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Protein expression following heat shock in the nervous system of *Locusta migratoria*

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ABSTRACT

There is a thermal range for the operation of neural circuits beyond which nervous system function is compromised. *Locusta migratoria* is native to the semiarid regions of the world and provides an excellent model for studying neural phenomena. In this organism previous exposure to sublethal high temperatures (heat shock, HS) can protect neuronal function against future hyperthermia but, unlike many organisms, the profound physiological adaptations are not accompanied by a robust increase of *Hsp70* transcript or protein in the nervous system. We compared Hsp70 increase following HS in the tissues of isolated and gregarious locusts to investigate the effect of population density. We also localized Hsp70 in the metathoracic ganglion (MTG) of gregarious locusts to determine if HS affects Hsp70 in specific cell types that could be masked in whole ganglion assays. Our study indicated no evidence of a consistent change in Hsp70 level in the MTG of isolated locusts following HS. Also, Hsp70 was mainly localized in perineurium, neural membranes and glia and prior HS had no effect on its density or distribution. Finally, we applied 2-D gels to study the proteomic profile of MTG in gregarious locusts following HS; although these experiments showed some changes in the level of ATP-synthase β isoforms, the overall amount of this protein was found unchanged following HS. We conclude that the constitutive level of Hsps in the tissues of locusts is high. Also the thermoprotective effect of HS on the nervous system might be mediated by post-translational modifications or protein trafficking.

1. Introduction

Nervous systems are regularly exposed to high temperatures either from environmental sources or as a consequence of pathology, and cellular mechanisms that preserve neuronal function during hyperthermia would be highly beneficial. It is well established that previous exposure to sub-lethal high temperatures can protect cells, tissues and organisms from death via a process known as the *heat shock response* (HS) (Bukau et al., 2006; Kultz, 2005; Jaattela, 1999; Lindquest, 1992). In many organisms this effect is mediated by over-expression of a set of highly conserved proteins, named heat shock proteins (Hsps), which are molecular chaperones that assist unfolded proteins to regain their functional structure (Richter et al., 2010). There is considerable information about the localization and functional role of Hsps in the mammalian nervous system (Mayer and Brown, 1994) and among these Hsp70 is the most widely studied and the most inducible following hyperthermic stress (Daugaard et al., 2007).

Hsp70 has several constitutive and protective roles in nervous tissue (for reviews see: Franklin et al., 2005; Brown, 2007; Lu et al., 2010). In particular, Hsp70 localizes to synapses in the mammalian brain after hyperthermia (Bechtold et al., 2000) and presynaptic transgenic upregulation of Hsp70 protects the operation of larval neuromuscular junctions in Drosophila at high temperatures (Karunanithi et al., 2002; Xiao et al., 2007; Klose et al., 2008). The molecular complexity of synaptic transmission renders it particularly vulnerable to abiotic stressors, and illustrates the need to protect this vital process. Nevertheless, Hsp induction in the nervous system is injury-specific (anoxia vs. hyperthermia; Krueger et al., 1999), region-specific (cerebellum vs. cortex; Foster et al., 1995) and cell-type specific (glia vs. neurons; Pavlik et al., 2003; Franklin et al., 2005). Moreover, whereas motoneuronal vulnerability to stress can be ascribed to failure to activate a heat shock response in cell culture (Bruening et al., 1999; Batulan et al., 2003), increased levels of Hsp70 are sufficient but not necessary to protect synaptic parameters from hyperthermia in neonatal mouse brain slices (Kelty et al., 2002). In addition, increased transcription of Hsp70 mRNA is not necessarily accompanied by increased levels of Hsp70 protein indicating post-transcriptional regulation of the response to hyperthermia (Krueger et al., 1999). It is likely that full protection requires the coordinated up-regulation of a combination of Hsps and experimental manipulation of single Hsps can

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be less effective or ineffective compared to endogenous physiological mechanisms (Batulan et al., 2006; Mileva-Seitz et al., 2008). Also the experimental elimination of HS-induced Hsp70 can be compensated by up-regulation of alternative Hsps (Hsp83, Hsp40) to protect synaptic transmission (Neal et al., 2006). Thus, the neural HS response is complex and the role of Hsp70 in thermoprotection is still unclear.

The migratory locust, *L. migratoria*, is native to the semiarid regions of Africa and Asia and provides an excellent model system for detailed investigations of neural phenomena. It is well established that prior HS protects behavior and different aspects of its neural operation, at the cellular and circuit level (Robertson, 2004a,b; Money et al. 2005, 2009; Armstrong et al., 2006; Rodgers et al., 2007; Garlick and Robertson, 2007). Notably, the first description of HS-mediated protection of synapses from hyperthermia was in *L. migratoria* (Dawson-Scully and Robertson, 1998). Some features of the HS response of locusts are known but there are discrepancies in the literature. For example, measuring newly synthesized proteins in the cultured tissues of *L. migratoria* following HS has shown a remarkable increase in the production of six putative Hsps (Whyard et al., 1986), whereas quantifying Hsp70 levels has shown constitutive expression and a modest (~2-fold) increase in response to HS measured in fat bodies and there was no significant increase in *Hsp70* mRNA after HS in neural tissue or fat bodies (Qin et al., 2003). These features are considered well-suited for the locust's biology and life-style (Qin et al., 2003) and are consistent with those of other thermotolerant insects (Elekonich, 2009), but they fail to account satisfactorily for the HS-induced protection of neuronal properties, physiology and behavior.

Our goal was to investigate more fully the molecular response to HS in locusts and we focused on neural tissue in the metathoracic ganglion (MTG) because it is known that circuits in this ganglion are protected by prior HS. In particular we examined *Hsp70* transcript after different stresses (HS or non-HS) and compared the distribution of Hsp70 in MTG before and after HS. It was also speculated that gregarious locusts have already encountered some level of stress by living in a crowded colony and having to compete for limited food resources; hence, the constitutive level of Hsp70 in their tissues is high (Wang et al., 2007). Therefore, to eliminate the effect of such background Hsp70, a number of isolated animals were examined for their Hsp70 response by quantitative western blots following stress. Finally, a proteomic approach was taken, to more broadly examine the protein expression in the MTG following HS. Any change in the accumulation of proteins, Hsps or non-Hsps, was monitored in an attempt to discover pathways underlying the HS response in the locust nervous system.

