

## HEAT SHOCK-INDUCED THERMOPROTECTION OF HINDLEG MOTOR CONTROL IN THE LOCUST

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### Summary

**Functional neuromuscular connections are critical for appropriate behavioural responses, but can be negatively affected by increases in temperature. We investigated the effects of heat shock on the thermosensitivity of a neuromuscular pathway to the hindleg tibial extensor muscle of *Locusta migratoria*. We found that exposure to heat shock induced thermoprotection of both neuromuscular transmission and extensor muscle contraction by (i) increasing the upper temperature limit for failure, (ii) improving recovery following heat-induced failure and (iii) stabilizing excitatory junction potential amplitude and duration and extensor muscle contraction force at high temperatures. Furthermore, the heat-shock-induced thermoprotection of extensor muscle contraction was not attributable to a protective effect on intrinsic**

**components of muscle contraction. Finally, the use of jumping as a locomotor strategy to avoid capture, a behavioural response dependent upon functionally competent neuromuscular connections at the hindleg tibial extensor muscle, became less sensitive to temperature following heat shock. We conclude that the natural stress response of the locust stabilizes neuromuscular signalling during temperature stress, and that this can underlie a thermoprotection of muscle contraction force and thus alter the thermosensitivity of an escape behaviour critical for survival.**

Key words: insect, neuromuscular junction, temperature, thermotolerance, behaviour, *Locusta migratoria*.

### Introduction

Heat shock, an exposure to high but sublethal temperature, protects cells, tissues and organisms from a subsequent exposure to a normally lethal high-temperature stress. There is a growing consensus that the induced thermoprotection acts *via* a natural cellular stress mechanism mediated by upregulation of heat shock protein (hsp) synthesis (Kiang and Tsokos, 1998; Sharp et al., 1999) and that the hsps impart protection not only from temperature but also from many other physiological stresses (Parsell and Lindquist, 1993; Feder and Hofmann, 1999). Considering the success of heat shock (hsp70 induction) in reducing the extent of cell death as a consequence of ischaemia in experimental models of stroke (Yenari et al., 1998), heat-shock-induced stress protection may have possible therapeutic implications. Although it is well documented that the survival of nervous tissue during stress is increased by previous heat shock treatment, it is unclear whether neural signalling pathways and their resultant behavioural outputs are similarly protected during stress or whether heat shock is simply minimizing tissue damage. To determine whether neuroprotective effects were induced by heat shock in an animal model that encapsulates both signalling and behaviour, we studied the effect of heat shock on neuromuscular transmission, muscle contraction and a behavioural response

for capture avoidance in the migratory locust *Locusta migratoria*.

In their natural environment, locusts can be exposed to ambient temperatures in excess of 40 °C (Uvarov, 1966). A typical heat shock response has been previously described in locusts. Whyard et al. (1986) reported that exposure to a humid environment at 45 °C for 3 h induced thermotolerance in locusts, correlated with a strong upregulation in hsp expression. Previous research has demonstrated that heat shock protects synaptic transmission within the locust flight system by reducing the thermosensitivity of synaptic delay and excitatory postsynaptic potential (EPSP) amplitude (Dawson-Scully and Robertson, 1998). A similar preservation of signalling has also been characterized in *Drosophila melanogaster* larvae as a thermoprotection of neuromuscular excitatory junctional currents (EJCs) following prior heat shock (Karunanithi et al., 1999). If heat shock does protect neuromuscular transmission, a fundamental question is whether the effects of heat shock would still be evident downstream of transmission as a reduced thermosensitivity of (i) the output of the muscle (i.e. force of contraction) and/or (ii) a behaviour dependent upon the generation of sufficient motor force. The hindleg of the locust provides an excellent

model system for testing all components of this question. The tibial extensor muscle of the hindleg is innervated by the fast extensor tibiae (FETi) motor axon, a phasic excitor, which initiates a rapid extension of the hindleg tibia by stimulating spiking excitatory junction potentials (EJPs) in individual muscle cells (Hoyle, 1978). Extracellular stimulation of nerve 5, which contains the FETi motor axon, induces a contraction of the extensor muscle and a single rapid extension of the hindleg. Thus, the FETi model allows an investigation of heat-shock-induced effects on the thermosensitivity of neuromuscular transmission and the force of muscle contraction. Furthermore, because the hindleg extensor muscle is a critical component for jumping, walking and kicking, an investigation of the effects of heat shock on the behavioural responses of the animal can also be undertaken.

Using intracellular recordings of extensor muscle fibres in the hindleg of *Locusta migratoria* and near-isometric tension recordings of the hindleg during tibial extension, we have been able to characterize heat-shock-induced thermoprotection. Here, we describe the protective effects of heat shock on both neuromuscular transmission and the force of hindleg tibial extension and show that prior heat shock enables the proper functioning of the hindleg neuromuscular system at elevated temperatures. We also provide evidence that the use of jumping to avoid capture, an important behavioural response intrinsically dependent on functionally competent neuromuscular connections, is less sensitive to environmental temperature effects following heat shock.

## Materials and methods

### Heat shock

Mature locusts *Locusta migratoria* (at least 2 weeks past the imaginal moult) were collected from a crowded colony (25–30°C; 16h:8h light:dark photoperiod) maintained at Queen's University. Heat shock locusts were placed into a humid incubator at 45°C for 3h, which induces thermotolerance in our colony (Robertson et al., 1996). Control locusts were kept for the same period in a similar environment at room temperature (25–30°C). Experiments were performed within 1–6h of the heat shock treatment.

