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Photoperiod-induced plasticity of thermosensitivity and acquired thermotolerance in *Locusta migratoria*

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Summary

The mechanisms by which different life histories affect neural circuits are largely unknown. We show that the thermosensitivity and thermotolerance of neural circuit operation are affected in a complex dynamic fashion by photoperiod, prior heat experience and the sex of the animal. We compared thermosensitivity and thermotolerance of ventilatory motor pattern generation in locusts reared under two photoperiods (12:12 and 16:8; i.e. 12 h:12 h and 16 h:8 h L:D, respectively) before and after heat shock pre-treatment (HS: 3 h, 45°C) in order to determine the effect of daylength on properties of neural function. We monitored central pattern generator (CPG) output electromyographically from muscle 161 in the second abdominal segment during ramped increases in temperature and also measured the time taken for the circuit to fail at high temperatures and the time taken to recover on return to room temperature. There were effects of photoperiod, heat pre-treatment and the sex of the animal on ventilatory rate, time-to-failure and time-to-recovery. The ventilatory motor pattern of 16:8 and 12:12 locusts responded differently to increasing and maintained high temperature stress in both control and heat shocked locusts. We found that 12:12 locusts were generally more robust than 16:8 locusts: they lived longer, they showed greater tolerance to high temperatures, and they recovered more quickly from temperature-induced circuit failure. A faster ventilatory rate in 12:12 animals at high temperatures may have accelerated evaporative cooling to mediate improved temperature tolerance.

Key words: central pattern generator, heat shock, insect, life history, locust, *Locusta migratoria*, photoperiod, thermosensitivity, thermotolerance, ventilation.

Introduction

Many organisms are faced with natural environmental stresses such as extreme temperatures that can impair the operation of vital neural circuits before these conditions directly cause cell and tissue death (Robertson, 2004a). Therefore, survival depends on the ability of neural circuits to adapt to temperature fluctuations, allowing the animal to carry out important behaviours properly. It is well established that prior exposure to a high but sub-lethal temperature stress (heat shock: HS) leads to a state of improved thermotolerance in which the animal can continue to generate motor patterns and thus behave normally under previously non-permissive conditions (Robertson et al., 1996; Barclay and Robertson, 2000; Newman et al., 2003; Robertson, 2004a; Robertson, 2004b; Money et al., 2005). The mechanisms mediating acquired thermotolerance have not been fully elucidated; however, it is conceivable that the short- and long-term phenotypic changes induced by a prior stress are influenced by environmental variables such as photoperiod.

In poikilothermic animals, continued behaviour at normally lethal temperatures indicates improved performance of the nervous system. Development of thermotolerance is especially important for poikilothermic animals that lack the physiological mechanisms for maintaining a stable body temperature, and more so for those poikilotherms that are exposed to large fluctuations in ambient temperature on a regular basis. The locust *Locusta migratoria* is poikilothermic and native to sub-Saharan Africa. In their desert ecology, *L. migratoria* are routinely exposed to extremes in heat that can surpass 40°C, and internal temperature can be as much as 10–12°C above ambient during vigorous activities such as flight (Weis-Fogh, 1956). In order to cope in this environment, it is likely that the locust has evolved physiologically relevant ways of protecting neural function from heat stress. Thus, the locust is an excellent model for studies of thermosensitivity, thermotolerance and the HS response. Moreover, its relatively simple nervous system provides a unique opportunity for examination at the cellular level in live and semi-intact preparations.

One mechanism believed to underlie induced thermoprotection of neural circuits is enhanced expression of heat shock proteins (Hsps). *Locusta migratoria* adults exposed

to 45°C for 0.5–4.5 h experience coincident enhanced Hsp expression and improved thermotolerance during a subsequent, normally lethal temperature stress (Whyard et al., 1986). A twofold increase in expression of heat shock protein 70 (Hsp70) was found in locusts after being exposed to HS (Qin et al., 2003). Hsps have a number of roles in the cell, including protein chaperoning (Feder and Hofmann, 1999) and cytoskeletal stabilization (Feder, 1996; Liang and MacRae, 1997), and it has been suggested that Hsps may have a direct or indirect role in downregulation of K^+ currents as seen in HS locusts (Ramirez et al., 1999). Induction of Hsps and other aspects believed to be involved in thermotolerance are metabolically expensive and acquired thermotolerance is thus subject to energetic constraints. In an effort to minimize costs for animals that are exposed to widely fluctuating temperatures, it is a reasonable supposition that the HS response is plastic.

The benefits of having a plastic HS response are many. For example, plasticity allows an organism to modify the strength of its HS response in order to maximize protection against thermal damage while minimizing the metabolic costs incurred (Parsons, 2003). Evidence that the HS response is plastic has been demonstrated in intertidal animals that experience huge fluctuations in daily temperature as a consequence of where they live (Buckley et al., 2001; Halpin et al., 2004). For example, natural levels of Hsp72 and the threshold induction temperature of this protein were found to be greater in mussels (*Mytilus californianus*) inhabiting higher rocky intertidal areas, compared to mussels of the same species inhabiting the lower edges of the vertical distribution (Halpin et al., 2004). This study provides evidence that variation in physical conditions of closely spaced microhabitats can result in different natural levels of protein damage and a different HS response, depending on thermal history. Other studies have investigated the relationship between acclimation temperatures and natural levels of thermotolerance (Willhite and Cupp, Jr, 1982; Moseley, 1997). Our goal was to determine if daylength modulates the strength of the HS-mediated protection in the locust.

Photoperiod is a significant environmental variable that has been shown to affect cold tolerance (Kim and Song, 2000), heat stress resistance (Sorensen and Loeschcke, 2004), and Hsp70 level (Sorensen and Loeschcke, 2004) of insects. We used photoperiod to probe and challenge a crucial neural circuit in the locust to determine how the HS response is affected. A 12 h:12 h light:dark (L:D) photoperiod was considered the natural state and a longer daylength photoperiod of 16 h:8 h light:dark (L:D) was chosen arbitrarily as a more stressful daylength. We first measured phenotypic traits and lifespan to determine long-term effects of daylength, then we compared the ability of the nervous system to withstand heat stress in locusts reared under each photoperiod. We used ventilatory motor pattern generation as a model system to investigate plasticity of thermosensitivity and acquired thermotolerance in response to variation in photoperiod.

Ventilation is a crucial motor activity to locusts and is an appropriate neural circuit for investigation of the effects of photoperiod on acquired thermotolerance. Ventilation is under the control of a central pattern generator (CPG) located in the metathoracic ganglion (MTG) (Hustert, 1975; Bustami and Hustert, 2000). Ventilatory networks are sensitive to many different kinds of stress, including changes in internal concentration of gases such as CO_2 (Gulinson and Harrison, 1996; Henderson et al., 1998) and changes in pH (Snyder et al., 1980), as well as temperature changes (Banks et al., 1975; Lighton and Lovegrove, 1990; Henderson et al., 1998; Newman et al., 2003; Tryba and Ramirez, 2003). Locusts ventilate discontinuously when relaxed, but employ continuous ventilation when stressed, which makes it easy to determine when motor pattern generation has failed and recovered. The frequency of ventilatory bursts increases with increased heat. This is believed to be an adaptive advantage for the animal by increasing evaporative cooling, which in turn dissipates heat and lowers internal body temperature (Prange, 1990; Prange, 1996).