2. Materials and methods

2.1. Animals

Animals were taken from a crowded colony of *L. migratoria migratorioides* maintained in the Department of Biology at Queen's University. Cages were 40 × 40 × 50 cm in size and contained 150–200 animals. Locusts were reared under a 12:12 h (light:dark) circadian regime at room temperature (25 ± 1 °C) and humidity of 23 ± 1%. Animals also received radiant heat from a 40 W light bulb installed in each cage. Locusts were fed fresh wheatgrass seedlings and a dry mix of wheat bran, torula yeast and milk powder. To control for age, animals used in this study were collected immediately following final ecdysis and raised in a separate cage for 3 weeks. Only adult males were used in these experiments.

A colony of isolated animals (solitarious-like phase polymorphism) was reared by separating 1st instar hoppers from the gregarious colony. These animals, which could be considered as dissocians (the transient form segregated from gregarious phase), were separated as soon as they hatched. This procedure has the same effect on the animal's morphometrics as the more invasive procedure of washing and separating the eggs (data not shown). Morphology was measured by monitoring the animals F/C ratio, where F is the length of the femur and C is the head capsule width. Larger F/C ratios identify animals with increasingly solitarious morphology.

2.2. Experimental treatments and dissection

Heat shock: Animals were heat shocked in a perforated plastic container in a humid incubator (45 °C) for 3 h while a control group (CON) was kept in a similar container at 22 ± 1 °C. After this period, all animals recovered at room temperature (22 ± 1 °C) for 1 h and were then dissected (Robertson et al., 1996).

Anoxia: Animals were exposed to a pure nitrogen atmosphere at room temperature (22 ± 1 °C) for 2 h and subsequently allowed to recover at normal atmosphere for 1 h (Wu et al., 2002). Control animals were kept in a normal atmosphere for 3 h.

Metathoracic ganglia (MTG) were excised, rinsed with cold standard locust saline (in mM: 147NaCl, 10KCl, 4$CaCl_2$, 3NaOH, 10 HEPES buffer, pH 7.2) and blotted dry on a clean lint-free wipe. Muscle tissue was taken from m112, numbered according to Albrecht (1953). Tissues were immediately frozen in liquid nitrogen and stored at −80 °C for no longer than two weeks.

2.3. 2-D gel electrophoresis

Total protein extraction: Sixty micro liters of a lysis buffer composed of 7 M urea, 2 M thiourea, 1% CHAPS, 1% ASB-14 and 1% Triton X-100 were used per ganglion. Cell disruption was facilitated by sonication, centrifuged for 20 min at 15,000*g*, and the supernatant collected and used immediately.

Sequential protein extraction: Proteins were extracted in different phases using a sequential extraction kit (Cat. No. 163–2100, Bio-Rad). Twenty MTG were suspended in 60 μl of the first reagent.

Total protein concentration of the samples was determined using the Bio-Rad protein assay (Cat. No. 500–0002) based on the method of Bradford. All substances used in the 2-D gel lysis buffer or RIPA buffer had a compatible concentration considering our serial dilutions. Absorbance was measured at 595 nm in an ELx800 Universal Microplate Reader (BioTek Instruments, Inc.). ReadyStrip IPG strips from Bio-Rad were used to separate proteins according to their isoelectric point (pI). Isoelectric focusing (IEF) was conducted in a Protean IEF Cell (Cat. No.165–4001, Bio-Rad) for 15 min at 250 V, 6 h at 500 V, 2 h at 2000 V and for a total of 100,000 Vh at 10,000 V. IEF for both CON and HS samples were performed simultaneously and in the same unit.

Immediately after isoelectric focusing, IPG strips were incubated in the first equilibration buffer (6 M urea, 2% SDS, 50 mM Tris–HCl pH 8.8, 20% glycerol supplemented with 2% DTT) for 15 min followed by incubation in the second equilibration buffer supplemented with 5% iodoacetamide for another 15 min. IPG strips were mounted on a large format polyacrylamide gel. For preparing 12% gels, 30% Duracryl 37.5:1 (Cat. No. 80–0148, Genomic Solutions) was used. Electrophoresis was carried out in a Protean II xi Cell (Cat. No. 165–1811, Bio-Rad) at 20 °C and 50 V overnight followed by a constant current of 24 mA per gel until the bromophenol blue reached the bottom of the gel. SDS–PAGE for both CON and HS samples were run side by side.

Gels were silver stained and scanned in a GS-800 Calibrated Densitometer (Bio-Rad) with 500 dpi resolution and analyzed using PDQuest 2-D Gel Analysis Software V7.1.0 (Bio-Rad). Automated spot detection and matching by PDQuest was followed by manual corrections. Spot quantities were normalized based on

the total quantity in valid spots on the gel and shown in ppm. CON and HS gels were assigned to different replicate groups and three different analysis sets were created: a qualitative analysis set including all the spots only present in one replicate group, a quantitative set including all the spots whose average quantity had changed above two fold in HS group compared to the CON and a statistical analysis set including the spots whose change was significant based on student's *t*-test while the significance level was set at 0.05. A Boolean analysis set was then built from the union of these three analysis sets. The latter was used to create a quantity table of the spots on each individual gel which was used in further statistical analyses.

2.4. In gel tryptic digestion

Spots were excised from four or five gels using a pipet tip. A blank piece of gel was used as the negative control. Some pieces of a reference gel containing 2 pmol of bovine serum albumin (BSA) were also processed in parallel as a positive control. Gel pieces were silver-destained and then twice washed with solutions of 50 mM ammonium bicarbonate and 100% acetonitrile. Gel proteins were reduced by incubation in 10 mM DTT at room temperature for one hour, followed by a 30 min 55 mM iodoacetamide incubation in the dark. Twenty micro liters of Sequencing Grade Modified Trypsin (Cat. No. V5111, Promega) with a concentration of 20 ng/μl was added to each tube and incubated on ice for 15 min. After discarding this enzyme solution, gels were incubated in 20 μl of 40 mM ammonium biocarbonate at 37 °C overnight. Peptides were extracted once using 100 μl of 2% acetonitrile and 1% formic acid and then twice in 100 μl of 50% acetonitrile and 1% acid formic for 5 min each with sonication. The total of 320 μl extract from each sample was concentrated to 10 μl in a SpeedVac centrifuge (Cat. No. SPD121P, Savant Instruments) and desalinized using OMIX C18 Pipette Tips (Cat. No. A5700310 K, Varian).

