

Stress Response in Infective Larvae (L3) of the Parasitic Nematode *Haemonchus Contortus* is Accompanied by Enhanced Expression of Heat Shock Proteins (HSP 70)

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Abstract: The study was undertaken to investigate the effects of temperature and moisture stresses on protein profile and characteristics in the free living third larval stages (L₃) of *Haemonchus contortus*. Before and after temperature and moisture stress treatment, the L₃ were tested for the induction and expression of Heat-Shock Proteins (HSPs). Proteins were analyzed by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and immunoblot analysis with alkaline-phosphatase conjugated anti HSP 70 antibody. The stress resulted in an altered protein pattern. It was also revealed that stress treatment did not alter the signals obtained with the anti HSP 70 antibody, but the amount of HSP70 as estimated by densitometry differed between pre and post stress samples. This response in the free living third stage larvae (L₃) of *H. contortus* may be aimed at protecting the parasite against molecular damage and ensuring survival during stressful periods.

Keywords: *Haemonchus*, heat shock proteins, moisture, stress, temperature

INTRODUCTION

Parasites encounter stressful conditions throughout their life cycles both in their free living and parasitic phases. Under conditions where temperatures and moisture exceed the normal growth range, the free living infective larvae of gastrointestinal nematodes experience stress due to the damaging effect of heat and desiccation on intracellular macromolecules. It is presumed that the stressed larvae undergo not only morphological and behavioural adjustments (Siamba *et al.*, 2009; Womersley *et al.*, 1998), but also engages physiological mechanisms such as the expression of heat shock proteins to counteract lethal effects of stresses (Maresca and Carratu, 1992).

Stress conditions that lead to hypobiosis in nematodes, such as elevated environmental temperatures and reduced moisture have been found to elicit changes in biomolecular profiles. For example, a study by Kooyman and Eysker (1995) using two-Dimensional Gel Electrophoresis (2-DGE) showed that there were qualitative and quantitative differences in proteins associated with inhibited development in *Haemonchus contortus*. The 2-DGE gels showed a large number of resolved polypeptides, of which the majority remained unaltered during the prolonged periods of conditioning. Upon closer examination, it became clear that 7 polypeptides showed differences. Another study with *Ostertagia ostertagi* L3 using SDS-PAGE and densitometric analysis showed that two 75.4 and 70 kDa

protein bands exceeded the control profile by 4.5 and 44 fold, respectively (Dopchiz *et al.*, 2000). However, the nature of the proteins induced during stress remains unclear. Therefore, the hypothesis in the current study was that besides other macromolecules, the proteins induced during conditioning for hypobiosis are Heat Shock Proteins (HSP).

MATERIALS AND METHODS

Study site: The study was carried out between 2007 and 2008 at the Kenya Agricultural Research Institute, Naivasha (1700 m asl), which has a semi-arid climate with strong desiccating winds (up to 13 m/sec) during dry seasons. The area receives about 750 mm of rainfall annually in two seasons: the Long Rains between March and June and the Short Rains between October and November. The period between December and February is usually dry and hot with maximum temperature of 28-35°C while the period between July and October is cool and dry with average maximum temperature of 18-25°C.

Experimental parasites: *Haemonchus contortus* monoculture used in the study was established as described by Siamba *et al.* (2009) and maintained by regular passage through parasite-free small East African Goats (*Capra hircus*). Infective (L3) larvae of *H. contortus* for the experiments were obtained by culturing faecal material from donor goats artificially infected with

Table 1: Experimental design

Moisture	Temperature	
	Low	High
Moist	T ₁ (LM)	T ₂ (HM)
Dry	T ₃ (LD)	T ₄ (HD)

T₁: Low temperature, high moisture (LM); T₂: High temperature, high moisture (HM); T₃: Low temperature, low moisture (LD); T₄: High temperature, low moisture (HD)

parasite monoculture. Faeces were cultured at 27°C for 10 days. The third stage larvae (L3) was then acquired from the faecal material as described by Hansen and Perry (1990).

Stress conditions and procedures: One hundred and forty-eight disposable weighing dishes (41×41×8) 3mm (Neolab(r)-Karl-Kolb GmbH and Co.kg, Scientific Technical Supplies, Dreieich, Germany) were evenly filled with 10 g of fine laboratory grade sand, with water field capacity of 39.3% similar to the soils representative of the study site, as a substrate for the larvae. About 5000 *H. contortus* L3 larvae aliquots in 4 mL of distilled water (dH₂O) were dispensed in each of the 100 dishes ensuring that the distribution in the sand substrate was as even as possible. Four millilitres of plain dH₂O was dispensed in each of the remaining 48 dishes.