### Intracellular preparation

Locusts were pinned to a cork board ventral side up, and a small window was made in the ventral thoracic wall to expose the metathoracic ganglion (Hoyle and Burrows, 1973). The hindleg of the locust was held rigid within a Plasticine moulding (Fig. 1A), allowing ventral dissection of the leg and excision of the cuticle and flexor muscle (Fig. 1B). The axon of the FETi motoneuron, originating in the metathoracic ganglion and innervating the tibial extensor muscle in the locust hindleg, was stimulated with just-suprathreshold square voltage pulses (0.5 ms duration) at 1 Hz using a suction electrode placed on the severed end of nerve 5 within the thorax. Intracellular recordings were made from muscle cells innervated by the FETi motoneuron using glass

microelectrodes (filled with 2 mol l<sup>-1</sup> potassium acetate, resistance 60–80 MΩ). The hindleg was superfused with saline (147 mmol l<sup>-1</sup> NaCl, 10 mmol l<sup>-1</sup> KCl, 4 mmol l<sup>-1</sup> CaCl<sub>2</sub>, 3 mmol l<sup>-1</sup> NaOH, 10 mmol l<sup>-1</sup> Hepes buffer; pH 7.2), the temperature of which was controlled using a Nichrome heating coil around the inlet pipette leading from a reservoir maintained at room temperature. Saline temperature was monitored with a copper/constantan thermocouple (0.2 mm diameter, BAT-12, Sentsortek, Clifton, NJ, USA) located adjacent to the intracellular penetration.

### Muscle force preparation

The locust was pinned and dissected as in the intracellular experiments. The tibia of the hindleg was held perpendicular to the femur by a metal restraint. A thread tied around the tibia extended through a hole in the metal restraint and was attached to the myograph force transducer (F-2000, Narco Biosystems, Houston, TX, USA) (Fig. 1A). The myograph force transducer was moved away from the body of the animal until the thread was taut, and tibial extension force was measured nearly isometrically by the myograph transducer as tension exerted on

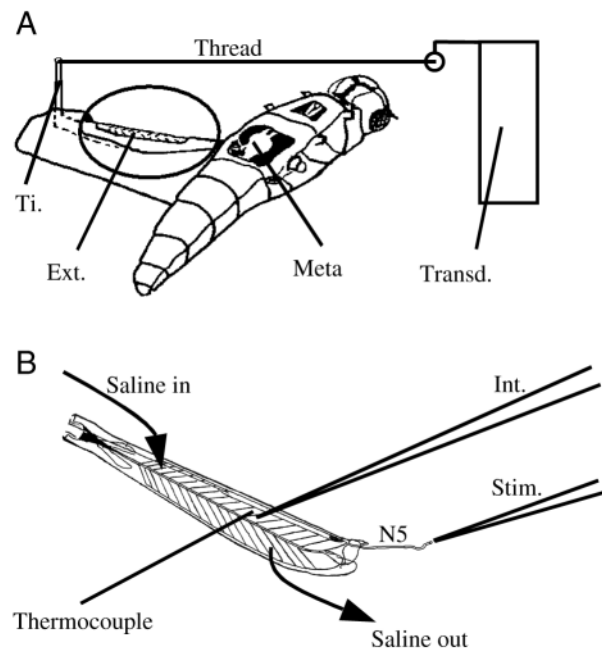


Fig. 1. Experimental arrangements. A preparation similar to that described by Hoyle and Burrows (1973) was used to expose the metathoracic ganglion. The hindleg was held rigid (see Materials and methods), and the tibia was made to extend by extracellular stimulation on the cut end of nerve 5 in the thorax. To record the force of hindleg tibial extension (A), a thread, extending from the force transducer to the tibia, was tied taut around the tibia. In force measurements and intracellular recordings (B), the hindleg was dissected and temperature-controlled saline flowed across the extensor muscle. A thermocouple was placed adjacent to the intracellular electrode to record the temperature at the neuromuscular junction. Ti., hindleg tibia; Ext., extensor muscle; Meta, metathoracic ganglion; Transd., force transducer; Int., intracellular electrode; Stim., stimulating electrode; N5, nerve 5.

the thread when the extensor muscles were stimulated to contract. The tension exerted on the thread was converted by the myograph into an electrical signal and recorded as a deflection from the baseline tension onto a physiograph (MK-III, Narco Biosystems, Houston, TX, USA). Note that, because the force of tibial extension is calculated as tension on the thread tied around the tibia, all measured forces had to be normalized to the initial force of tibial extension at room temperature for each preparation. The tibial extensor muscle fibres were stimulated to contract either (i) by extracellular stimulation of the FETi axon or (ii) by direct stimulation of the muscle to determine whether any possible effects were solely the result of the protection of neuromuscular transmission or of a further protection of intrinsic muscle contractile properties. For direct stimulation, the nerve was completely removed to prevent the stimulating current from evoking any presynaptic depolarization. Stimulation was accomplished by placing the electrode in direct electrical contact with the extensor muscle. The hindleg extensor muscle was visually inspected during the experiments, and direct stimulation appeared to evoke consistent contraction of the entire extensor muscle.

#### *Experimental procedure*

Excitatory junction potential or muscle force was recorded from each preparation with the saline inflow initially held at room temperature (approximately 22 °C). Recordings were made continuously while the temperature of the saline was gradually raised (at approximately 5 °C min<sup>-1</sup>) until stimulation failed to evoke an event. Following failure, the saline heater was immediately switched off and, because unheated saline continued to perfuse the hindleg, the preparation was quickly cooled back to room temperature. Complete cooling required approximately 1–2 min. Recording continued for 15 min post-failure to measure a time to recovery. Recovery was defined as having occurred when the recorded event became discernible again (a minimum of twice the noise level, at an appropriate latency phaselocked after the stimulus), independent of preparation temperature. It should be noted that this definition only indicates the return of a recordable event and does not take into account the functional efficacy of that event. After 15 min, if no discernible event had returned, the preparation was defined as having not recovered.

#### *Behavioural experiments*

Locusts had their wings clipped just distal to the wing hinge to prevent flight and were placed in an insulated cage (0.68 m<sup>3</sup>). The ambient cage temperature was gradually increased from room temperature (approximately 26 °C) by 6 °C increments using a space heater placed against an opening in the insulation of the cage and two heat lamps situated inside the cage. Each temperature change required approximately 5 min, and the temperature was then held constant for a minimum of 5 min. At each ambient temperature, the animal was monitored for (i) its natural movement and (ii) its behavioural response for capture avoidance. Conditions for capture avoidance were

artificially created by the experimenter loudly tapping the surface behind the animal and then quickly attempting to grasp the animal. Behavioural response was scored as jumping, walking, positional movement or no movement at all.