2.5. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Digested proteins were spotted on a 100-well MALDI target plate using two different matrices: α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB), (Sigma–Aldrich, Cat. No. C2020, G5254). An external calibration was applied with each matrix using a standard four-peptide mix of Renin, ACTH, GFP and Ang1 (Cat. No. R8129, A0673, F3261 and A9650, Sigma). The mass spectra were acquired in a Voyager-DE PRO Biospectrometry Workstation (Applied Biosystems) and analyzed using Data Explorer V4.0 while an internal calibration, based on the trypsin autolysis peaks, was also conducted. Monoisotopic masses in the spectrum were used to perform a search in SwissProt database using Mascot Peptide Mass Fingerprint search engine. One trypsin missed cleavage was allowed and the mass tolerance was set at 50 ppm. Carbamidomethyl (C) and oxidation (M) were considered as the fixed and variable modifications, respectively. The accuracy of protein match was assessed at $p < 0.05$.

In certain cases the remaining sample from MALDI-TOF experiments, which was not less than 8 μl, was subjected to Q-TOF LC/MS/MS analysis. The spectra were acquired with a Waters Q-TOF Global Ultima instrument which was operated in data-dependent acquisition mode using MassLynx 4.0. The inlet chromatography was performed on a Water's CapLC-XE using a Dionex Pepmap C18 column. The linear gradient was 5% acetonitrile to 40% acetonitrile over 40 min. The data was processed under MassLynx 4.0 and searched in Swissprot database through Mascot MS/MS Ion search engine. One missed cleavage was allowed and carbamidomethyl (C) and oxidation (M) were considered as the fixed and variable modifications, respectively. Error tolerances were set at 100 ppm and 0.2 Da for the parent mass and fragment mass, respectively.

2.6. Semi-quantitative RT-PCR

Total RNA was extracted from meso- and meta-ganglia and 1.7 μg were reverse-transcribed to cDNA with the SuperScriptII reverse transcriptase (Cat. No. 18,064–014, Invitrogen) in a 20 μl reaction. PCR was conducted in a final 25 μl reaction volume containing 5 μl of a given dilution of the reverse-transcription reaction, 0.4 μM of each primer, 200 μM dNTPs and 1 unit of Taq polymerase (Cat. No. M0267L, New England Biolabs). Two sets of primers were used, one as described in Qin et al., 2003 (AY178988) and another from Wang and Kang, 2005; (AY299637). Ten micro liters of PCR reaction was loaded on 1.7% agarose gel. The PCR product was quantified by measuring the integrated optical density (IOD) with GeneTools (SynGene).

2.7. Western blot

RIPA buffer was used to extract proteins with Triton X-100 (1.5%) and protease inhibitor (2%) (Cat. No. P-2714, Sigma–Aldrich) freshly added to the buffer before use. Tissues from three or four animals were used for each replicate. Tissues were homogenized and incubated on ice for 45 min. The lysate was then centrifuged at 15,000*g* for 10 min and the supernatant was used for western blotting after determining its protein concentration.

Proteins were denatured in SDS loading buffer at 100 °C for 5 min. For ATP-synthase β studies, 8 or 4 μg of the total protein were loaded on a 12% SDS–PAGE for obtaining the optimum band intensity. To quantify Hsp70, these numbers were changed into 80 and 40 μg loaded on a 7.5% SDS–PAGE. Both resolving and stacking (4%) gels were prepared by 30% acrylamide/bis 37.5:1 (Cat. No. 161–0158 Bio-Rad). Prestained SDS–Page Standard Broad Range (Cat. No. 161–0318, Bio-Rad) was used as the molecular weight marker. Proteins were separated in the SDS gel at 150 V for 1 h and transferred to the nitrocellulose membrane (Cat. No. RPN203D, Amersham Biosciences) for 1 h at 100 V in the transfer buffer (25 mM Tris, 192 mM glycine and 10% methanol). Membrane was blocked in Blotto solution (5% non-fat dry milk and 0.05% Tween-20 in TBS) for 1 h at room temperature. It was then incubated at 4 °C overnight in a solution of either polyclonal anti-ATP synthase β (1:2000; Cat. No. AS05 085, Agrisera), polyclonal anti-Hsp70, against recombinant human Hsp72 (1:5000; Cat. No. ADISPA-812 Stressgen), or monoclonal anti-Hsp 70, (1:1000; Cat. No. ADISPA-810 Stressgen). The mapped epitope for the monoclonal anti-Hsp 70 is in the region of amino acid residues 436–503 of human Hsp70. Protein BLAST shows 92% identity between locust and human Hsp70 within this region. After several washes in TBS-T (TBS, 0.1% Tween-20), membranes were incubated in HRP-conjugated goat polyclonal anti-rabbit IgG (1:10,000; Cat. No. DC03L, Calbiochem) at room temperature for 1 h. β-actin, as the internal loading control, was detected by the primary antibody against a peptide containing amino acid residues 20–33 in the actin sequence (1:10,000; Cat. No. A5060, Sigma–Aldrich). The specific bands were visualized by Immobilon Western Chemiluminescent HRP Substrate (Cat. No. WBKLS0100, Millipore. The band intensities for ATP-synthase β or Hsp70 were normalized to the actin band in the same lane while the background intensity had been subtracted from all the values.

2.8. Immunohistochemistry

Metathoracic ganglia were fixed, immediately upon excision, in 4% paraformaldehyde at 4 °C overnight and cryoprotected for 48 h in a solution of 30% sucrose in PBS. Ganglia were then embedded in

cryomatrix (Cat. No. 67,69,006, Thermo Scientific) and frozen in chilled 2-methylbutane. Serial cryosections (10 μm) were prepared from the tissue horizontally by a Shandon Cryotome (Thermo Fisher Scientific Inc.) and mounted on Superfrost-Plus slides (Cat. No. 12–550-15, Fisher Scientific). Sections were stored at −20 °C until use.