Operation of the ventilatory CPG in locusts can be protected against high temperature stress if subjected to a prior HS. Prior stress reduced whole cell K^+ currents (Ramirez et al., 1999), which would result in prolonged action potentials and reduced accumulation of extracellular potassium. Following a HS treatment of 3 h at 45°C, adult male locusts were capable of maintaining a ventilatory rhythm significantly longer at sustained high temperatures than animals that had not received this treatment (Newman et al., 2003). Additionally, HS animals recovered more quickly following hyperthermic failure, had a lower incidence of failure and a higher incidence of recovery during subsequent stress.

We examined frequency of ventilatory bursts, thermosensitivity, and thermotolerance of the ventilatory motor pattern during increasing temperature stress and the effect of a prior HS on these parameters in locusts reared under 16:8 and 12:12 L:D regimes. We defined thermosensitivity as the immediate response to changing temperature, and thermotolerance as the ability to cope with high temperature stress, i.e. time-to-failure and time-to-recovery of the motor pattern in response to increasing and constant high temperature stress. We hypothesized that animals from different photoperiods would be uniquely thermosensitive, and that thermotolerance of 16:8 and 12:12 HS animals would be different.

Materials and methods

Insects

Male and female *Locusta migratoria migratorioides* (R. and F.) were reared in two crowded colonies maintained in the Department of Biology at Queen's University. The two colonies differed in photoperiod, one with 16 h:8 h light:dark and the other with 12 h:12 h light:dark. Each cage was individually lit with a 40 W light bulb. Room temperature was maintained at 25±1°C, with a constant humidity of 23±1%. Cage temperature in the 16:8 and 12:12 colonies fluctuated over a 10°C range (from 25°C up to 35°C) during hours of

light, and temperature varied within cages depending on proximity to the light bulb. Animals were provided with carrots and wheat seedlings daily.

Lifespan and general morphology

Lifespan was monitored for male and female *L. migratoria* raised in 16:8 and 12:12 photoperiods to determine if the difference in daylength led to a difference in this fundamental life history trait. Animals were checked daily and dead ones were counted and removed. At 2–3 weeks of adult age, a total of 24 males and 24 females were removed from these cages, and basic body measurements were taken. These animals were not returned to the colony. Body measurements included mass, posterior femur length and head capsule width. Femur length (*F*) and head capsule width (*C*) were measured using Vernier calipers with 0.1 mm precision to generate the *F*/*C* ratio, generally regarded as the most reliable diagnostic indicator of phase in locusts (Dirsh, 1951; Dirsh, 1953). At 54 days of adult age, the lifespan experiment was terminated, and survivors were counted. Raw data were transformed to generate a Kaplan–Meier survival curve of cumulative survival probabilities (SigmaPlot 8.0, SPSS Inc., Chicago, IL, USA) Starting sample sizes were: 12:12 males, *N*=47; 12:12 females, *N*=61; 16:8 males, *N*=57; 16:8 females, *N*=57.

Experimental treatments

Male and female locusts, approximately 1–4 weeks of adult age, were randomly chosen from the 16:8 and 12:12 colonies and subjected to either a control or HS pre-treatment. Locusts that received HS pre-treatment were placed in a 2 liter ventilated plastic container in an incubator (45°C) for 3 h. A beaker of water was also placed in the incubator to maintain high humidity in order to prevent desiccation and minimize evaporative cooling of animals during pre-treatment. Control animals were placed in a similar container and left for 3 h at room temperature (21±2°C). All animals were allowed 1–5 h of recovery following pre-treatment and all experiments were performed between 13:00 h and 18:00 h to avoid potential time-of-day effects. Experiments on animals receiving different pre-treatments were interspersed over time with each other.

Animals were divided into eight experimental groups that allowed examination of the response of the ventilatory CPG to thermal stress depending on prior exposure, the sex of the animal, and photoperiod: (1) 12:12 control males, *N*=8; (2) 12:12 control females, *N*=8; (3) 16:8 control males, *N*=7; (4) 16:8 control females, *N*=12; (95) 12:12 HS males, *N*=9; (6) 12:12 HS females, *N*=8; (7) 16:8 HS males, *N*=8; (8) 16:8 HS females, *N*=9.

Experimental set-up

Following removal of legs, wings and pronotum, ventilatory muscle 161 was exposed by making a dorsal midline incision and pinning the locust open onto a corkboard, dorsal side up. The gut, air sacs and fat bodies were removed.

A Peri-Star peristaltic pump (World Precision Instruments Inc., Sarasota, FL, USA) was used for perfusion of standard locust saline into the body cavity which contained (in mmol l^{-1}) 147 NaCl, 10 KCl, 4 $CaCl_2$, 3 NaOH, and 10 Hepes buffer (pH 7.2) (all chemicals were from Sigma-Aldrich, Oakville, ON, Canada). The saline flow was directed onto the MTG where the ventilatory CPG is located and flowed through the animal toward the posterior end. Saline passed through a glass pipette wrapped in Nichrome™ wire, and the temperature of the saline was controlled by varying the amount of current passed through the wire. Temperature at the ganglion was monitored using a thermocouple connected to a digital thermometer (BAT-12; Physitemp Instruments Inc., Clifton, NJ, USA).

An extracellular recording of the ventilatory motor pattern was obtained by placing a 0.1 mm diameter copper wire, insulated except at the tip, onto abdominal muscle 161. The recording was digitized using a DigiData 1200 Series Interface (Axon Instruments Inc., Union City, CA, USA) (e.g. Fig. 1) and displayed using Axoscope 9.0. The preparation was grounded by placing a silver wire in the posterior tip of the abdomen.

Protocol

The preparation was allowed to stabilize for 20 min at room temperature, which fluctuated over a 4°C range (from 19°C up to 23°C). Then the internal temperature was raised in a ramped manner by approximately 5°C min^{-1} from ambient to 45°C. Temperature was held at 45°C for 30 min or until failure of motor patterning. At this time, temperature was allowed to return to room temperature and observation continued for 30 min or until recovery of motor pattern. Failure was characterized as the cessation of electrical activity in the extracellular recording and the lack of visible contractions of the ventilatory muscles. Recovery was defined as the first visible sign of rhythmical abdominal contractions.