#### *Data analysis*

Intracellular recordings and temperature traces were digitized and recorded onto VHS videotape for analysis using Brainwave analysis software (Datawave Technologies, Longmont, CO, USA). For each animal, EJPs were analyzed for amplitude (measured from the baseline to the peak of the event), duration (event measured at half-maximal amplitude), time-to-peak (measured from the beginning to the peak of the event) and latency (measured from the stimulus artefact to the beginning of the event). The dataset consisted of 10 control and nine heat shock animals, although three animals (two control and one heat shock) were not included in EJP amplitude, time-to-peak and duration measurements because of inconsistency in the stability of intracellular penetration throughout these individual experiments. Individual EJP parameters were measured continuously over the range of temperatures, and these results were later averaged per animal in 5 °C bins, such that each animal contributed one value per temperature bin. Muscle tension was recorded directly onto a physiograph and measured by hand. For each animal, the contraction was analyzed for force (measured as the peak of physiograph deflection) and duration (event measured at half-maximal amplitude). The dataset consisted of 10 control and 10 heat shock animals (for extracellular nerve stimulation experiments) and 12 control and 12 heat shock animals (for direct muscle stimulation experiments). The force of tibial extension was also measured continuously over the temperature range, and these results were also averaged per animal in 5 °C bins. Force measurements were also made at discrete time points (every 2 min) post-failure. Behavioural experiments were conducted twice with 10 control and 10 heat shock animals (first dataset) and 16 control and 16 heat shock animals (second dataset). Significant differences ( $P < 0.05$ ) between control and heat shock animals were assessed using two-way repeated-measures analysis of variance (ANOVA) (intracellular and tension results) and *post-hoc* comparison of means using either a Tukey test or an unpaired *t*-test where applicable.

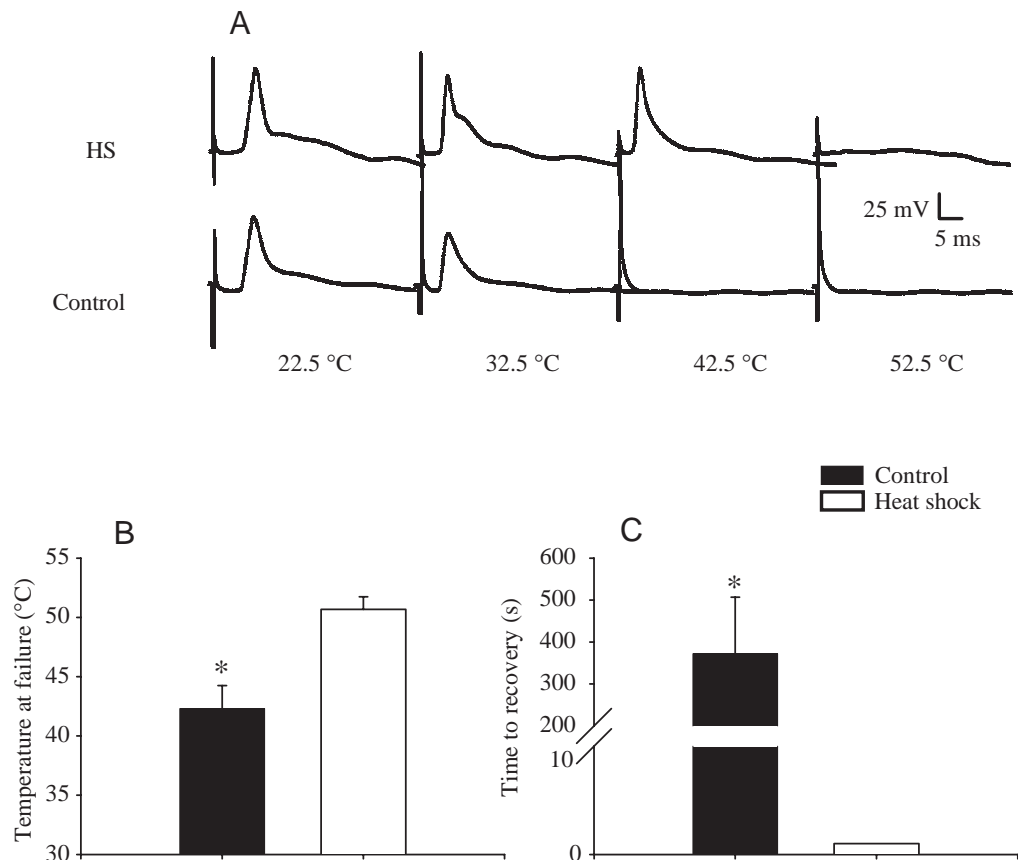
Behavioural results were assessed using contingency table analysis ( $\chi^2$ -test). All values are reported as means  $\pm$  S.E.M.

## **Results**

### *Intracellular experiments*

Excitatory junction potentials in locust hindleg extensor muscle were recorded intracellularly at various temperatures, and characteristic changes in EJP parameters were elicited in both control and heat shock animals with an increase in temperature: a decrease in temporal parameters (latency, time-to-peak, duration at half-maximal amplitude) and a decrease in amplitude (Fig. 2A). The temperature at which neuromuscular

Fig. 2. Thermoprotection of neuromuscular transmission following prior heat shock (HS). Excitatory junction potentials (EJPs) were evoked by extracellular stimulation of the fast extensor tibiae (FETi) motor neuron. (A) Representative traces of EJPs recorded from locust hindleg extensor tibia muscle fibres. Note that in this trace there is some movement artefact associated with the hindleg tibial extension which did not affect the parameters measured. (B) The temperature at which EJPs failed to be evoked is increased in heat shock animals. (C) Following heat-induced failure of neuromuscular transmission, the recovery time required before EJPs could be successfully evoked was decreased in heat shock animals. Values are means  $\pm$  S.E.M. In this and subsequent figures, significant differences between control and heat shock animals assessed using a *t*-test are denoted with an asterisk ( $P < 0.05$ ).



transmission failed was increased by 8.37 °C by prior heat shock (Fig. 2B). Failure occurred at  $50.67 \pm 1.08$  °C in heat shock animals in comparison with  $42.30 \pm 1.96$  °C in controls, and this increase was found to be significant (*t*-test,  $t = 3.63$ , *d.f.* = 17,  $P < 0.01$ ). Time to recovery following failure was decreased by heat shock (Fig. 2C). Each heat shock animal required only 1 s (with no variation at all) for resumption of neuromuscular transmission, which was a significant improvement over the value of  $372 \pm 134$  s for control animals (*t*-test:  $t = 3.82$ , *d.f.* = 12,  $P < 0.01$ ). Each heat shock preparation recovered the instant the temperature fell back below the temperature at which failure originally occurred (i.e. before the preparation had time to cool). In addition, the percentage of animals that actually demonstrated neuromuscular junction recovery was reduced in control animals (56 %) compared with those previously exposed to heat shock (100 %).