For immunostaining, sections were subjected to antigen retrieval by three washes in 0.25% Triton X-100 in PBS (PBS-T). Subsequently, tissues were blocked in a solution of 10% normal goat serum (Cat. No. G9023, Sigma Aldrich) and PBS-T for 1 h at room temperature. Sections were then transferred to a solution of polyclonal anti-Hsp70, against recombinant human Hsp72 (1:500; SPA-812, Stressgen) and 3% normal goat serum in PBS-T. After incubation in primary antibody for 48 h, slides were three times rinsed in PBS-T, each for 10 min and then incubated in a solution of FITC-conjugated goat polyclonal anti-rabbit IgG (1:300; Cat. No. F9887 Sigma Aldrich) and 3% normal goat serum in PBS-T for 2 h. Ten microliters of DAPI (1:10,000, Cat. No. D8417 Sigma–Aldrich) were added to the slides prior to the coverslip mounting. CON and HS slides were immunostained at the same time. A control slide was always processed without primary antibody to verify non-specific signals not originating from antibody-target binding. Another control was applied by replacing the primary antibody with a pre-immune rabbit serum in the same concentration; this approach allows monitoring non-specific fluorescence caused by the other serum IgGs. Control slides were analyzed with the same exposure time and within the same intensity range as the slides receiving the anti-Hsp70.

Images were taken by a fluorescent Leitz microscope (Leica Microsystems) and a CCD Sensicam camera and analyzed using SlideBook version 4.2 (Intelligent Imaging Innovation). Images from CON and HS slides were captured using the same exposure time and then analyzed within the same intensity range. Quantitative analyses were carried out by manually selecting the regions of interest and comparing their mean intensities between CON and HS tissues.

2.9. Statistical tests

All statistical analyses were performed with SigmaPlot 9.01 (Systat Software, Inc.). Two-tail student *t*-tests were used to assess intensity differences between CON and HS groups of 2-D gels. Mann–Whitney test was used as the non-parametric test when the normality test failed. Two-tail *t*-tests were also used to compare CON and HS data in immunohistochemistry experiments while multiple comparison of the Hsp70 expression in various parts of metathoracic ganglion was performed using one-way ANOVA followed by Holm-Sidak test. F/C measurements for gregarious and solitarious animals were also evaluated by unpaired *t*-tests. Differential expression of proteins measured by western blots was analyzed by paired *t*-test to compensate for day to day technical variations. A confidence interval of 95% was considered to assess the significance of differences in all the tests. Data were plotted using Microsoft Excel. Error bars represent standard error of means.

3. Results

Preliminary experiments using a variety of different cellular stressors demonstrated that none was effective at reliably and significantly increasing transcript levels of *Hsp70* measured using semi-quantitative RT-PCR. There was no significant increase in *Hsp70* transcript in the metathoracic ganglion (MTG) following HS. The observed results after anoxia, lipopolysacharide injection and hunger were similarly also not significant (data not shown). Hence we decided to focus our studies on the Hsp70 protein and thereafter on the whole protein profile of MTG.

3.1. Rearing of isolated animals induced solitarious-like phase morphology

The lack of a significant HS response in *L. migratoria* at the protein expression level could be attributed to a high constitutive level of HS related proteins in the tissues of studied animals. Living in crowded conditions could be one reason for having a constant high level of Hsps (Wang et al., 2007). Therefore, it was hypothesized that the environmental stresses such as HS or hypoxia will change the protein profile of the tissues in solitarious or isolated locusts more profoundly than the gregarious animals.

Locusts were raised in isolation staring from the 1st instar. Solitary juveniles (hoppers) raised in this condition were brown, developing some level of green pigmentation as adults in the areas of their body which are normally yellow in gregarious phase animals. These green areas were still not as large and intense as it is in the extreme solitaries. The pronotum, with its high convex crest, had noticeably transformed toward solitarious. F/C ratio is an index which is widely used to characterize phase state in locusts was also used to evaluate the solitarious state of the animals, with F being the femur length and C the head capsule width (Hoste et al., 2002). A significant shift was observed on the F/C of both isolated males and females compared to their gregarious parents (male: greg. = 3.06 ± 0.03, iso. = 3.46 ± 0.01, female; greg. = 2.93 ± 0.04, iso. = 3.25 ± 0.03; unpaired *t*-test, $P < 0.001$). Comparing these numbers with the published F/C for solitarious animals shows that the current procedure has driven animals' morphology significantly toward solitarious. In another analysis, current F/C values were compared with the published numbers for isolated locusts that were raised by separating and washing the eggs (Hoste et al., 2002). These analyses prove both procedures equally efficient to change the morphometrical indices in male and female locusts (exp.; male = 3.46 ± 0.01, female = 3.25 ± 0.03, pub.; male = 3.35 ± 0.15, female = 3.15 ± 0.16).

3.2. The effect of stress on Hsp70 expression in the tissues of gregarious and isolated L. migratoria

The expression of Hsp70 in both ganglion and muscle of the gregarious and isolated animals was examined by quantitative western blots. Animals were subjected to either HS or anoxia and compared to a control condition. Both of these treatments have been reported to have the same thermoprotective effect on the nervous system function of *L. migratoria* (Newman et al., 2003). Neither of these treatments changed the level of Hsp70 significantly in the muscle or MTG of gregarious locusts compared to controls, as detected using a polyclonal antibody (Fig. 1). Similarly, using a monoclonal antibody against the inducible form of Hsp70 showed no difference between control and HS. These results were consistent with our RT-PCR result as well as the previous ELISA experiments measuring Hsp70 in gregarious animals (Qin et al., 2003). Likewise in isolated locusts, Hsp70 level never changed beyond 1.5 times following anoxia or HS in either ganglion or muscle (Fig. 1). These changes were not significant (paired *t*-test).

