h). Sugar and water were provided ad libitum. During the first 6 days post-emergence, flies were provided with beef liver as a protein source to allow normal oogenesis and embryonic development. On the eleventh day following emergence, a 50 g packet of liver was provided as a substrate for larviposition. On the day following larviposition, larvae were transferred to fresh packets of liver at a density of approximately one larva per g of liver. These packets were placed in plastic tubs lined with a 2 cm layer of sawdust into which the larvae were allowed to wander and pupate. Following pupariation, puparia were sifted from the sawdust and stored in Petri dishes until adult emergence.

Behavioural assessments. The method used to assess the proboscis extension reflex was a modified form of that described by Dethier (1976). Within 12 h following emergence or manual extraction, S. crassipalpis were affixed to wooden toothpicks with a drop of hot melt glue placed on their scutellum. Care was taken to leave all other parts of the fly's body free from the adhesive. Following attachment, flies were allowed to recover in an upright position for 30 min at 25°C.

Flies (n = 27) from each of the treatment groups were subsequently tested, at 25°C, by applying small drops of sucrose solutions to the prothoracic tarsi and labellum via a capillary tube attached to a plastic syringe. To ensure that the observed responses were not being elicited by the activation of water receptors, each fly was first tested with distilled water. If a fly responded with a proboscis extension, it was allowed to drink to satiety. Subsequently, all flies were tested with increasingly concentrated sucrose solutions (0.125, 0.5, 1.0%). Between each test the tarsi and proboscis of each fly were rinsed with distilled water to remove residual sucrose.

Each fly's performance of the proboscis extension reflex was rated as not extending, partially extending, or fully extending. Flies could have exhibited partial extension in one of two ways: by extending the rostrum while the haustellum and labellum remain flexed, or by extending the labellum while the remainder of the proboscis remained flexed.

Within 12 h following emergence or manual extraction, the ability of flies from the three groups to perform normal grooming was assessed following a modified form of the procedure outlined by Phillips et al. (1993). Under ambient laboratory conditions (c. 22°C, 60% relative humidity), three sets of ten flies, one from each of the three treatment groups, were placed in 50 ml plastic centrifuge tubes containing 5 mg of dust (Reactive Yellow 86, Sigma Chemical Co.) and gently shaken for 10 s. Excess dust was removed from the flies by covering the tube with a screen and tapping it into a Petri dish. Each group was divided in half and each subgroup was placed in a clean, ventilated, grooming chamber also constructed from a 50 ml plastic centrifuge tube.

During the first 5 min following dust application, the time each fly spent grooming, or attempting to groom, was recorded. At 2 and 8 h following dust application, the flies were scored for

success in grooming. Success was defined as the percentage of body parts cleared relative to control flies.

**Electrophysiology.** Prior to obtaining physiological measurements, flies were immobilized on ice, pinned left side down, and the TTM, TTMs, and thoracic ganglia exposed. Dissections consisted of removing the right lateral thoracic cuticle and other overlying tissue. To minimize the effects of anoxia, care was taken to keep the spiracles on the contralateral side of the thorax dry and unobstructed. If resting membrane potential or conduction velocity were to be measured, the thoracic cavity was filled with *Sarcophaga* ringleans solution (Mitchell & Itagaki, 1992: pH 6.9). During measurement of neuromuscular latency, calcium-free ringer solution was used to reduce muscle contraction and body movement. This solution had the same composition as normal saline except that calcium was replaced by magnesium.

Intracellular recordings from single TTM fibres were obtained using 3 M KCl-filled glass microelectrodes (resistance 15–30 MΩ). Intracellular recordings were always made from the central portion of the muscle. Resting membrane potentials were recorded as the voltage change observed on the oscilloscope (Tektronix) upon electrode withdrawal from stable fibres.

Paired extracellular recordings were used to calculate the conduction velocity of motor neurones in the TTM. One suction electrode was placed at the base of the DMN as it emerged from the thoracic ganglion. The second electrode was placed on the finer and more delicate TTM distal to its bifurcation from the DMN. Action potentials in the TTMs of control or rapidly cold-hardened *S. rassipalis* were elicited by tactile stimulation of hairs on the abdomen, or deflection of the ipsilateral antenna with an eyelash brush. Because this method of stimulation was ineffective in cold-shock injured flies, action potentials from this treatment group were elicited by gently pressing on the abdomen or head with a wooden applicator stick. These signals were amplified by an AC amplifier (AM Systems), visualized on an oscilloscope, and recorded for later analysis on a computer (Apple Macintosh Powerbook 160) via a MacLab data acquisition system (AD Instruments). Action potentials common to recordings from both electrodes were interpreted as activity of the TTM motor neurones and used to calculate conduction velocity.