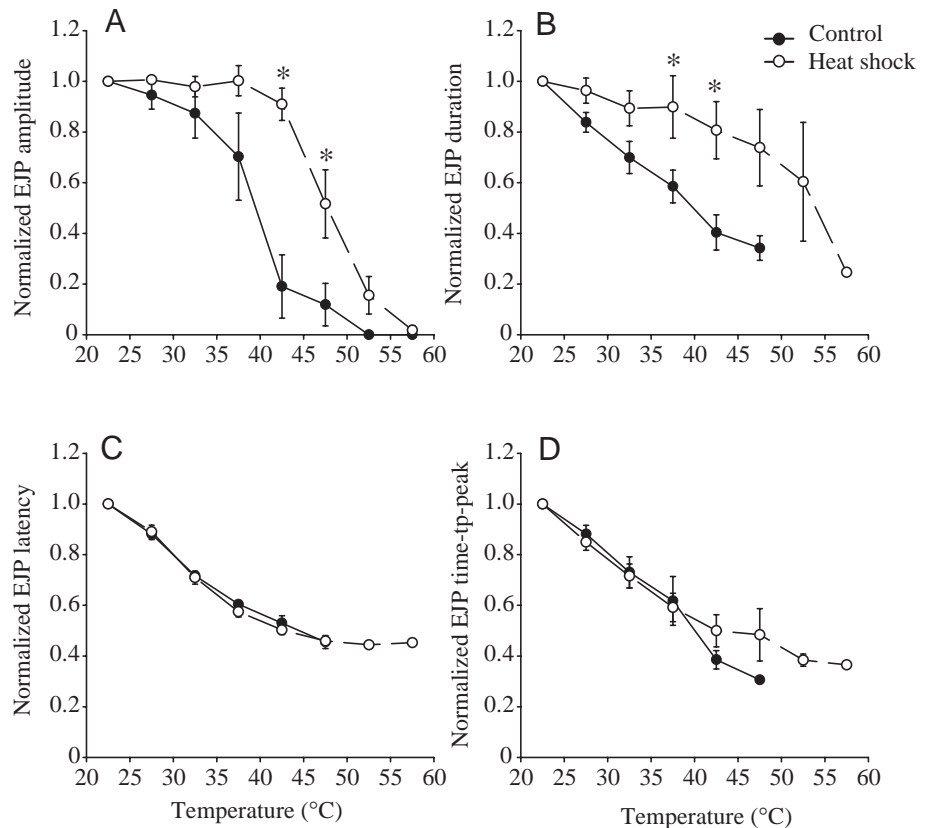
The effect of temperature on individual EJP parameters was also altered in animals subjected to prior heat shock. The amplitude of EJPs decreased less in response to a temperature increase in heat shock animals than in controls (Fig. 3A). Using a two-way repeated-measures ANOVA assessment, there was a significant effect of the heat shock treatment ( $F = 13.32$ , *d.f.* = 1,  $P < 0.01$ ) and a significant effect of temperature ( $F = 48.20$ , *d.f.* = 6,  $P < 0.01$ ). In addition, the protective effect of heat shock was shown to be potentiated at higher temperatures (two-way repeated-measures ANOVA,  $F = 5.67$ , *d.f.* = 6,  $P < 0.01$ ). The duration of the EJP also decreased less in response to an increase in temperature in heat

shock animals (Fig. 3B). There was a significant effect of the heat shock treatment (two-way repeated-measures ANOVA,  $F = 6.25$ , *d.f.* = 1,  $P = 0.03$ ) and a significant effect of temperature ( $F = 17.12$ , *d.f.* = 5,  $P < 0.01$ ). The protective effects of heat shock were also potentiated at higher temperatures for EJP duration (two-way repeated-measures ANOVA,  $F = 3.71$ , *d.f.* = 5,  $P < 0.01$ ). Although EJP latency and time-to-peak were strongly decreased by a temperature increase in both control and heat shock animals, there were no statistical differences between the two experimental groups (Fig. 3C,D).

#### Muscle force experiments

As force of muscle contraction is dependent on EJP amplitude and duration, we next investigated whether the protection evident in intracellular events also protected the output of the muscle. The force of locust hindleg tibial extension was recorded at various temperatures, and the expected changes in tibial extension force were found in both control and heat-shock-exposed animals with an increase in temperature: a decrease in the force of extension and in all temporal parameters (Fig. 4A). Under nerve-stimulated conditions, the temperature at which failure occurred was increased 5 °C by heat shock (Fig. 4B), failure occurring at  $57.40 \pm 0.95$  °C in heat shock animals, but at only  $52.20 \pm 1.05$  °C in controls. However, under direct-stimulated conditions, the temperature at which failure occurred differed by only 2 °C between control ( $56.20 \pm 0.83$  °C) and heat shock ( $58.30 \pm 0.65$  °C) preparations. Using a one-way ANOVA