3.3. Comparing localization pattern of Hsp70 in the metathoracic ganglion of control and heat shocked locusts

We next investigated the possibility of local, albeit small, protein changes in the ganglion following HS by doing immunohistochemistry (IHC) on horizontal sections from metathoracic ganglion. In the sections stained with Hsp70 antibody, the highest immunofluorescence belonged to the cytoplasm of perineurial and

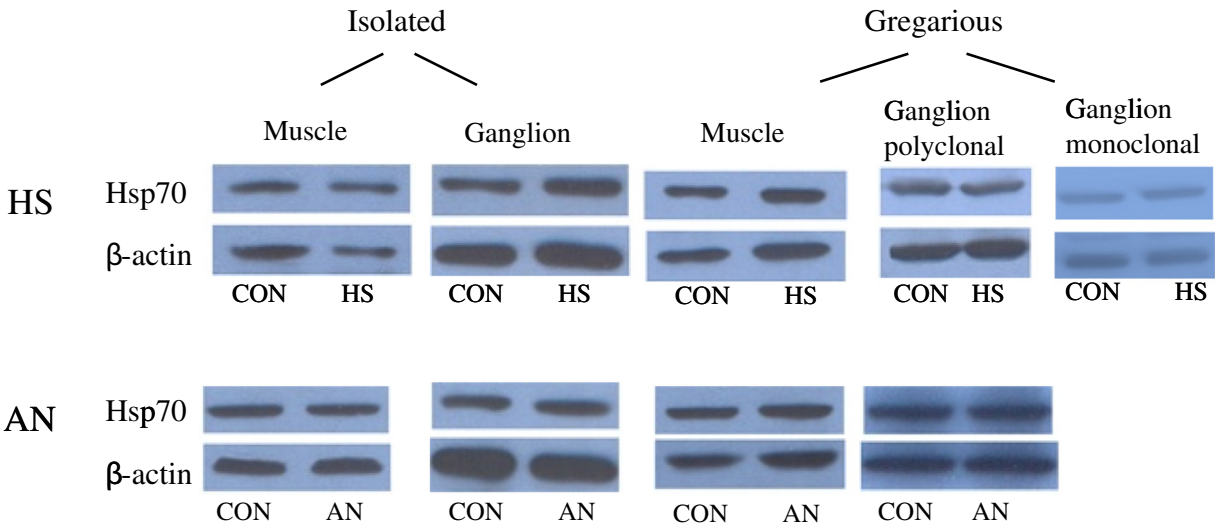


Fig. 1. Hsp70 level in the ganglion and muscle of isolated and gregarious locusts was measured by western blots following heat shock or anoxia (AN), separately. Hsp70 and β-actin were detected by polyclonal and monoclonal antibodies against recombinant human Hsp70 and a conserved peptide in the N-terminal of actin, respectively. Data were normalized to the β-actin level. Images shown represent one of the two (gregarious) or three (isolated) replicates. Hsp70 expressions in the tissues of CON and HS were not significantly different in any group (paired *t*-test).

tracheal cells. The fluorescent intensity of neuronal nuclei was the faintest signal, very close to the background intensity. Therefore, this region was used for normalizing the intensity in the other areas. These experiments showed that there was an appreciably higher level of Hsp70 in the cytoplasm of glial cells compared to the neurons, whereas Hsp70 was more localized in the membrane of neurons. In the core of the ganglion which was, in general, heavily stained with the Hsp70 antibody, there was an irregular distribution of brighter spots. This pattern could be due to the higher concentration of protein in the membranes of the neural processes.

Immunostained slides prepared from CON and HS tissues were visually analyzed under equal conditions. According to these analyses, HS did not induce a clear change in the Hsp70 expression pattern throughout the metathoracic ganglion. Additional quantitative analyses performed by measuring the fluorescence intensity in different areas of the tissue (perineurium, trachea, neuron cytoplasm, neuron nuclei, glial cells and core of the ganglion) confirmed the lack of a significant difference between CON and HS tissues stained with Hsp70 antibody (unpaired *t*-test; Fig. 2).

3.4. 2-D gel electrophoresis

Quantitative analysis for five replicates of 2D gels revealed only two spots whose intensity was significantly different between CON and HS gels ($P = 0.032$ for spot 1, Mann–Whitney test and $P = 0.028$ for spot 2, unpaired *t*-test), the protein content of these spots, however, was insufficient for identification by MALDI-TOF analyses and the acquired spectra did not contain any peaks. Since both spots were among the faintest ones on the gel and their intensity was close to the lower limit of the silver staining dynamic range (Smales et al., 2003), more experiments were needed to confirm that the observed change is associated with HS.

Results of total protein extractions suggested that the possible proteomic changes following HS might be in very low-abundance proteins. In order to intensify the corresponding spots on the gel, tissue homogenates were sequentially extracted using lysis buffers with different solubilizing capacities. Results of protein concentration assays and subsequent 2-D gels showed that nearly three quarters of the proteins were extracted in the first fraction, while the majority of the remaining proteins were extracted by the second buffer and very few proteins were found in the third extract. Since there were not many spots on the gels resulting from the third extract and the existing spots were not obviously different between CON and HS gels, this extract was examined only once. Studying three replicates of the first extract also did not yield any significant difference between CON and HS groups (unpaired *t*-test). However, in three replicates of the second extract, five spots showed an increased intensity in HS gels (Fig. 3). Although this difference between CON and HS gels was not statistically significant (unpaired student *t*-test), further steps were taken to identify these proteins.

MALDI-TOF analyses showed that these spots all contained the same protein which was the subunit β of ATP-synthase (Online Resource). Spots 1, 2 and 3 appeared on the gel with a molecular weight around 55 kD which is the expected size of ATP-synthase β. However, spots 4 and 5 were located at ~40 kD on the gels. Such spot multiplicity could be caused by post-translational modifications (PTMs) in the horizontal dimension or protein degradation in vertical dimension. In order to confirm that the proteins located in the two molecular weight levels are all ATP-synthase β or its derivatives, the digested samples from spots 2 and 5 were also subjected to LC/MS/MS analysis; both proteins were again identified as ATP-synthase β (Mowse scores; 134 and 53, sequence coverages; 7.2%, 2.3% for spots 2 and 5, respectively).