Fig. 1 demonstrates that ventilation is a series of rhythmic electrical bursts in the abdominal muscles. Ventilatory frequency (mean ± s.e.m.) was calculated by averaging the reciprocal of the cycle period for all cycles of each animal during the first 20 min while the locust was allowed to acclimatize at room temperature and at every 5°C increase in temperature up to and including 45°C. Frequency at 40°C was used rather than frequency at 45°C in some of our analyses because there was a low incidence of failure of the motor pattern by the time the temperature ramp reached 40°C. Time-to-failure was measured as the time at failure minus the time at the beginning of the temperature ramp. Time-to-recovery was measured as the time at recovery minus the time at failure. Animals that did not fail before 30 min at 45°C or recover within 30 min at room temperature were not included in the time-to-failure and time-to-recovery analyses.

Statistical analyses

Data were plotted using SigmaPlot 8.0 using the mean as the measure of central tendency and standard error (s.e.m.) to describe dispersion about the mean. Two-way RM-ANOVAs, *t*-tests, *z*-tests, and Pearson Product Moment Correlations were performed using SigmaStat 3.0 statistical analysis software (SPSS Inc.). For the analysis of data shown in Fig. 3 and Fig. 4,

we used JMP IN 5.1 statistical analysis software (SAS Institute Inc., Cary, NC, USA) to perform ANOVAs with three subject factors (photoperiod, pre-treatment and the sex of the animal). *Post hoc* Tukey tests were performed to determine which groups drove the main effects. A 95% confidence interval was used to determine significance among means.

Results

Morphological differences in locusts from the two colonies

12:12 females weighed significantly more than 16:8 females (*t*-test, t=2.857, P=0.006, d.f.=46) (Fig. 2A). There was no significant difference in body mass between 16:8 and 12:12 males (*t*-test, t=0.1, P=0.9, d.f.=46) (Fig. 2A).

12:12 males had a greater femur length and slightly reduced head capsule width compared to 16:8 males (data not shown), leading to a significantly greater *F*/*C* ratio (*t*-test, t=2.6, P=0.01, d.f.=46) (Fig. 2B). There was no significant difference in the *F*/*C* ratio between 16:8 and 12:12 females (*t*-test, t=0.13, P=0.89, d.f.=23) (Fig. 2B).

Effect of photoperiod on longevity

We found significant differences in survival probability between groups (Kaplan–Meier Survival Analysis, Gehan-Breslow test statistic=21.06, P<0.001, d.f.=3) (Fig. 2C). Pairwise comparisons revealed that 12:12 females and males had a significantly higher survival probability than 16:8 females and males, respectively (Holm–Sidak pairwise comparisons: 12:12 females *vs* 16:8 females, t=15.2, P<0.01; 12:12 males *vs* 16:8 males, t=8.8, P<0.01). There were no sex differences in survival probability within each photoperiod (Holm–Sidak pairwise comparisons: 12:12 females *vs* 12:12 males, t=0.33, P=0.57; 16:8 females *vs* 16:8 males, t=2.66, P=0.1).

Effect of photoperiod on temperature sensitivity of ventilatory motor pattern generation before and after HS

Frequency of ventilatory bursts increased as internal temperature was increased in all groups (Fig. 3A,B). There was an effect of photoperiod on ventilatory frequency during increasing temperature in control animals (Fig. 3A). In addition, there was an effect of photoperiod on thermosensitivity, i.e. the slope of the relationship between ventilatory rate and temperature, in both control and HS locusts (Fig. 3A,B). Frequency of ventilatory bursts during a ramped increase in temperature was significantly higher in 12:12 control locusts than in 16:8 control locusts (two-way RM-ANOVA, P<0.001, $F_{(1,59)}$=23.457) (Fig. 3A). The ventilatory rhythm of 16:8 and 12:12 control locusts also responded differently to increasing temperature stress (significant interaction between temperature and photoperiod: two-way RM-ANOVA, P<0.001, $F_{(5,59)}$=15.611) (Fig. 3A). There was no main effect of photoperiod on frequency of ventilatory bursts during a ramped increase in temperature in HS locusts (two-way RM-ANOVA, P=0.148, $F_{(1,61)}$=2.309); however, the ventilatory rhythm of 16:8 and 12:12 HS locusts responded differently to increasing temperature stress (significant interaction between temperature and photoperiod: two-way RM-ANOVA, P=0.025, $F_{(5,61)}$=2.785) (Fig. 3B).

16:8 and 12:12 locusts responded differently to constant high temperature stress before and after HS (Fig. 3C–F). 12:12 locusts ventilated significantly longer than 16:8 locusts when internal temperature was increased to and held at 45°C (16:8 control males and females *vs* 12:12 control males and females: *t*-test, t=–3.994, P<0.05, d.f.=29; 16:8 HS males and females *vs* 12:12 HS males and females: Mann–Whitney Rank Sum Test, t=266.000, P<0.05) (Fig. 3C,D). 12:12 locusts had a significantly shorter time-to-recovery of the ventilatory motor pattern following hyperthermic failure than 16:8 locusts (16:8 control males and females *vs* 12:12 control males and females: *t*-test, t=3.547, P<0.05, d.f.=24; 16:8 HS males and females *vs* 12:12 HS males and females: Mann–Whitney Rank Sum Test, t=120.000, P<0.05) (Fig. 3E,F).

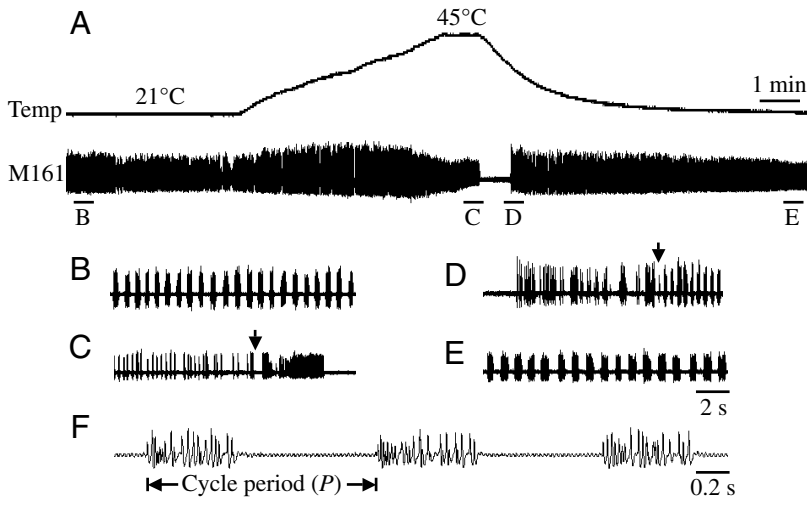

Fig. 1. Recording of the ventilatory rhythm at room temperature, at hyperthermic failure, and at recovery of ventilatory motor pattern generation in the locust. (A) Simultaneous recording of the temperature of the superfusing saline at the MTG (Temp) and the ventilatory motor pattern recorded from muscle 161 in the second abdominal segment (M161). Areas marked B–E are shown in expanded form below. (B) A section of the ventilatory rhythm during the first 20 min while the preparation was allowed to stabilize at room temperature. (C) Heat-induced failure of motor pattern generation. The arrow indicates where failure of motor patterning was determined. The burst of electrical activity following hyperthermic failure of the rhythm is typical and usually marks the cessation of all electrical activity. (D) Post-stress recovery of the ventilatory rhythm. The arrow indicates where recovery of the rhythm was determined. (E) The rhythm returns to normal shortly following recovery. (F) Expansion of bursts displaying intraburst activity. Mean ventilatory frequencies were obtained by calculating the inverse of the cycle period (1/*P*) for all bursts and calculating the average of these values.