Fig. 3. Thermoprotection of excitatory junction potential (EJP) parameters following prior heat shock. (A) Normalized EJP amplitude thermosensitivity was significantly reduced in heat shock preparations. Amplitudes were normalized at each temperature to that at room temperature (approximately 22.5 °C) for each experiment. There are significant differences between individual temperature bins in the 40–45 °C range ( $t$ -test,  $t=5.12$ ,  $d.f.=14$ ,  $P=0.0002$ ) and the 45–50 °C range ( $t=2.51$ ,  $d.f.=14$ ,  $P=0.03$ ). (B) Normalized EJP duration thermosensitivity was also significantly reduced in heat shock preparations. There are significant differences between individual temperature bins in the 35–40 °C range ( $t$ -test,  $t=2.25$ ,  $d.f.=14$ ,  $P=0.04$ ) and the 40–45 °C range ( $t=2.38$ ,  $d.f.=10$ ,  $P=0.04$ ). (C) Normalized EJP latency. (D) Normalized EJP time-to-peak. No significant differences in thermosensitivity of EJP latency and time-to-peak were evident between control and heat shock animals. Values are means  $\pm$  S.E.M.



assessment, there was a significant variation in failure temperature between treatments ( $F=9.13$ ,  $d.f.=3$ ,  $P<0.01$ ). Using a *post-hoc* multiple pairwise comparison (Tukey test) of failure temperatures between treatments, nerve-stimulated controls failed at a significantly lower temperature than any other of the experimental preparations (nerve-stimulated heat shock, direct-stimulated control and direct-stimulated heat shock) and all other preparations were not significantly different from each other. Similar results were obtained when comparing recovery in all groups (Fig. 4C). One-way ANOVA showed significant variation in time to recovery between experimental preparations ( $F=7.04$ ,  $d.f.=3$ ,  $P<0.01$ ), nerve-stimulated control animals requiring a significantly longer recovery time compared with the other three groups (*post-hoc* Tukey test). The other three experimental groups were not significantly different from each other.

Prior heat shock protected the hindleg tibia during extension by reducing the decrease in force with increasing temperature under nerve-stimulated conditions (Fig. 5A). Assessed using two-way repeated-measures ANOVA, there was a significant effect of temperature ( $F=63.85$ ,  $d.f.=7$ ,  $P<0.01$ ) and a significant interaction between heat shock and temperature ( $F=4.99$ ,  $d.f.=7$ ,  $P<0.01$ ). Under nerve-stimulated conditions, the force of the tibial extension during recovery following heat-induced failure was increased for heat shock preparations compared with controls (Fig. 5B). There was found to be an effect of prior heat shock treatment (two-way repeated-measures ANOVA,  $F=5.31$ ,  $d.f.=1$ ,  $P=0.03$ ), a significant effect of recovery time ( $F=4.25$ ,  $d.f.=5$ ,  $P<0.01$ ) and a

significant interaction between heat shock and recovery time ( $F=3.27$ ,  $d.f.=5$ ,  $P<0.01$ ). As previously observed with failure temperature and recovery time, the effects of prior heat shock are not evident under direct-stimulated conditions. In addition, heat shock does not protect direct-stimulated hindleg tibial force from the effects of temperature (Fig. 5C) or improve recovery following heat-induced failure (Fig. 5D). The force of tibial extension under direct-stimulated conditions is only 10% of the original force after 10 min following failure, whereas under nerve-stimulated conditions, the force of tibial extension in heat shock animals recovers to 20% of the original force. Furthermore, although there was a decrease in the duration of tibial extension with increasing temperature, we found no significant difference between control or heat shock preparations, regardless of the mode of stimulation (data not shown).

#### Behavioural experiments

We investigated whether a behavioural response that is partially dependent upon the extensor muscle would also be protected from the effects of temperature by heat shock. Locusts were placed in an enclosed environment that had its ambient temperature increased. At various discrete temperatures, the animals were first tested for their natural behavioural response (Fig. 6A). Each animal was classified as exhibiting movement (jumping, walking or positional movement) or not moving at all. As ambient temperature was increased, the percentage of animals exhibiting movement increased. Using three-dimensional contingency analysis

Fig. 4. Thermoprotection of tibial extension following prior heat shock (HS). Extension was evoked indirectly either by nerve stimulation (NS) of the fast extensor tibiae (FETi) motor neuron or by direct stimulation (DS) of the extensor muscle. Note that tibial extension force was measured relative to initial force only (see Materials and methods). (A) Representative traces of the force of locust hindleg tibial extension under nerve stimulation conditions. (B) Temperature at which the hindleg tibia fails to extend upon stimulation. (C) Recovery time required before stimulation evokes tibial extension following heat-induced failure. Nerve-stimulated control preparations are impaired in both their upper temperature limit for tibial extension and their ability to recover following failure in comparison with nerve-stimulated heat shock animals and both conditions of direct-stimulated animals (control and heat shock). Values are means  $\pm$  S.E.M.