3.5. Quantitative western blot for ATP-synthase β

Results of 2-D gels following sequential extraction of proteins led to the assumption that ATP-synthase β is over-expressed at high temperatures to fulfill the energy demand in the cells during stressful conditions. However, spot multiplicity in the 2-D gels did not allow precise estimation of the overall protein level. To investigate this issue, quantitative western blot was employed. A polyclonal antibody, recognizing a highly conserved part of the protein sequence, detected two bands on western blots of the locust ganglion extract: one band at around 55 kD (the expected MW of ATP-synthase β) and the other one about 30 kD (Fig. 4). Since some form of protein degradation was also observed in 2-D gels, both bands were taken into account: the intensity of individual bands as well as the overall intensity in both bands were measured and normalized to the β-actin. According to the results of three replicates, assessed by paired *t*-test, the overall level of ATP-synthase β did not change appreciably following HS.

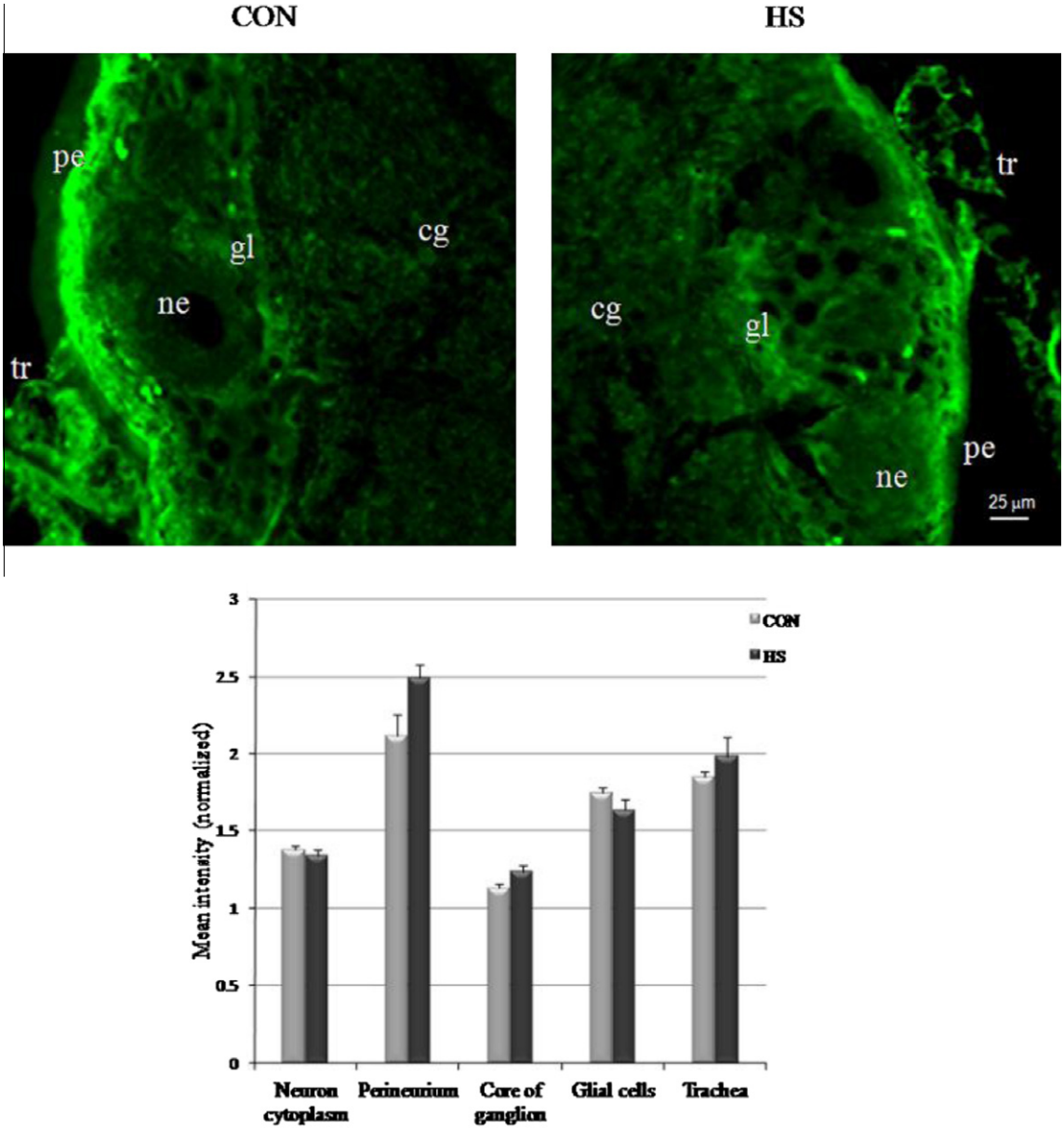


Fig. 2. Heat shock does not affect Hsp70 expression throughout the metathoracic ganglion. a. Horizontal sections of MTG (CON, HS) immunostained by polyclonal anti-Hsp70 (ne; neuronal cell body, cg; core of the ganglion, pe; perineurium, gl; glial cells, tr; trachea) b. Fluorescence intensities in different parts of ganglion were normalized to the intensity in neuron nucleus. No significant change was observed in these values due to heat shock (unpaired *t*-test). In both CON and HS groups fluorescent intensity in perineurium was significantly higher than the other parts (asterisk). Also glial cells and trachea displayed a significantly stronger signal than the core of the ganglion (neural processes) and neuron cytoplasm (daggers) ($P < 0.05$: one-way ANOVA followed by Holm-Sidak method).

This antibody was also used for immunoblotting a 2-D gel resulting from protein fraction 2. Only the three spots with the higher molecular weight (~55 kD) were detected by the antibody. Referring to the MALDI spectra obtained from the two lower spots, it can be observed that they do not have the part of sequence containing epitope which is located after amino acid 368 (Online Resource). Therefore, it was confirmed that the spots 4 and 5 contain a partial sequence of the protein and could be a product of protein degradation.

3.6. Proteomic analysis of the metathoracic ganglion following longer recovery times from HS

It is also possible that the change in protein expression which has started during HS accumulates over time (Diller, 2006). In other words, while some proteomic changes are not detectable after one hour recovery, they may become significant after a few hours. To investigate this matter, tissues were also examined after longer recovery times including 8, 12 and 24 h. Total protein extracted from these tissues, as well as the control and 2 h-recovery tissues, were separated on 17 cm IPG strips (pH5–8) and 12% SDS page. Samples were all processed in parallel. There were no obvious differences among the first replicates of these gels, and thus experiments were not repeated (data not shown).