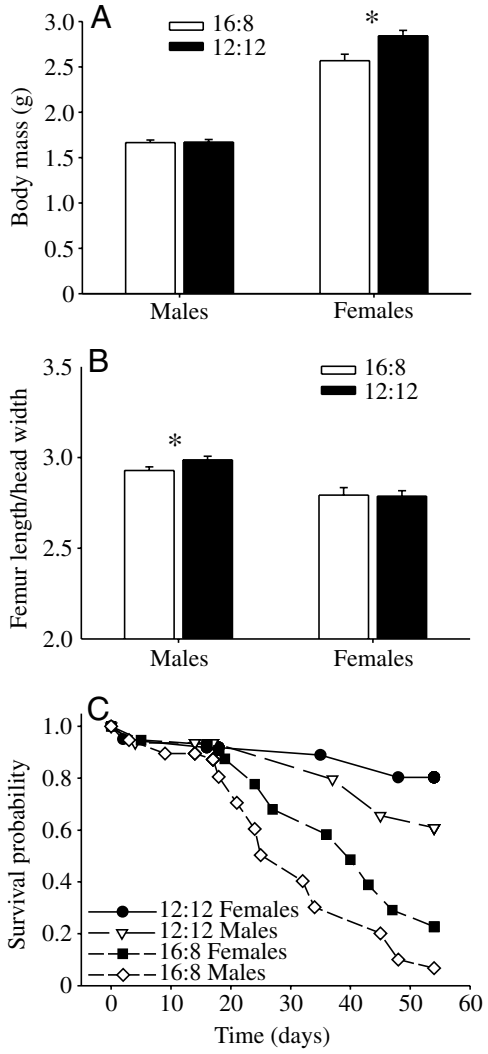

Fig. 2. Locusts reared under 16 h:8 h L:D and 12 h:12 h L:D regimes differed in morphology and lifetime survival probability. (A) 12:12 females (N=24) weighed significantly more than 16:8 females (N=24). (B) 12:12 males (N=24) had a significantly greater F/C ratio than 16:8 males (N=24). (C) 12:12 locusts had a significantly higher survival probability over time than 16:8 locusts (starting sample sizes: $N_{12:12}$=111, $N_{16:8}$=113). Significant differences are denoted by asterisks.

Percentages of locusts that failed and recovered

Failure of the ventilatory motor pattern occurred during the temperature ramp before 45°C in some instances, and some animals continued to generate a rhythm for 30 min at 45°C. The percentage of locusts whose motor pattern failed before 40°C, 45°C and after 30 min at 45°C as well as the percentage of locusts whose ventilatory rhythm recovered before 30 min at RT following hyperthermic failure are significant measures of thermotolerance and are presented for each group in Table 1.

There was a small percentage of locusts whose motor pattern failed before 40°C in four out of the eight groups (Table 1). Overall, a higher percentage of all 16:8 locusts (5/36=14%) failed before 40°C compared to all 12:12 locusts (2/33=6%); however, there were no significant differences among groups (z-tests, P>0.05).

The total percentage of locusts whose ventilatory rhythm failed before the temperature ramp was complete increased from 40 to 45°C (Table 1). Overall, a higher percentage of all 16:8 locusts (12/36=33%) failed before 45°C compared with all 12:12 locusts (6/33=18%). A significantly lower percentage of 12:12 control females (0%) failed before 45°C than 16:8 control females (42%) (z-test, z=2.109, P=0.035) and 16:8 HS males (50%) (z-test, z=2.309, P=0.021) (Table 1). There were no other significant differences among groups (z-tests, P>0.05).

The ventilatory motor pattern failed before 30 min at 45°C in a significantly greater percentage of all 16:8 control locusts (19/19=100%) than all 12:12 control locusts (12/16=75%) (z-test, z=2.316, P=0.021). The ventilatory motor pattern failed before 30 min at 45°C in a significantly greater percentage of all 16:8 HS locusts (17/17=100%) than all 12:12 HS locusts (13/17=76%) (z-test, z=2.128, P=0.033). All 12:12 females, both control and HS, were significantly more likely to tolerate increasing and constant high temperature stress at 45°C compared to the other three control and HS groups, respectively (z-tests: 12:12 control females *vs* 12:12 control males, z=2.309, P=0.021; 12:12 control females *vs* 16:8 control females, z=2.739, P=0.006; 12:12 HS females *vs* 12:12 HS males, z=2.426, P=0.015; 12:12 HS females *vs* 16:8 HS females, z=2.426, P=0.015) (Table 1). Thus, 12:12 animals were significantly more likely to withstand increasing and constant high temperature stress than 16:8 animals, further evidence of improved thermotolerance.

Another important measure of thermotolerance is the percentage of locusts whose ventilatory rhythm recovered before 30 min at room temperature following hyperthermic failure (Table 1). There were some instances in which recovery of motor patterning following hyperthermic failure did not occur before 30 min at room temperature (RT) (Table 1). Of the locusts whose ventilatory rhythm failed before 30 min at 45°C, there was 100% recovery before 30 min at RT in all groups except for 12:12 control males (63%) and 16:8 control females (83%) (Table 1). There were no significant differences in percentage of recovery among groups (z-tests, P>0.05).

Effect of photoperiod, pre-treatment, and the sex of the animal on ventilatory rate at room temperature and during high temperature stress

We compared mean ventilatory frequencies at 20°C (Fig. 4A), 40°C (Fig. 4B), and the average change in ventilatory frequency from 20 to 40°C (Fig. 4C) in each group to determine if there were effects of photoperiod, pre-treatment, or sex on these parameters.