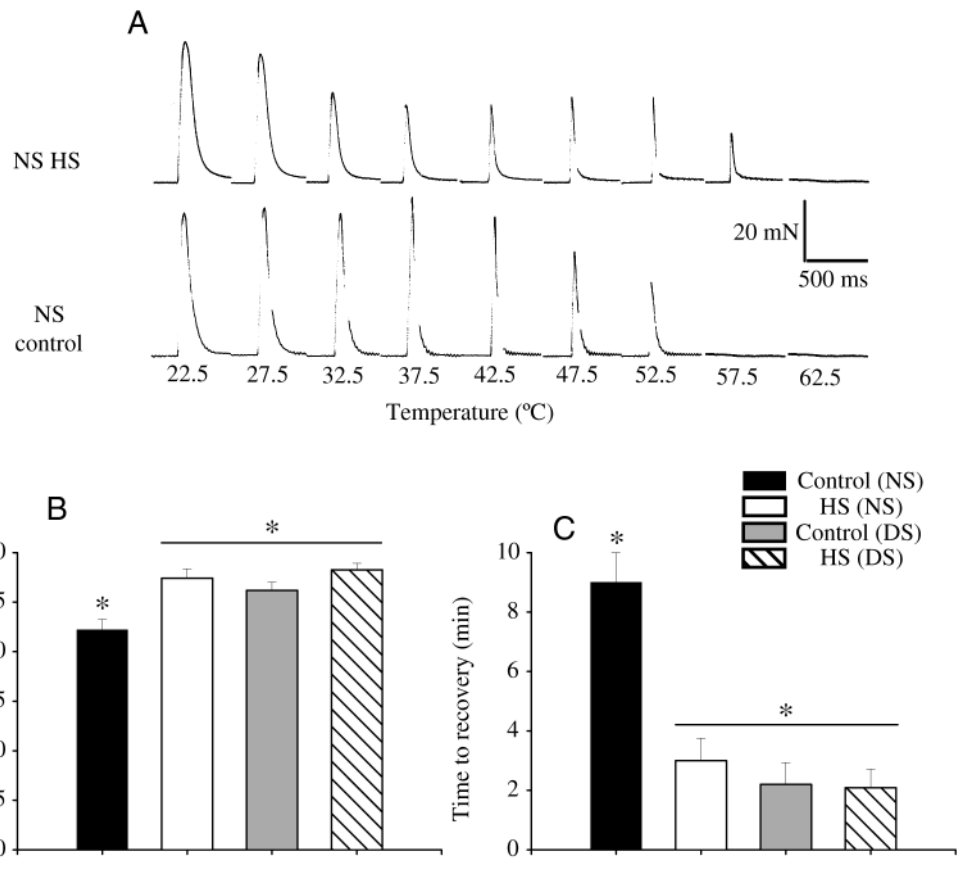
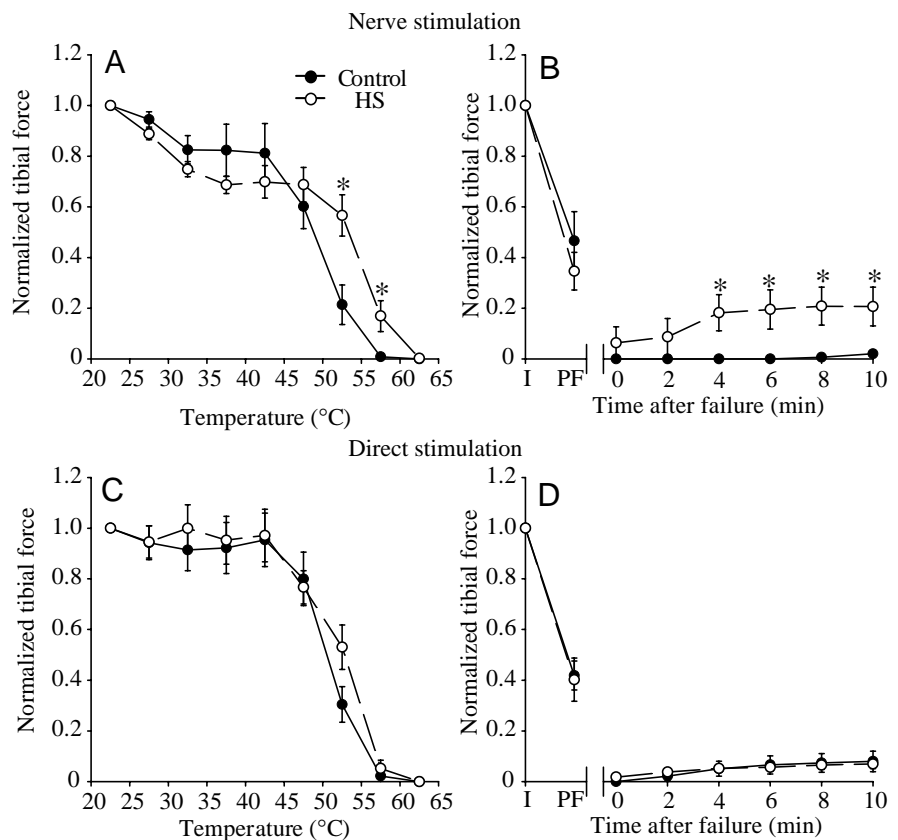


Fig. 5. Thermoprotection of force of hindleg tibial extension following prior heat shock (HS) under nerve-stimulated (NS) conditions. (A) Thermosensitivity of normalized force of tibial extension under nerve-stimulated conditions was significantly reduced in heat shock preparations. There are significant differences between individual temperature bins in the 50–55 °C range ( $t$ -test,  $t=3.12$ , d.f.=18,  $P=0.006$ ) and the 55–60 °C range ( $t=2.61$ , d.f.=18,  $P=0.02$ ). (B) The time course of recovery of normalized force of tibial extension under nerve-stimulated conditions following heat-induced failure was also significantly reduced in heat shock preparations. There are significant differences between control and heat shock preparations after a recovery period of 4 min ( $t$ -test,  $t=2.56$ , d.f.=18,  $P=0.02$ ), 6 min ( $t=2.53$ , d.f.=18,  $P=0.02$ ), 8 min ( $t=2.68$ , d.f.=18,  $P=0.02$ ) and 10 min ( $t=2.34$ , d.f.=18,  $P=0.03$ ). (C) Normalized force of tibial extension of the extensor muscle fibres under direct stimulation conditions. (D) Time course of recovery of normalized force of tibial extension under direct stimulation conditions following heat-induced failure. No significant differences between control and heat shock preparations were found for direct stimulation conditions. I, initial; PF, pre-failure. Values are means  $\pm$  S.E.M.