Measurement of neuromuscular latency combined the use of extracellular and intracellular recording techniques. Activity of the TTMs motor neurones was recorded extracellularly with a suction electrode placed at the base of the nerve following its emergence from the DMN. The occurrence of end plate potentials in fibres of the TTM was recorded using glass microelectrodes as described above. Attempts were made to place the extracellular and intracellular electrodes at equivalent intervals in all animals.

**Results.**

**Behavioural assessments.**

The proboscis extension reflex was severely impaired in cold shocked flies (Table 1). Even when the most concentrated sucrose solution (1 M) was applied to the proboscis, only five of twenty-seven flies in the cold-shock treatment group responded with a complete extension whereas all control flies exhibited a complete reflex (*P* < 0.0001, Chi-square test). However, 67% of cold-shock injured flies responded to this treatment with a partial proboscis extension. Even if flies were simply rated either as responding (i.e. partially or fully extending) or not responding to sucrose, cold-shocked flies still responded significantly less than control or rapidly cold-hardened flies (*P* < 0.001, Chi-square test). All partial proboscis extensions in this experiment were the result of rostrum movements produced while the remaining segments were kept flexed. Similar results were obtained in response to tarsal sucrose application. Although the average level of response decreased as the concentration of the sucrose solution decreased, it did so to a much greater extent for cold-shocked flies than for control or rapidly cold-hardened flies (Table 1).

Control and rapidly cold-hardened flies spent 92.4 ± 5.1% and 94.1 ± 2.3% respectively of the first 5 min following dust application grooming. In contrast, injured flies spent only 12.4 ± 8.3% of this period attempting to groom (i.e. exhibiting elevated levels of leg and body movement that were ineffectual in removing dust). 8 h after application of dust, cold-shocked flies had not removed any dust from their bodies; in contrast, control and rapidly cold-hardened flies had cleaned more than 60% of their body parts.

**Table 1.** Percentage of flies exhibiting one of three levels of response to proboscs or tarsal application of various sucrose solutions. For both tarsal and proboscs application, the response of cold-shocked flies was significantly impaired when compared to control or rapidly cold-hardened flies (for each of the six treatment/sucrose concentration groups: Chi-square tests produced *P* < 0.001). In each case the sample size was twenty-seven flies.

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<th>Treatment</th>
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<tr>
<td>Tarsal application 1.0 M</td>
<td>Control</td>
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Electrophysiology

Three physiological comparisons were made between the treatment groups: (1) resting membrane potential of the TTM, (2) conduction velocity of the three motor axons in the TTMs, identified as slow, medium and fast with respect to their ability to propagate action potentials, and (3) the latency between the firing of these axons and intracellular recording of end plate potentials in the TTM.

The mean resting membrane potential (−41.6 ± 15.7 mV) of TTM fibres in cold-shock injured S. crassipalpis was significantly less negative than that of control (−65.9 ± 3.6 mV, P = 0.0275) or rapidly cold-hardened flies (−62.3 ± 6.3 mV, P = 0.0469) (Fig. 1). Although the mean value for cold-shock injured flies was low, a number of fibres were found to retain resting potentials similar to those of control fibres.

Cold shock was also associated with a significant decrease in the conduction velocities of the three motor neurones of the TTMs relative to controls (slow: P = 0.0313, medium: P = 0.011, fast: P < 0.01; unpaired t-tests). These decreases were prevented by rapid cold-hardening. Although conduction velocities for TTM motor neurones of S. crassipalpis in this treatment group were not significantly different than those of controls, all three exhibited significantly higher values than their cold-shock injured counterparts (slow: P = 0.0023, medium: P = 0.0054, fast: P = 0.023; unpaired t-tests) (Fig. 2).

Although it was possible to elicit neuromuscular activity in cold-shock injured flies, these animals seemed to require greater stimulation than did control or rapidly cold-hardened flies. Physiological responses of control and cold-hardened flies could be elicited by lightly brushing an antenna or abdominal bristles with an eyelash brush. In contrast, such responses could only be elicited from cold-shocked flies by slightly compressing the animal’s head or abdomen with the tip of a wooden applicator stick.

End plate potentials were readily obtained from the TTMs of control and rapidly cold-hardened flies. These flies exhibited similar neuromuscular latencies ranging from 0.8 to 1.4 ms. In contrast, neuromuscular transmission was impaired in cold-shocked flies, in which end plate potentials were only occasionally observed in the TTM when hyperpolarizing current was passed into its fibres.

Fig. 1. Effects of cold shock and rapid cold-hardening on the mean resting membrane potential of tergotrochanteral muscle fibres in Sarcophaga crassipalpis. Rapid cold-hardening prevented the significant decrease in resting membrane potential associated with cold shock (*P < 0.05, unpaired t-test). Each value represents the average of ten fibres from each of ten flies ± standard error of the mean.