The dishes were randomly assigned to four equal treatments (25 seeded dishes each) groups (T₁, T₂, T₃ and T₄). The 48 un-seeded dishes were also equally distributed to the treatments such that each treatment had 25 seeded and 12 un-seeded dishes.

The dishes were subjected to either low or high temperature/moisture treatments in a completely randomised design in a 2×2 factorial treatment structure as described by Siamba *et al.* (2009) (Table 1).

Recovery of larvae from substrate and processing of samples for SDS-page: Infective larvae were extracted from sand using high molarity sucrose solution as described by Freckman *et al.* (1977). Recovered larvae in the test tube were enumerated by serial dilution count (MAFF, 1987) to standardize the larvae for SDS-PAGE as follows: Aliquots from each treatment estimated to carry approximately 3000 each, were dispensed in microtubes and allowed to settle. The volumes were adjusted to 0.5 mL suspension. The larvae were ultrasonically homogenised (sonicated) using a Sonoplus HD 2070 ultrasonic Homogenizer (Bandelin, Germany) for 1 min at an amplitude of 10 microns at maximum power (70 W). The process was repeated twice with intervals of 25 sec' rest on ice.

Electrophoresis on SDS-page: One dimensional Polyacrylamide Gel Electrophoresis (PAGE) was carried out using standard Hoefer 250 vertical slab gel unit (Hoefer scientific inc. USA), using a discontinuous buffer system described by Laemmli (1970). Samples were

denatured by heating to 100°C for 5 min in a reducing environment of 2% SDS, 0.1M Tris-HCL (pH 6.8), 5% mercaptoethanol, 10% glycerol and 0.001 bromophenol blue.

A 12% polyacrylamide resolving slab gel ((10% SDS, 30% acrylamide, 1M Tris (pH 8.8), 10% APS and TEMED.) supplemented with freshly prepared 10% Ammonium Persulphate (APS) followed by N, N, N', N'-Tetramethylethylenediamine (TEMED) was overlaid with 5% stacking gel (10% SDS, 30% acrylamide, 1M Tris (pH 6.8), 10% APS and TEMED). The samples diluted in a Tris (pH 6.8) sample buffer (0.1M Tris-HCl, 2% SDS, 10% glycerol, 0.2 M 2-mercaptoethanol and 0.1% bromophenol blue) and 100 µL (equivalent to a 300 larval protein) were then loaded per lane. The current was set at 30 mA and electrophoresis monitored using 0.1% bromophenol blue. The protein fractions were visualized by staining with 0.10% Coomassie Brilliant Blue R 250 (Sigma, B-0149) and eventually destained as described by Johnston and Thorpe (1987). Molecular weight standard mixtures (MW 4,000-240,000, Invitrogen) loaded alongside the samples were used for calibrating the gel. Digital images of the destained gel were transferred to a computer and thereafter, analyzed by UN SCAN IT gel software (Silk Scientific, USA) to determine the relative molecular weights of individual bands and area integration analysis to estimate the relative quantities of individual bands.

Transfer of proteins to Nitrocellulose membranes (NC): Electrophoretic elution technique described by Towbin *et al.* (1979) was used to transfer proteins onto a sheet of Nitrocellulose membrane (NC) using a transfer-Blot cell apparatus (Hoeffer Scientific instruments, San Francisco, Model SE 600) filled with transfer buffer. Electrophoretic transfer of the proteins was carried out for 1.5 h at constant voltage of 100V at 4°C. The NC membrane was removed and briefly air dried at room temperature before probing.

Probing for heat shock proteins: Following transfer, the reactive sites on NC membrane were blocked using a 5% non-fat milk-Tris Buffered Saline Tween (TBST) solution overnight at room temperature while shaking. The blocking solution was removed and the membrane rinsed twice in TBST. Five (5) mL of alkaline-phosphatase conjugated anti HSP 70 antibody (Stressgen reagents, USA, conjugate was diluted 1:2000 in 5% milk-TBST) was added and incubated at room temperature for 30 min while shaking. Following incubation, the membrane was washed in 4 changes of TBST at room temperature with each wash lasting 5 min. The membranes were then developed by addition of 500 µL of BCIP/NBT substrate (KPL in brown bottle, stored at 4°C). The development was stopped after 15 min by rinsing the membrane with dH₂O.