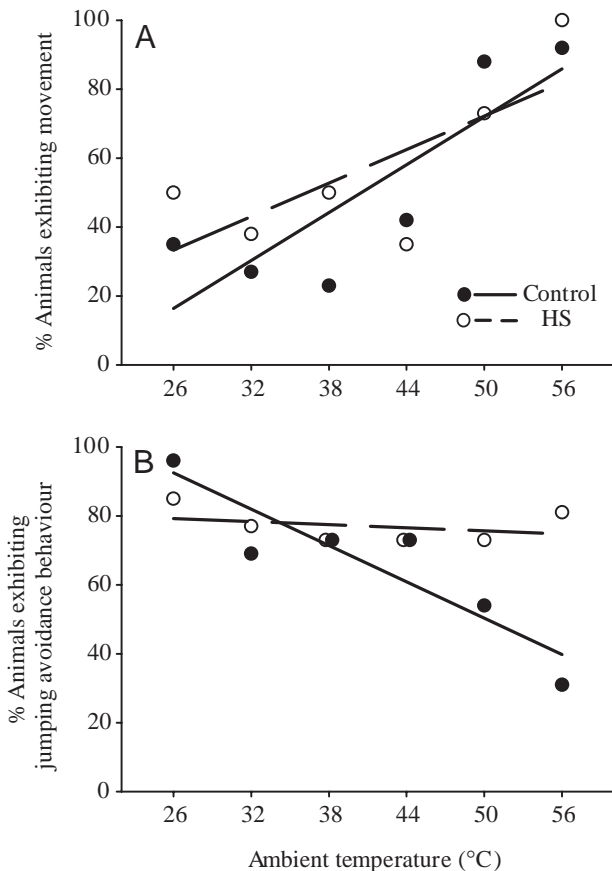


Fig. 6. Thermoprotection of escape behaviour following prior heat shock. Animals were scored at each temperature for (A) their natural propensity for movement and (B) their use of jumping to escape capture, a behavioural response dependent upon sufficient contraction strength of the extensor muscle. The propensity for movement was dependent on ambient temperature in both control and heat shock animals. However, jumping as an avoidance response was not contingent upon temperature in heat shock animals, whereas it was significantly dependent upon ambient temperature in controls. Both A and B give linear regressions through the behavioural results with respect to temperature for control and heat shock animals.

(Sokal and Rohlf, 1981), we found an effect of one of the experimental variables, heat shock or temperature, on the natural propensity for movement of a locust ( $\chi^2=93.62$ , d.f.=10,  $P<0.01$ ). This allowed us to deconstruct into two separate  $\chi^2$ -tests. These tests indicated that the increase in movement was contingent upon the temperature increase in both heat shock ( $\chi^2=35.39$ , d.f.=10,  $P<0.01$ ) and control ( $\chi^2=57.24$ , d.f.=10,  $P<0.01$ ) animals. Each animal was also monitored for its behavioural response to avoid capture (Fig. 6B). Animals were classified as using jumping or walking as a locomotor strategy to escape capture. With an increase in temperature, the use of jumping as an escape response decreased for control animals, but not for heat shock locusts. Using three-dimensional contingency analysis, we found an effect of one of the experimental variables, heat shock or temperature, on the behaviour of jumping to avoid

capture ( $\chi^2=42.63$ , d.f.=10,  $P<0.01$ ), allowing us to deconstruct into two separate  $\chi^2$ -tests. These tests indicated, however, that jumping as an avoidance behaviour was not contingent upon temperature in heat shock animals ( $\chi^2=3.90$ , d.f.=10,  $P>0.90$ ), whereas it was contingent upon temperature in control animals ( $\chi^2=29.10$ , d.f.=10,  $P<0.01$ ) for which jumping as an avoidance behaviour decreased with elevated temperatures.

## Discussion

The locust hindleg and its tibial extensor muscle are critical for many behavioural responses, such as jumping, walking and kicking (Burrows, 1996). Jumping is one possible escape behaviour to avoid capture rapidly and effectively, and an impairment in extensor muscle function could pose a serious risk to the animal. Temperature is a natural environmental stress that, when increased, can negatively affect synaptic transmission and muscle contraction (Rall and Woledge, 1990; Janssen, 1992). At extremely high temperatures, transmission and contraction can fail completely, thus disrupting the behaviour of the animal. We have demonstrated that prior heat shock significantly increases the thermotolerance of neuromuscular transmission, muscle contraction and an escape behaviour in the locust. Heat shock increases the temperature at which neuromuscular transmission and nerve-evoked muscle contraction fail and decreases the recovery time for resumption of transmission and contraction following heat-induced failure. Intracellular measurements of EJPs revealed that heat shock reduced the thermosensitivity of EJP amplitude and duration, without affecting latency or time-to-peak. Heat shock also decreased the thermosensitivity of muscle contraction force. We investigated the effects of temperature on a behavioural response that is reliant on functional neuromuscular connections and found that the use of jumping as a locomotor strategy to avoid capture was less affected by a temperature increase following prior heat shock.

### *Increased stability of the neuromuscular junction*

Synaptic transmission is affected by increases in temperature, which is particularly important for poikilothermic animals that must be able to preserve adequate function during fluctuations in environmental temperature (Montgomery and MacDonald, 1990). Whereas prior exposure to heat shock is known to increase the thermotolerance of organismal survival (Parsell and Lindquist, 1993; Feder et al., 1996; Robertson et al., 1996), heat shock has also recently been shown to reduce the thermosensitivity of axonal conduction velocity (Gray and Robertson, 1998), to protect synaptic transmission during heat stress (Dawson-Scully and Robertson, 1998; Karunanithi et al., 1999) and to decrease the time to recovery for synaptic transmission following heat-induced failure (Dawson-Scully and Robertson, 1998). We show here that prior exposure to heat shock protected the neuromuscular pathway in the locust hindleg by altering the thermosensitivity of neuromuscular transmission (i) by increasing the upper temperature limit for

transmission (Fig. 2B), (ii) by decreasing the recovery time following heat-induced failure of transmission (Fig. 2C) and (iii) by reducing the thermosensitivity of EJP amplitude and duration (Fig. 3A,B). Because heat shock had no noticeable effect on muscle contraction under direct-stimulated conditions, we believe heat shock is acting solely at the synapse and having no effect on excitation–contraction coupling and/or contraction itself within the locust tibial extensor muscle. Thus, the stabilization of the neuromuscular pathway by heat shock underlies the similar protection of tibial extensor muscle contraction seen under nerve-stimulated conditions (Figs 4B,C, 5A,B), permitting the pathway to operate at higher temperatures up to the limit at which the muscle itself fails. The upper temperature limit (56–58 °C) and recovery requirements (2–3 min) for the tibial extensor muscle would then be unaffected by prior heat shock and dictated solely by the intrinsic thermosensitivity of the muscle itself. However, when the muscle is activated *via* its normal nerve-stimulated pathway, as it would be during natural behaviour within its environment, the limiting factor for contraction in the locust extensor tibial muscle would still be the neuromuscular junction. Thus, we believe that heat shock-induced thermoprotection of the neuromuscular junction acting to reduce the thermosensitivity of both transmission and tibial extensor muscle contraction would preserve the neuromuscular pathway at elevated temperatures where its function would normally have failed.