Fig. 2. Effects of cold shock and rapid cold-hardening on conduction velocities of the three motor axons (slow, medium, and fast) innervating the tergotrochanteral muscle of Sarcophaga crassipalpis. For each motor neurone, cold shock was associated with a significant decrease in mean conduction velocity (*P < 0.05, unpaired t-test) which was prevented by rapid cold-hardening.

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Discussion

As found in previous studies (Lee & Denlinger, 1985; Yocum et al., 1994), the effects of cold shock of pharate adult S. cressidapalis were manifested as an inability of the adults to emerge during eclosion. When extricated manually, cold-shocked flies exhibited impaired proboscis extension reflexes and grooming behaviours. Physiological examination of these flies indicated that cold-shock damage affected the resting membrane potential of TTM fibres, neural conduction and neuromuscular transmission. Because the physiological data were obtained from the portion of the neuromuscular system controlling the jump response, such damage could not be directly correlated with the observed behavioural alterations. However, the behavioural changes that were observed seemed to be the result of neuromuscular damage. Taken as a whole, these results indicate that the neuromuscular system is a primary site of cold-shock injury.

Injured flies rarely exhibited a complete proboscis extension reflex, even when tested with labelled application of a 1.0 M sucrose solution. Rather, most individuals would extend their rostrum while their haustellum and labellum remained flexed. This type of response was reported by Dethier (1976) for flies responding to a weak stimulus. Thus, cold shock may increase the threshold for activation of the reflex, as would occur if the chemosensory cells within hairs on the labellum and tarsi were damaged. A similar effect might be observed if any component of the sensory-motor pathway was damaged. However, if cold shock damaged the motor components of this reflex it is interesting that it would only affect those components controlling the haustellum and labellum, but not the rostrum.

Grooming ability was also compromised by cold shock. Although cold-shocked flies responded to dust application with brief periods of supra-baseline levels of limb twitching, their ability to produce complete leg sweeps was impaired. As was the case for the proboscis extension reflex, this alteration could have been a result of damage to the sensory apparatus responsible for initiation of grooming. If so, cold shock may have caused an increase in the behavioural threshold of activation for the complete reflex. However, as with the proboscis extension reflex, it is also possible that the nerve or muscle components of the motor system underlying the behaviour were affected.

Physiological data were obtained supporting the hypothesis that the observed behavioural impairments were due to cold-shock damage to the neuromuscular system. The average resting membrane potential of TTM fibres was decreased by cold shock injury, as were the mean conduction velocities of the motor neurones leading to these fibres. Although a decrease in conduction velocity does not in itself indicate a specific type of cellular injury, the possibility that such a slowing was due to a decrease in the electrochemical gradient of the involved axons must be considered in light of the effects of cold shock on TTM fibres.

For both muscle and nerve, decreases in resting membrane potential could have occurred through a number of mechanisms. One possibility is that damage to the cell membrane resulting from thermoelastic stress or phase transition of membrane lipids (Quinn, 1985; McGrath, 1987; Drobnis et al., 1993) decreased net membrane resistance. This effect would have impaired or eliminated the ability of the cell to maintain a normal electrochemical gradient. Another possibility is that the electrogenic ATPases, normally responsible for maintaining this gradient, were damaged (Lehning et al., 1994), inhibiting the ability of the cell to compensate for the flow of ions across the cell membrane.

The finding that end plate potentials were often observed in control and rapidly cold-hardened but only rarely seen in cold-shock injured flies, could indicate several forms of cellular injury. However, our observation of end plate potentials indicating neurotransmitter release, suggests that most components of the synaptic vesicle cycle (Sudhoff, 1995) in the nerve terminal remained intact. It is conceivable that the decrease in elicited neuromuscular transmission was caused by some factor preventing the localized Ca" influx required for vesicle release. Such factors could include a conduction block in regions of the nerve near the nerve terminals, or damage to Ca" channels. An alternative possibility is that a form of postsynaptic injury, such as a decrease in receptor functionality or density, or even in resting membrane potential, is decreasing the muscle fibre's response to neurotransmitter.

For all behavioural and physiological parameters assessed, rapid cold-hardening prevented the damages caused by cold shock. These data provide direct evidence that the physiological processes comprising rapid cold-hardening prevent damage to specific systems. At the cellular level this form of protection may defend against various forms of damage caused by cold shock; cryoprotectants (i.e. glycerol) accumulated during cold-hardening may act to maintain the membrane lipid bilayer or preserve protein structure and function (Quinn, 1985; Zachariae, 1985; Drobnis et al., 1993).

The results of this study support the hypothesis that the neuromuscular system is of prime susceptibility to cold-shock injury. Cold shock was found to cause behavioural and physiological damage which was prevented by rapid cold-hardening. The observed physiological alterations produced by cold shock might be explained by cellular effects such as thermoelastic stress and phase transition of membrane lipids as proposed by other authors.

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