RESULTS

As demonstrated in Fig. 1, protein electrophoresed on 12% acrylamide gel under reducing conditions in the presence of SDS resolved into at least 13 major peptide bands. There were few distinct differences in the banding patterns in stressed compared to the unstressed parasite notably the proteins observed at 46 and 32 kDa. The 46 kDa (heavy arrow) protein was detected only in the unstressed parasites while the 32 kDa (light arrow) appeared only in stressed larvae.

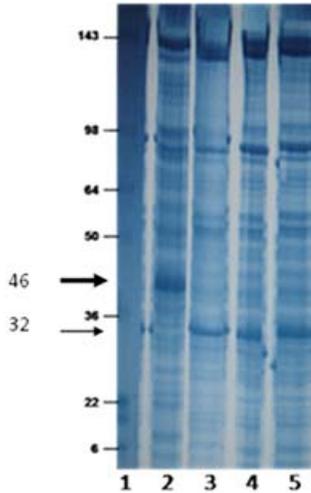


Fig. 1: Electrophoregram of coomassie blue stained SDS-polyacrylamide gel of sonicated *H. contortus* larvae subjected to temperature (lane 2) desiccation at low temperature (lane 4), desiccation at high temperature (lane 5) loaded along side molecular weight standards (lane 1) and unstressed larvae (lane 2)

Table 2: Relative quantities (as determined by UN SCAN IT gel digitizing computer software) of resolved proteins of different relative molecular weights

Protein (kDa)	LM	HM	HD	LD	Nature of difference
92	12.82	-	10.08	10.84	Quantitative/qualitative
80	15.43	14.81	27.49	30.23	Quantitative
60	5.2	4.7	13.47	12.76	Quantitative
46	20.9	-	-	-	Qualitative
32	6.13	14.55	19.97	20.28	Quantitative

However further image analysis of the electrophoregram using UN SCAN IT^(r) gel computer software (Fig. 2) not only confirmed the qualitative differences but also revealed quantitative differences in the banding pattern of unstressed larvae compared to stressed larvae. There were at least 21 detectable protein peaks in unstressed larvae compared to 18, 19 and 13 in treatment T₂, T₃ and T₄ respectively. Differences were noted at several regions designated as a, b, c and d in Fig. 2 corresponding to locations indicated by arrows on the electrophoregram at 32, 46, 80 and 92 kDa respectively.

Area integration analysis of area a, b, c and d, revealed quantitative differences as shown by the relative density of individual bands in Table 2. Bands with relative molecular weights of 80, 60 and 32 kDa, more than doubled in stressed compared to the unstressed larvae. On the other hand, the protein of 46 kDa in the unstressed larvae was undetectable in stressed parasites.

Immunoblotting analysis was carried out on larval samples separated by SDS PAGE and electrotransferred to nitrocellulose paper. When probed with alkaline-phosphatase conjugated anti HSP 70 antibody (Stressgen reagents, USA), the antibody was reactive with proteins that included a number of closely spaced but distinct

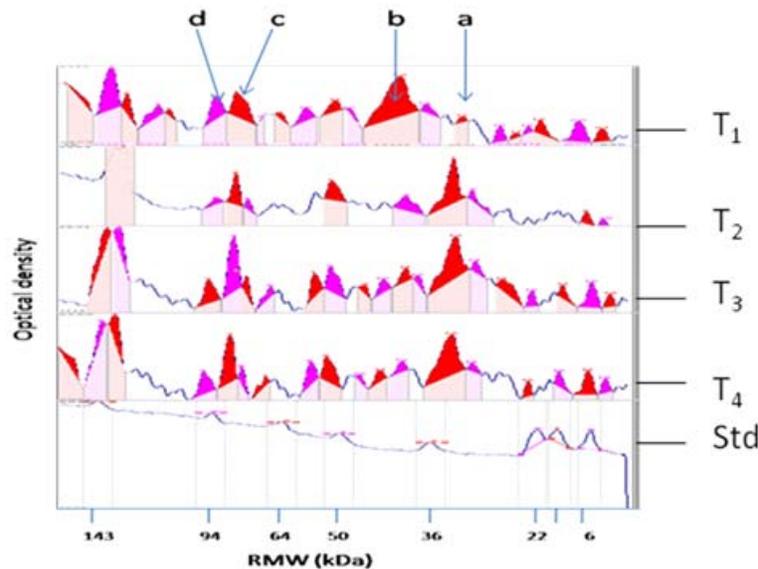


Fig. 2: Area integration showing the peaks a, b, c and d corresponding to peptides of approximate molecular weight 32, 46 and between 80-92 kDa, respectively