There were significant differences in ventilatory rate at 20°C among groups (three-way ANOVA, P=0.0006, $F_{(7,56)}$=4.3823) (Fig. 4A). Statistical analysis revealed a main effect of pre-treatment on ventilatory rate at 20°C (three-way ANOVA, P=0.0425, $F_{(1,56)}$=4.3108), which is difficult to interpret because there was a significant interaction between pre-treatment and sex (three-way ANOVA, P=0.0002,

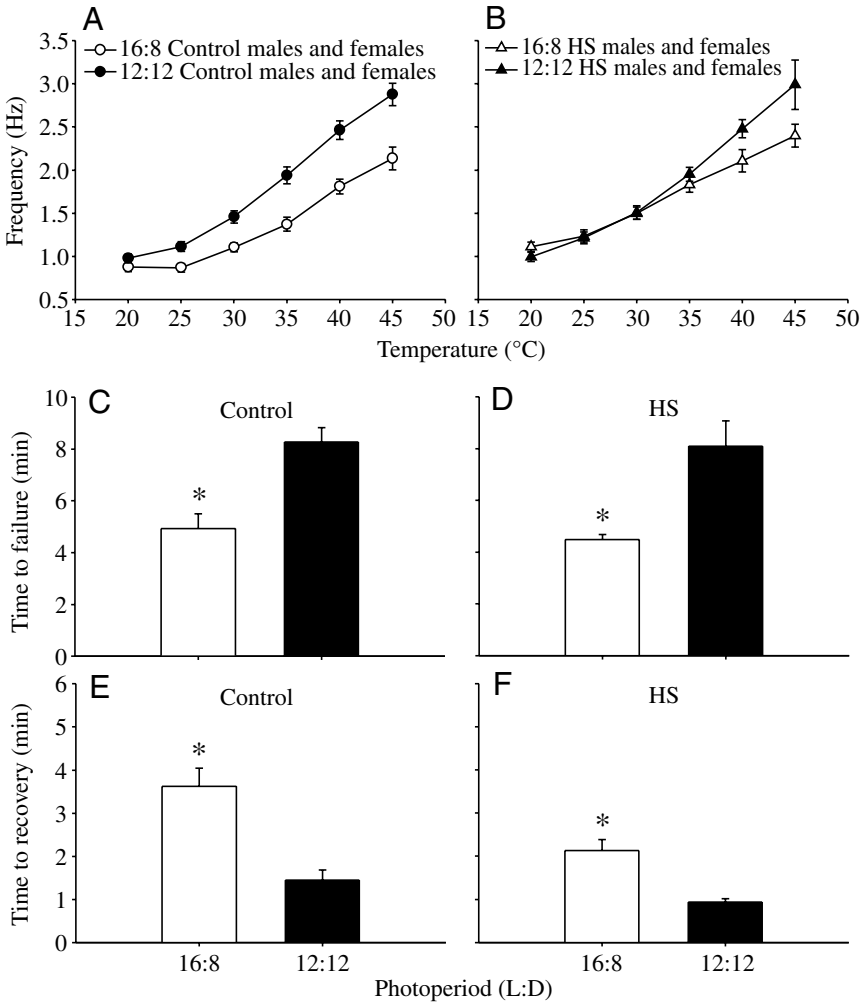

Fig. 3. Thermosensitivity to increasing heat stress, time-to-failure and time-to-recovery of the ventilatory motor pattern in control and HS locusts. There was a main effect of photoperiod on ventilatory rate during a temperature ramp in control locusts (Control: $N_{16:8}$=19, $N_{12:12}$=16) (A), but not in HS locusts (HS: $N_{16:8}$=17, $N_{12:12}$=17) (B). The ventilatory motor pattern of 16:8 and 12:12 locusts responded differently to increasing temperature in the control condition (A) and after a HS (B). 12:12 control males and females (N=12) ventilated significantly longer than 16:8 control males and females (N=19) when temperature was increased to and held at 45°C (C), and a similar difference was found between 12:12 HS males and females (N=13) and 16:8 HS males and females (N=17) (D). 12:12 control males and females (N=9) had a significantly shorter time-to-recovery following hyperthermic failure than 16:8 control males and females (N=17) (E), and a similar difference was found between 12:12 HS males and females (N=13) and 16:8 HS males and females (N=17) (F). Asterisks indicate a significant difference between 16:8 and 12:12 locusts. Error bars indicate s.e.m.; some in A,B may be hidden behind symbols.

$F_{(1,56)}$=15.5389). The main effect of pre-treatment was driven by a significant effect of pre-treatment on ventilatory rate at 20°C in females (control females *vs* HS females: *post hoc* Tukey test, P<0.05). 16:8 control females had a significantly lower ventilatory rate at 20°C than 16:8 control males, 16:8 HS males, 16:8 HS females, and 12:12 HS females (*post hoc* Tukey tests, P<0.05).

There were significant differences in ventilatory rate at 40°C among groups (three-way ANOVA, P<0.0001, $F_{(7,50)}$=6.6099) (Fig. 4B). Statistical analysis revealed a main effect of

Table 1. *Percentages of locusts whose ventilatory rhythm failed before 40°C, 45°C, and before 30 min at 45°C, and recovered within 30 min at room temperature*

Groups	Starting sample sizes	% Failed			
		Before 40°C	Before 45°C	Before 30 min at 45°C	Before 30 min at RT
CON					
12:12 ♂	8	0	25	100	63
12:12 ♀	8	0	0	50	100
16:8 ♂	7	14	14	100	100
16:8 ♀	12	17	42	100	83
HS					
12:12 ♂	9	0	22	100	100
12:12 ♀	8	25	25	50	100
16:8 ♂	8	25	50	100	100
16:8 ♀	9	0	22	100	100

CON, control; HS, heat shocked; RT, room temperature.