4. Discussion

Exposure to extreme but sub-lethal temperatures can protect neural operation during subsequent high temperatures but the exact mechanisms underlying this response are not understood. It has been speculated that up-regulation of Hsps, especially of

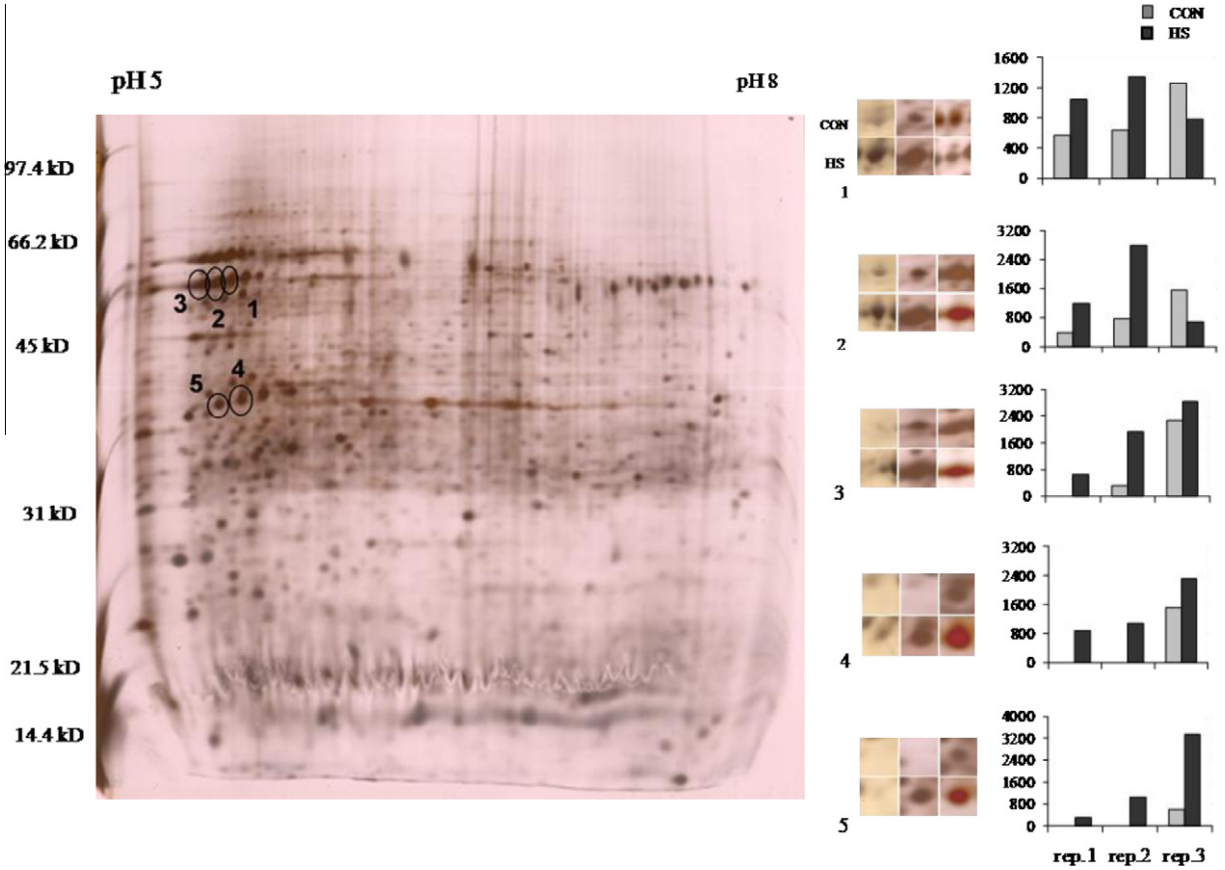
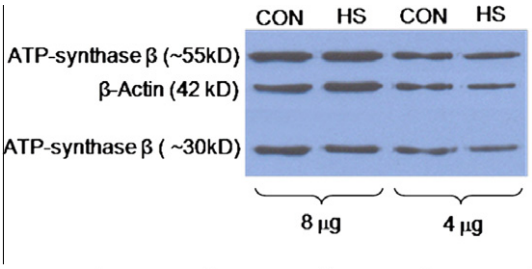


Fig. 3. The protein map obtained from metathoracic ganglion of *L. migratoria* by 2-D gel electrophoresis. Proteins were extracted in three different fractions according to their solubility properties (Bio-Rad sequential extraction kit). The lysis buffer was designed to extract proteins with an intermediate solubility. Five spots appeared with an increased intensity in HS gels compared to CON. Image subsets and graphs on the right show the spot intensity in individual replicates. These changes were not statistically significant (unpaired *t*-test). The gel image on the left is one of the three replicates (HS).



ATP-syn. β increase	~55 kD band	~30 kD band	55 kD + 30 kD bands
Rep. 1	1.00	1.1	1.80
Rep. 2	1.46	0.70	1.27
Rep. 3	1.09	0.91	1.01
Mean	1.18	0.90	1.36
P value	0.30	0.27	0.49

Fig. 4. ATP-synthase β was immunoblotted using a polyclonal primary (against a recombinant peptide conserved across the protein from different species) and a goat HRP-conjugated anti-rabbit secondary antibodies. The antibody detected two bands at around 55 and 30 kD. The intensity of putative ATP-synthase β bands was normalized to the β-actin band visualized by a rabbit anti-β-actin (against a peptide covering amino acids 20–33). Each band's intensity in addition to the overall intensity of both bands was considered in quantitative analysis. Numbers in the table represent the ratio of protein expression in HS tissues to CON. Heat shock did not induce any significant change in the level of ATP-synthase β in MTG (Paired *t*-test).