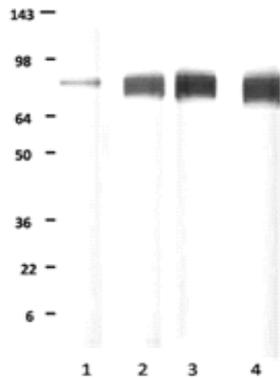


Fig. 3: Immunoblot analysis of resolved protein for HSP 70 of *H. contortus* infective larvae (L₃) unstressed (lane 1), temperature stressed (lane 2), desiccated at low temperature (Lane 3) and desiccated at high temperature (lane 4)

bands between 70-80 kDa (Fig. 3). The signal was detected in all treatments, but it was however conspicuously weak in the unstressed larvae compared to the stressed parasites. These bands corresponded to some of the abundant proteins detected by Coomassie Blue staining of the same samples

DISCUSSION

This study was based on the hypothesis that reprogramming and expression of genes coding for stress induced proteins and energy metabolism enzymes alters the protein and other macromolecules profiles of stressed infective larvae (L₃) of *Haemonchus contortus*. This was evaluated by means of SDS-PAGE, immunoblot and densitometric analysis.

Accordingly, the present study has demonstrated changes in protein levels possibly in response to underlying biological changes induced by stress. In accordance with the results of Kooyman and Eysker (1995) and Dopchiz *et al.* (2000), this study has confirmed that few major (qualitative) changes occur at the protein level upon stress. However, there were considerable changes in quantities of the proteins. The expression of some specific proteins was enhanced in stressed larvae. The observed differences in their abundance may represent the responses towards acclimation to stressful conditions. The changes may involve up or down regulation of genes coding for specific biomolecules such as Heat Shock Proteins (HSP), enzymes and other regulatory proteins as indicated by Horowitz (2002). This adjustment could result in extended thermal comfort zone by utilizing thermotolerant enzymes. Such enzymes have recently been reported to be expressed in *Ostertagia circumcincta* subjected to increasing temperatures (upto 45° C) (Walker *et al.*,

2007). These glycolytic enzymes, including hexokinase, pyruvate kinase and malate dehydrogenase may be similarly expressed in *H. contortus* for the same purpose. This behaviour may also partially explain the ability of the parasites to survive in the host animal despite the drastic change in temperature from around 22°C in the environment to 40°C in the rumen. In addition, expression of genes encoding for enzymes for synthesis of protective sugars and other macromolecules most likely contributes to the quantitative changes in proteins profile. Heat induced activation of trehalose-6-phosphate synthase for example has been reported (Jagdale *et al.*, 2005) and has been found to precede the accumulation of trehalose.

In addition, results of immunoblotting analysis using anti HSP 70 confirmed the presence of heat shock proteins in stressed as well as unstressed larvae. However the observed quantitative variations of the immunoreactive molecules revealed by densitometry analysis indicated that this/these molecules are both constitutive and induced. Earlier studies have established that HSP 70 is a class of approximately 70 kDa proteins encoded by multigene family expressed under a variety of physiological conditions in mammals. There are at least four distinct proteins in the HSP70 group (HSP72, HSP73, HSP75 and HSP78), Among these proteins are the 'constitutive' HSP 73 and the 'inducible' HSP 72 (Welch and Feramisco, 1984). Since HSP are phylogenetically conserved proteins (Bardwell and Craig, 1984; Craig, 1985; Liu *et al.*, 1989; Van Leeuwen, 1995), it is likely that these two HSP (HSP 73 and HSP 72) constituted the proteins detected before and after temperature and moisture stress

As constitutive glycoproteins, HSP act as molecular chaperons or proteases that have a number of intracellular functions. Chaperones are involved in the assembly and folding of oligomeric proteins, whereas proteases such as the ubiquitin-dependent proteasome mediate the degradation of damaged proteins (Hendrick and Hart, 1993) but as stress induced proteins, they are involved in maintaining the secondary structure of essential proteins during stress conditions (Pockley, 2001).

CONCLUSION

From the foregone discussions, the observed response in the free living third stage larvae (L₃) of *H. contortus* may constitute part of biochemical responses aimed at protecting the parasite against molecular damage and ensuring survival during stressful periods.

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