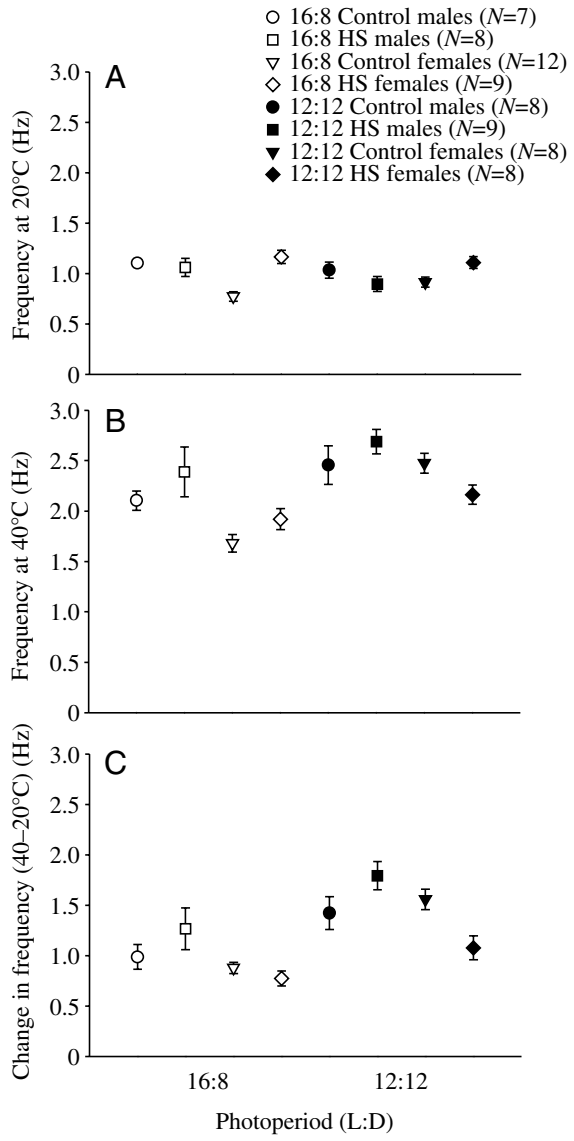

Fig. 4. Mean ventilatory frequency at 20°C and 40°C, and the average change in frequency from 20°C to 40°C in each group. (A) There was a significant interaction between the effects of pre-treatment and sex on ventilatory rate at 20°C, which was driven by a significant difference between control and HS females. 16:8 control females had a significantly lower ventilatory rate at 20°C than 16:8 control males, 16:8 HS males, 16:8 HS females, and 12:12 HS females. (B) There were main effects of photoperiod and sex on ventilatory rate at 40°C. 16:8 control females had a significantly lower ventilatory rate at 40°C than 16:8 HS males, 12:12 control males, 12:12 HS males, and 12:12 control females. 16:8 HS females had a significantly lower ventilatory rate at 40°C than 12:12 HS males. (C) There was a main effect of photoperiod on the change in ventilatory frequency from 20 to 40°C, and there was a significant interaction between the effect of sex and pre-treatment, which was driven by a significant difference between HS males and females. 12:12 HS males had a significantly higher change in ventilatory rate from 20 to 40°C than 16:8 control males, 16:8 control females, 16:8 HS females, and 12:12 HS females. 12:12 control males and 12:12 control females had a significantly higher change in ventilatory rate from 20 to 40°C than 16:8 control females and 16:8 HS females. Error bars may be hidden behind symbols.

photoperiod (three-way ANOVA, P=0.0001, $F_{(1,50)}$=17.0251) and a main effect of sex (three-way ANOVA, P=0.0013, $F_{(1,50)}$=11.6802) on ventilatory rate at 40°C. There were no statistically significant interactions between photoperiod, pre-treatment, and sex. 16:8 control females had a significantly lower ventilatory rate at 40°C than 16:8 HS males, 12:12 control males, 12:12 HS males, and 12:12 control females (*post hoc* Tukey tests, P<0.05). 16:8 HS females had a significantly lower ventilatory rate at 40°C than 12:12 HS males (*post hoc* Tukey test, P<0.05).

There were significant differences in the change in ventilatory rate from 20 to 40°C among groups (three-way ANOVA, P<0.0001, $F_{(7,49)}$=8.6146) (Fig. 4C). Statistical analysis revealed a main effect of photoperiod (three-way ANOVA, P<0.0001, $F_{(1,49)}$=26.9272) and a main effect of sex (three-way ANOVA, P=0.0028, $F_{(1,49)}$=9.9118) on the change in ventilatory rate from 20–40°C, which is difficult to interpret because there was a significant interaction between sex and pre-treatment (three-way ANOVA, P=0.0018, $F_{(1,49)}$=10.9587). The main effect of sex was driven by a significant effect of sex on the change in ventilatory rate from 20 to 40°C in HS locusts (HS females *vs* HS males: *post hoc* Tukey test, P<0.05). 12:12 HS males had a significantly higher change in ventilatory rate from 20 to 40°C than 16:8 control males, 16:8 control females, 16:8 HS females, and 12:12 HS females (*post hoc* Tukey tests, P<0.05). 12:12 control males and 12:12 control females had a significantly higher change in ventilatory rate from 20 to 40°C than 16:8 control females and 16:8 HS females (*post hoc* Tukey tests, P<0.05).

Effect of photoperiod, pre-treatment, and the sex of the animal on time-to-failure and time-to-recovery of the ventilatory motor pattern

We compared time-to-failure of ventilatory motor pattern generation in response to increasing and constant high temperature stress (Fig. 5A) and time-to-recovery of motor patterning (Fig. 5B) in each group to determine if there were effects of photoperiod, pre-treatment, or sex on these parameters.

There were significant differences in time-to-failure among groups (three-way ANOVA, P<0.0001, $F_{(7,53)}$=6.3637) (Fig. 5A). Statistical analysis revealed a main effect of photoperiod (three-way ANOVA, P<0.0001, $F_{(1,53)}$=28.0393) on time-to-failure. There were no statistically significant interactions between photoperiod, pre-treatment and sex. 12:12 control males had a significantly longer time-to-failure of their ventilatory rhythm than 16:8 HS males, 16:8 control females, and 16:8 HS females (*post hoc* Tukey tests, P<0.05). 12:12 HS males had a significantly longer time-to-failure of their ventilatory rhythm than 16:8 HS males and 16:8 control females (*post hoc* Tukey tests, P<0.05). 12:12 HS females had a significantly longer time-to-failure of their ventilatory rhythm than 16:8 control females (*post hoc* Tukey test, P<0.05).

There were significant differences in time-to-recovery among groups (three-way ANOVA, P<0.0001, $F_{(7,48)}$=10.2533) (Fig. 5B). Statistical analysis revealed a main

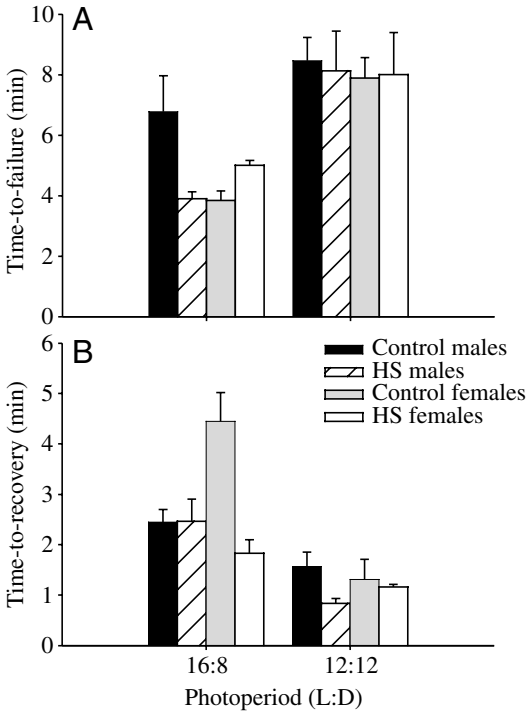

Fig. 5. Time-to-failure during increasing and maintained high-temperature stress, and time-to-recovery following hyperthermic failure of motor patterning, in each group. (A) There was a main effect of photoperiod on time-to-failure. 12:12 locusts had a significantly longer time-to-failure than 16:8 locusts. (B) There was a significant interaction between the effects of photoperiod, pre-treatment and sex on time-to-recovery. In general, 16:8 animals had a longer time-to-recovery than 12:12 animals, but this difference was mainly driven by the 16:8 control females, whose time-to-recovery was significantly longer than all other groups. 16:8 HS males had a significantly longer time-to-recovery than 12:12 HS males.