#### *Alteration to behaviour*

In the locust, the use of jumping as a behaviour was found to be contingent upon the ambient temperature only in those animals that had not been subjected to prior heat shock (Fig. 6B). As temperature increased, control locusts increasingly utilized walking as a behavioural response to avoid capture, whereas heat shock animals consistently jumped at all temperatures examined. This is in contrast to the increased propensity of the animal for movement at higher temperatures for both control and heat shock animals (Fig. 6A). The obvious explanation for a reduction in the use of jumping is the inability of the locust to generate sufficient force in the extensor muscle in order to jump. This hypothesis would predict visible qualitative evidence of failed jumping attempts by control locusts at extreme temperatures. However, instead of failing at an attempt to jump, control locusts changed their behavioural response by using a walking escape manoeuvre. This might indicate that temperature affects the higher-order neural processing initiating escape behaviour and alters the behavioural response for capture avoidance at extreme temperatures at which jumping would be impaired. Under this hypothesis, heat shock could prevent this by preserving the neural pathway for initiating jumping or *via* sensory feedback from the hindleg, indicating the protected physiological state of the neuromuscular connections involved in jumping. While both jumping and walking in locusts require a tight coordination of neural circuitry and utilize numerous muscle fibres in a regulated manner (Heitler and Burrows,

1977a,b; Burrows, 1996) and escape manoeuvres depend on rapid induction of a complex motor output, whether heat shock could simultaneously affect all synaptic connections is not addressed by these experiments. Furthermore, although heat shock did reduce the thermosensitivity of jumping in the locust, there are possible sites of protection other than the extensor muscle. First, the reduction in jumping in control locusts could be due to a failure of afferent sensory input from the periphery initiating a jumping response. This is unlikely because the animals did not stop their capture avoidance response, they only changed that response from jumping to walking, a less effective avoidance behaviour. In addition, there was an evident increase in motility at high temperatures, which is in agreement with a reported increase in afferent input due to temperature increases in the grasshopper central nervous system (Abrams and Pearson, 1982). Second, the reduction in utilization of jumping behaviour could be a consequence of an increase in energy requirements to maintain cellular health during the temperature stress. Jumping in adult locusts requires 14 mJ of total stored energy in both metathoracic legs (Bennet-Clark, 1975). If energy were depleted during exposure to extreme temperatures, it might become an energetic necessity for the animal to utilize walking, instead of jumping, as an escape response. Under this hypothesis, it would be possible that heat shock is acting to preserve energy during a temperature stress, thus allowing the animal to jump to escape capture at extreme temperatures. These other possibilities do not alter the correlation between a thermoprotection of both the hindleg extensor muscle and a behavioural response that is critically dependent upon a functional extensor muscle. Thus, heat shock-induced thermoprotection allows the animal to continue to utilize jumping, the most rapid and effective response to avoid capture, at temperatures at which it would normally not be able to jump.

The behavioural results also point to a mismatch in the functional temperature range for the locust hindleg. While heat shock animals demonstrated strong jumping behaviour at ambient temperatures as high as 56 °C, and even control animals showed some jumping behaviour, both neuromuscular transmission and tibial extensor muscle contraction would already have failed. However, ambient temperature might not directly represent the temperature at the neuromuscular junction. The elevation in ambient temperature may require longer to equilibrate the neuromuscular junction completely to the new temperature. This still would not explain the difference in failure temperature of neuromuscular transmission and tibial extensor muscle contraction, where the recorded temperature should be the same. Extensor muscle contraction involves co-contraction of all tibial extensor muscle fibres and, although the temperature increase clearly affects neuromuscular transmission, the recorded temperature might not be uniform for all muscle fibres. Temperature was increased by a saline flow over the muscle surface where the temperature measurements and intracellular penetrations were made. The temperature for fibres deeper in the muscle may have been significantly lower than at the muscle surface. During tibial



extensor muscle force measurements, there remains the possibility that a subset of deeper fibres at a lower temperature is generating sufficient force for hindleg tibial extension. In addition, it cannot be discounted that intracellular penetration by the recording microelectrode could contribute to the early failure of neuromuscular transmission at temperatures lower than that of the entire muscle fibre.

#### Mechanism of heat-shock-induced thermoprotection

Neural signalling is a complex pathway, dependent upon multiple protein-protein interactions and a proper sequential execution of events. However, as temperature is increased, there is an increased tendency for these proteins to unfold and to bind improperly to each other, thus creating nonfunctional aggregates (Somero, 1995). Furthermore, high temperature can also increase membrane fluidity which, in turn, can inhibit normal membrane function (Vigh et al., 1998). Although the mechanism by which heat shock induces thermoprotection of the neuromuscular junction is not addressed by these results, heat shock could possibly act *via* heat shock protein stabilization of key signalling proteins (e.g. cysteine string proteins; Chamberlain and Burgoyne, 1997, 1998; Zinsmaier et al., 1994) or heat shock protein alteration of pre- or postsynaptic channel activity (e.g. K<sup>+</sup> channels; Ramirez et al., 1999). Heat shock could also exert its effect *via* increased production of trehalose (Neves and Francois, 1992; Becker et al., 1996), the main sugar in insect haemolymph. Trehalose has been shown to promote survival under conditions of extreme heat (Singer and Lindquist, 1998). This study has shown that an exposure to heat shock thermally protects neuromuscular transmission in the hindleg of the locust, an animal that would experience heat shock-like conditions naturally in its environment. Furthermore, this preservation of neuromuscular signalling at high temperatures can account for thermoprotection of both the force of muscle contraction and an important behavioural response critically dependent upon functional transmission. We conclude that the thermosensitivity of behaviour necessary for survival is reduced by the natural stress response of the locust during exposure to heat shock by stabilizing the neuromuscular pathway during a subsequent temperature stress.

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