Hsp70, is responsible for these events, since their protective effects in tissues during high temperatures are well established in most systems. In the locust, profound effects of HS on physiology and behavior are not accompanied by correspondingly robust increases of Hsp70 transcript or protein in the nervous system, as shown here. To resolve this discrepancy we investigated: (1) constitutive and inducible Hsp70 levels in control and HS locusts, as well Hsp70 levels of animals reared in isolation compared to the gregarious phase; (2) the location of Hsp70 protein in the MTG to determine if HS affected Hsp70 in specific cell types that could be masked in whole ganglion assays; and (3) the up- and down-regulation of other proteins by HS to identify potential alternatives to Hsp70. We found that there was no differential effect of rearing conditions on Hsp70 levels before or after HS. Also, Hsp70 was localized primarily in perineurium and glial cells but HS had no obvious effect on levels of expression in different cell types. Finally, 2D gel electrophoresis suggested that ATP-synthase β was up-regulated in response to HS but this was not confirmed by Western blot analysis. Hence our primary conclusion is negative; we could find no evidence to suggest that HS measurably affected protein levels in the MTG, particularly levels or cellular distribution of Hsp70. These data are consistent with a model whereby the protective effects of HS in this organism are mediated post-translationally, perhaps via phosphorylation or protein trafficking.

4.1. Hsp70 expression was not affected by the current HS treatment

Despite robust physiological HS effects in the whole animal (Robertson, 2004a), such Hsp70 responses prove elusive (as re-

ported here), or modest (as in Qin et al., 2003). *In vitro*, Whyard et al. (1986) has reported a significant increase in the synthesis of putative Hsps in locusts. However, applying ELISA to measure the Hsp70 in the tissues of HS animals yielded only a modest increase (Qin et al., 2003). Similar results were obtained by measuring the *Hsp70* transcript in tissues or by 2-D gel analysis of the total protein extracts from MTG. Interestingly, both control and HS animals showed similar expression of the inducible form of Hsp70. It could be that these animals have sufficiently high constitutive expression not to require strong induction mechanisms. Alternatively, it could be that this antibody has cross-reactivity with constitutive members in locust, although the antibody we used has been shown to be specific in other species (Chen and Brown, 2007). Given our currently limited knowledge of the Hsp70 family in locust, we can not distinguish these possibilities. However, *L. migratoria* is well adapted to the semiarid regions of equatorial Africa where the average day temperature is around 32 °C (Uvarov, 1966). Radiant heat from the sun or metabolic heat produced during flight can frequently push the internal body temperature to above 40 °C (Chapman, 1976). In this animal, the constitutive amount of HS-related proteins might be relatively high in which case any subtle change in the protein level due to the current protocol would be lost in a high background of existing protein. There is evidence that the level of Hsps is constitutively up-regulated in the tissues of animals inhabiting areas with extreme temperatures. This helps thermoadapted species to be constantly prepared for the harsh conditions of their environment and thus they do not display a drastic increase of the Hsps in their tissues following hyperthermia or other environmental stresses. The general adaptations to these stresses could be greatly modified in these species (Evgen'ev et al., 2007; Zatsepina et al., 2000; Elekonich, 2009).

Aside from being adapted to a harsh environment, the high population density could also be stressful to the animals and increase the level of Hsps in their tissues (Wang et al., 2007). Therefore we predicted that animals isolated for a substantial period of their life (except for the embryonic stage) might display a more significant increase of Hsps in their tissues following stress than the gregarious ones. Results of the current study, however, did not support this hypothesis. There was no consistent change in Hsp70 levels and the observed changes were always less than 2-fold. It remains a possibility that extreme solitaries might be different; the rearing procedure in this study shifted animals' morphometrics significantly toward solitarious, however other characteristics of phase change, including some of the molecular ones, occur over generations (Pener, 1991).

Our immunohistochemistry results show that the distribution pattern of Hsp70 in the horizontal sections of metathoracic ganglion was not affected by HS treatment. These observations reject the possibility of local over-expression of Hsp70 that had been assumed undetectable in the total protein extracted from tissue. The IHC experiments also provided some information about the distribution of constitutive Hsp70 in the metathoracic ganglion of locusts. According to these data, in both HS and CON tissues, the perineurium contains the largest amount of Hsp70. This tissue is an active barrier between hemolymph and the nervous system (Wigglesworth, 1959). Its role in K^+ homeostasis in the ganglion is also well established (Schofield and Treherne, 1985). Thus over-expression of Hsps with their well-known housekeeping activities, such as folding newly-synthesized proteins, in tissue with a high metabolic activity such as perineurium is not unexpected (Bukau et al., 2006). Our results also show that Hsp70 has a higher expression in glial cytoplasm compared to neuronal cytoplasm; this pattern resembles the differential expression of Hsp70 in glial cells and neurons of the rat brain following hyperthermia (Mccabe and Simon, 1993; Pavlik et al., 2003). Moreover, *in vitro* studies show that both Hsc70 and Hsp70 are secreted by glial cells and taken up by neurons (Guzhova et al., 2001). Our experiments indicate that, in neurons, Hsp70 accumulates in the cell membrane. This is consistent with the localization of both Hsc70 and Hsp70 (the latter following HS) in the membrane-associated lipid rafts isolated from mammalian brain (Chen et al., 2005).

We found that a multiple-spot pattern of ATP-synthase β on 2-D gels was different for CON and HS. However this difference was not statistically significant based on the existing replicates and subsequent western blots did not reveal any change in the level of this protein. Nevertheless the protein may have been modified by post-translational mechanisms (PTM) as a response to energy crises during stress. A tentative phosphorylation prediction for ATP-synthase β (using the fruit fly sequence and NetPhos 2.0 Server) shows multiple phosphorylation sites in its sequence (Blom et al., 1999). These phosphate groups are found to be critical in the assembly and function of ATP-synthase subunits (Del Riego et al., 2006). Our current data, however, are not sufficient to support whether HS is an inducing factor for specific PTMs, and this issue needs to be investigated using further techniques including phosphoproteomics.

5. Summary

In the nervous system of *L. migratoria* the robust physiological adaptations following HS are not associated with a significant increase of Hsps in MTG (Qin et al., 2003). Our data suggest that HS protects neural function through mechanisms that do not depend on increased protein expression; e.g. Na^+/K^+ ATPase or ion channels can be regulated by their post-translational modification through second messenger pathways. Phosphoproteomic analyses are suggested for future studies to identify possible kinase targets mediating HS response in the nervous system and to trace the underlying signal transduction pathways.

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