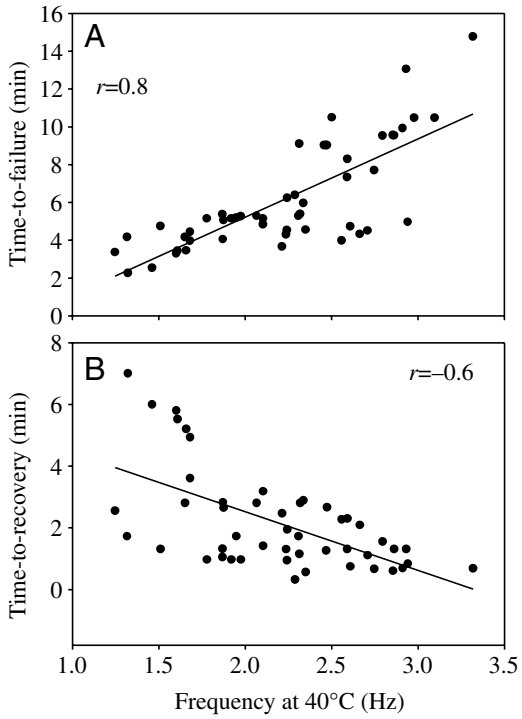

Fig. 6. Frequency of ventilatory bursts during high temperature stress was positively correlated to time-to-failure (A) and negatively correlated to time-to-recovery (B). Animals that ventilated more rapidly at 40°C could tolerate a greater duration of heat stress, and recovered more quickly following failure. All animals pooled: (A), N=51; (B), N=48.

effect of photoperiod (three-way ANOVA, P<0.0001, $F_{(1,48)}$=28.1415) and a main effect of pre-treatment (three-way ANOVA, P=0.0053, $F_{(1,48)}$=8.5216) on time-to-recovery. These main effects are difficult to interpret because there is a statistically significant interaction between photoperiod, pre-treatment and sex (three-way ANOVA, P=0.0095, $F_{(1,48)}$=7.2972). 16:8 control females had a significantly longer time-to-recovery than all other groups (*post hoc* Tukey tests, P<0.05) and 16:8 HS males had a significantly longer time-to-recovery than 12:12 HS males (*post hoc* Tukey test, P<0.05).

Time-to-failure and time-to-recovery are correlated to ventilatory frequency at 40°C

There was a strong and significant positive correlation between ventilatory frequency at 40°C and time-to-failure of the motor pattern (Pearson Product Moment Correlation, r=0.8, P<0.0001) (Fig. 6A).

There was a significant negative correlation between ventilatory frequency at 40°C and time-to-recovery of the motor pattern following failure (Pearson Product Moment Correlation, r=–0.6, P<0.0001) (Fig. 6B).

Discussion

This study demonstrates photoperiod-induced plasticity of morphological traits, longevity, thermosensitivity to increasing heat stress, and the ability to withstand and recover from maintained high temperature stress in *L. migratoria*. Differences in life history traits between animals of each photoperiod imply long-term consequences of daily light:dark hours that could influence overall fitness. Differences in ventilatory rate between 16:8 and 12:12 locusts could be due to the effect of photoperiod resetting any number of circadian oscillators. There was an effect of photoperiod on the time taken for the rhythm to fail during increasing and maintained high temperature stress. We found strong correlations between ventilatory frequency and the ability to tolerate and recover from high temperature stress, suggesting that these properties are linked. In addition to an effect of photoperiod, there were also effects of HS pre-treatment and the sex of the animal on ventilatory rate, suggesting that the HS response is plastic and that there can be sex-related variation in neural function.

Long-term effects of photoperiod

12:12 females weighed significantly more than 16:8 females, and 12:12 males had a significantly greater *F*/*C* ratio than 16:8 males. The *F*/*C* ratio is a reliable indicator of phase change in locusts (Dirsh, 1951; Dirsh, 1953), i.e. the ability of locusts to

exhibit density dependent polymorphism in characters such as development, morphometry, reproduction, behaviour, colour, etc. Thus, differences in this morphometric index suggest fundamental physiological differences in males from each photoperiod. 12:12 locusts had a higher survival probability than 16:8 locusts, and this difference increased with time. There were no significant differences in survival probability between males and females within each photoperiod, indicating that the differences in longevity were driven primarily by photoperiod. Photoperiod is a reliable seasonal cue for insects that has been shown to modify a variety of life history traits such as development time to adulthood (Nylin and Gotthard, 1998), body size (Uvarov, 1966; Uvarov, 1977; Tanaka and Okuda, 1996; Nylin and Gotthard, 1998) and sexual maturation (Uvarov, 1966; Uvarov, 1977; Tanaka et al., 1993; Tanaka and Okuda, 1996). Trade-offs between the above life history traits and longevity have also been examined (Parsons, 2004; Sorensen and Loeschcke, 2004). African migratory locusts are naturally adapted to a 12:12 photoperiod, so a longer daylength photoperiod of 16:8 could have inflicted a number of fitness consequences. The 16:8 colony could be considered a stressful habitat for a number of reasons. 16:8 animals were awake, active and exposed to light and heat (emitted from light bulbs inside cages) for longer periods than 12:12 animals. Increased daily activity increases metabolism, and increased exposure to light and heat results in more frequent and longer duration elevations in core temperature, potentially inducing stress and further increasing metabolism. Since 16:8 animals were exposed to a life-long cycle of extended light hours, longer bouts of activity and longer periods of high temperature, we conclude that the metabolic costs incurred resulted in accumulation of deleterious changes in cells and tissues, manifested as aging, and a more pronounced decrease in survival probability with time compared to 12:12 animals.

Effect of photoperiod on thermosensitivity of the ventilatory CPG

16:8 control locusts had a lower ventilatory frequency than 12:12 control locusts at each temperature during a temperature ramp. *L. migratoria* reared in long-day photoperiods (L:D 16 h:8 h) had lower oxygen consumption rates than those reared in short-day photoperiods (L:D 12 h:12 h) (Tanaka and Okuda, 1996). The ventilatory response to increasing thermal stress differed as a function of photoperiod in both control groups of locusts and HS groups of locusts. The differences in thermosensitivity of 16:8 and 12:12 locusts could be attributed to an effect of photoperiod on circadian rhythms, resulting in different coordination of metabolic processes.

Circadian clocks are adaptive for insects by generating rhythms that can be entrained to environmental cycles and/or by coordinating various internal processes. There is evidence that ventilation and metabolism have oscillations dependent on the time of day, suggesting that these processes are endogenously controlled (Saiki and Mortola, 1995; Peever and Stephenson, 1997). Peaks of oxygen consumption that occurred at certain times in a 24 h cycle and persisted during extended darkness were found in two species of cockroaches, *B. giganteus* and *B. craniifer* (Banks et al., 1975). A parallel morning–night difference in ventilation and metabolism was found in 6-day-old rats and hypoxia interferes with this coupling (Saiki and Mortola, 1995). If similar mechanisms operate in the locust, cyclic oscillations of metabolism and ventilation might explain differences in ventilatory rate of locusts reared under different daylength cycles.

It is unclear how circadian systems are organized and how clocks controlling different rhythms are related, but studies on *Drosophila* have provided evidence for expression of clock genes in both the brain and peripheral tissues (Giebultowicz, 1999). Many peripheral oscillators in insects are light-sensitive and capable of being synchronized by the sun, resulting in any number of independently entrained clocks that determine the physiological state of an animal. There are two light-entrainable circadian clocks in saturnid moths, one in the forebrain and another in the prothoracic gland, each independently coupled to the L:D cycle (Pittendrigh, 1993). Every known oscillating tissue in *Drosophila* can be reset by light (Plautz et al., 1997). There are numerous circadian oscillators in *Drosophila*, suggesting the presence of independent photoreceptive clocks throughout the fly with light as the master coordination signal (Plautz et al., 1997).

We presume that locusts have circadian organization and components analogous to that of *Drosophila*; however, this has not been thoroughly investigated. The rhythm of cuticle growth in locusts is light sensitive (Neville, 1967), suggesting an independent circadian mechanism located in epidermal cells that is entrained to L:D hours (Giebultowicz, 1999). If circadian clocks controlling ventilatory rhythms in locusts are synchronized by the sun, exposure to different L:D hours could result in different metabolic and ventilatory oscillations and thus a different resting ventilatory rate and response to increasing temperature, as seen in our 16:8 and 12:12 locusts.

Relationship between ventilatory frequency and tolerance to high temperature stress

12:12 locusts had a higher ventilatory frequency at 40°C and change in ventilatory frequency from 20°C to 40°C than 16:8 locusts. 12:12 locusts were also able to cope with high temperature stress and continue to ventilate for a longer period than 16:8 locusts. We found a strong positive correlation between time-to-failure and ventilatory frequency at 40°C such that these two variables almost always increased together. Increased ventilatory rate is an adaptive response to facilitate water loss and increase evaporative cooling, and to effectively dissipate body heat in insects (Prange, 1990; Prange, 1996). A smaller percentage of 12:12 animals failed during increasing and constant high temperature stress than 16:8 animals, evidence that animals from the 12:12 colony are better able to tolerate heat and maintain neural functions necessary for survival. We suggest that 16:8 animals were more vulnerable to high temperature stress due to the lack of thermoregulation afforded by fast abdominal pumping.

16:8 animals had a longer time-to-recovery than 12:12

animals, and we found that time-to-recovery almost always decreased as ventilatory rate at 40°C increases. The correlation between ventilatory rate at 40°C and time-to-recovery was not as tight as the correlation between ventilatory rate at 40°C and time-to-failure. We presume that increased ventilatory rate at high temperatures directly affected time-to-failure, whereas there does not appear to be any cooling mechanisms that could directly affect time-to-recovery, which would explain a less tight correlation. Time-to-failure and time-to-recovery were both improved in locusts that had a high ventilatory rate at 40°C, which suggests a linkage between time-to-failure and time-to-recovery, i.e. pleiotropic protective mechanisms.

Photoperiod-induced variation in the heat shock response of the ventilatory motor pattern

Although we found that 12:12 animals were better able to cope with heat stress in general than 16:8 animals, an interesting question is why we did not observe an overall increase in thermoprotection of the ventilatory motor pattern following HS as seen in previous studies (Newman et al., 2003). Acquired thermotolerance was observed most clearly in 16:8 females, the group that seemed to perform most poorly in the control condition compared to all other groups. 16:8 control females had the lowest ventilatory frequency at 20°C and 40°C, and ventilatory frequency increased at these temperatures in 16:8 HS females. 16:8 HS females were able to maintain motor pattern generation for a longer period during high temperature stress and were able to recover more quickly following failure compared to 16:8 control females. HS did not improve the response of 12:12 females to subsequent heat stress. Thus, our study demonstrates photoperiod-induced plasticity of the HS response.

It is not well understood how a prior HS induces long-term changes in cells, or how an organism's interaction with its environment modulates the HS response. One aspect believed to be involved in the stress response is the induction of Hsps. Although Hsps are molecular chaperones that alleviate stress and play a key role in inducible thermotolerance, they are energetically costly and can be harmful if overproduced (Krebs and Feder, 1997; Krebs and Feder, 1998). The functional consequences of Hsps are concentration dependent and the concentration depends on the level of stress in a habitat (Feder and Hofmann, 1999), which could very well have varied in the 16:8 and 12:12 colonies. Neuromodulators such as serotonin are also believed to be involved in the HS response (Hirashima and Eto, 1993; Newman et al., 2003). Serotonin plays a role in the modulation of many physiological processes (Osborne, 1996), and has been shown to mimic the effects of a HS on ventilation during subsequent temperature stress (Newman et al., 2003). Large fluctuations in serotonin levels were shown in the locust MTG following 4 h of crowding of solitarious animals (Rogers et al., 2004), indicating that these types of rapid changes in serotonin levels are possible in a short time-frame in response to important environmental stimuli, and, moreover, in the same ganglion that houses some of the neurons that are critical for generating the ventilatory rhythm. Serotonin levels in the optic lobes of crickets (*Gryllus bimaculatus*), which house bilaterally paired circadian pacemakers, fluctuate depending on the time of day, regulating sensitivity of neurons (Saifullah and Tomioka, 2002). The involvement of serotonin in the HS response and the evidence for daily rhythms of serotonin levels in insects could explain differences in the HS response of our 16:8 and 12:12 locusts. Thus, it is likely that conditions associated with daylength impose constraints on resources devoted to thermoprotection, such as Hsps and neuromodulators, and the way in which these are regulated.

This study provides further insight into phenotypic plasticity of neural function and the mechanisms underlying adaptation to environmental stress. We provide evidence that photoperiod can have profound effects on locusts in a number of ways. Animals reared under a photoperiod more closely resembling that of their natural habitat (L:D 12 h:12 h) are more thermosensitive and are able to maintain neural function during high temperature stress for a longer period than animals reared under a long-day photoperiod (L:D 16 h:8 h). This is likely due to improved heat loss mechanisms associated with fast abdominal pumping. 12:12 locusts live longer than 16:8 locusts, further evidence that light:dark hours can have a marked influence on insects. The effects of stress pre-treatment varied in animals reared under different photoperiods, indicating that the mechanisms underlying acquired thermoprotection are plastic. Our main conclusion is that neural circuit operation is plastic and environmental variables such as photoperiod modulate properties of neural function such that the ability to cope with stress is affected.

List of abbreviations

CPG	central pattern generator
F/*C*	femur length/head capsule width
HS	heat shock
Hsp	heat shock protein
L:D	light:dark
MTG	metathoracic ganglion
RT	room temperature

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