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Accumulation of trehalose in temperature and moisture stressed infective (L_3) and in hypobiotic (L_4) larvae of *Haemonchus contortus* in goats

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A study was conducted to investigate the accumulation of trehalose in infective third (L_3) stage of *Haemonchus contortus* induced for hypobiosis by exposure to gradual increase in temperature and decrease in moisture. Trehalose content in stressed L_3, hypobiotic (L_4) and mature *H. contortus* was estimated by densitometry. It was established that infective (L_3) larvae subjected to moisture stress accumulated significantly ($p = 0.037$) higher levels of trehalose compared to the unstressed controls. In addition, infection of goats with the stressed L_3 resulted in high proportion of larvae arrested at the fourth (L_4) larval stage as indicated by the number of L_4 recovered from the abomasal sub-mucosal tissue of goats slaughtered 24 days post infection. These hypobiotic larvae (L_4) had significantly ($p = 0.0024$) higher relative trehalose content than mature *H. contortus* recovered from the lumen of the abomasa. The high correlation of trehalose content in stressed larvae and hypobiosis suggested that trehalose metabolism could play a role in hypobiosis. It is suggested that further research should be conducted to confirm or refute this link between hypobiosis and trehalose metabolism.

Key words: Hypobiosis, trehalose, stressed, *Haemonchus*, larvae.

INTRODUCTION

Hypobiosis also known as inhibited or arrested development, is a temporary cessation of development at an early phase of parasitic existence in the host. It is a common phenomenon in parasitic nematodes particularly in the species of the family trichostrongylidae and plays an important role in the epidemiology of nematode infection (Gatongi et al., 1998; Eysker, 1997, 1998). In *Haemonchus contortus*, it is the early 4th stage (L_4) which becomes arrested and remains lodged in the abomasal mucosa for prolonged periods. Resumption of development occurs in response to yet unidentified stimulus but is thought to be spontaneous. The exact etiology of hypobiosis in nematodes remains obscure but there has been considerable speculations on it mechanism. Initially it was linked to acquisition of immunity (Martin et al., 1957; Michel, 1963; Soulsby, 1966). However, non immune developmental arrest has been described and the current standing is that this phenomenon could be ascribed to inherent developmental adaptations in the infective larval stages, either genetically or environmentally induced (Armour et al., 1969a; Eysker, 1997). To date, hypobiosis is always linked to events in the environment that are stressful to the free living pre-parasitic infective (L_3) larvae on pastures such as elevated temperatures and reduced moisture (Armour et al., 1969b; Connan 1971; Almeria et al., 1996). However, the underlying biochemical or physiological responses in the L_3 in such circumstances has not been fully studied. So far studies focusing on physiological and biochemical adaptations of many eukaryotic microorganisms such as nematodes to stressful conditions, have demonstrated accumulation of sugars including the non-reducing sugar, trehalose (Brown, 1978; Womarsley et al., 1998). Besides being a source of energy, this sugar stabilizes and protects important cellular components thus allowing organisms to

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Table 1. Experimental design and final stress levels.

	Temperature	
Moisture	**Low**	**High**
Moist	T_1	T_2
Dry	T_3	T_4

Key
T_1: Low temperature (27°C), high moisture (39.4%)
T2: High temperature (34.5°C), high moisture (39.4%)
T3: Low temperature (27°C), low moisture (2.6%)
T4: High temperature (34.5°C), low moisture (1.31%)

preserve biological activity through times of environmental stress (Crowe et al., 1987; Sun et al., 1996). Although there is no documented evidence that trehalose levels influence developmental arrest in ruminant parasitic nemetodes, a study by Vogel et al. (1998) using tobacco plants attributed retarded development in this plant to higher than normal levels of trehalose. However this lead has never been pursued further. Based on this background, it was hypothesized that *H. contortus* L_3 and L_4 accumulate trehalose in response to stress in the environment and in the host, respectively and that the high tehalose level influences hypobosis. The objective of this study therefore was to induce hypobiosis and determine the association of trehalose levels in L_3 and L_4 with hypobiosis of *H. contortus* in goats artificially infected with stressed L_3.

MATERIALS AND METHODS

Study site

The study was conducted at the National Animal Husbandry Research Centre- Naivasha, Kenya. The site, at an elevation of approximately 1700 m above sea level, has a semi-arid climate with strong desiccating winds (upto 13 m/s) during the dry season (Wariuru et al., 1998; Gatongi et al., 1998).

Haemonchus contortus monoculture and larvae (L_3) for experiments

H. 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 (L_3) larvae of *H. contortus* for the experiments were obtained by culturing faecal material from donor goats artificially infected with parasite monoculture. Faeces were cultured at 27°C for 10 days. The L_3 were separated from the faecal material as described by (Hansen and Perry, 1990),

Temperature and moisture stress conditions

The stress conditions typical of a semi arid environment used in the study were adopted from literature and analysis of weather data from the station at the study site. Temperature and moisture stresses were achieved using a programmable Binder cold/heat testing chamber (MK53, Neolab, Karl-Kolb GmbH and Co. kg Scientific, Technical supplies, Dreieich Germany). The incubator was found appropriate because it has automatic mechanisms to control the fan speed for required air current over the samples.

Stress procedures and experimental design

One hundred and forty eight (148) disposable weighing dishes (41 x 41 x 8) mm^3, (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 of the representative study site, as a substrate for the larvae. One hundred dishes were each seeded with approximately 5000 L_3 suspended in 4 ml of distilled water (dH_2O) ensuring that the distribution in the sand substrate was as even as possible. Four (4) ml plain dH_2O was dispensed in each of the remaining 48 dishes. The dishes were randomly assigned to four equal (25 seeded dishes each) treatments (T) groups (T_1, T_2, T_3 and T_4). The 48 unseeded dishes were also equally distributed to the treatments such that each treatment had 25 and 12 seeded and unseeded dishes, respectively. The dishes were subjected to either low or high temperature/moisture treatments in a completely randomized design with a 2 x 2 factorial treatment structure as shown in Table 1. Specific treatment procedures were as describe elsewhere (Siamba et al., 2009). Recovery of stressed larvae from sand was as described by Freckman et al. (1977).

Infection and maintenance of experimental animals

The infective (L_3) larvae recovered after stress sessions were used to infect experimental animals. Viability of L_3 in each treatment was estimated and used to adjust the dose rates to 3000 viable larvae. The experimental animals consisted of 20 Small East African (SEA) goats aged between 7 - 12 months. After a 10 day adaptation period, the animals were randomly assigned to 4 treatment groups (T_1, T_2, T_3 and T_4.), with 5 animals in each treatment. Animals in T_1, T_2, T_3 and T_4 were artificially trickle infected daily for 4 days with 3000 L_3 unstressed larvae (T_1), temperature stressed (T_2), moisture stressed (T_3) and combined temperature/moisture stressed (T_4), respectively. The animals were confined and maintained on a diet consisting of a mixture of commercially grown grass and Lucerne hay and offered clean water *ad libitum*. A faecal egg count was conducted every 7 days to monitor progress of infection. The animals were slaughtered 24 days after the last dose of infection. This was ample time to allow the parasite to develop to maturity.

Post mortem parasite counts from the abomasums

All experimental animals were slaughtered 24 days post infection and differential parasite counts for mature and arrested larvae was conducted as described by Hansen and Perry (1990).

Estimation of trehalose content of samples

Trehalose content in extracts of L3, arrested larvae (L4) and mature H. contortus was estimated by densitometry following TLC.

Processing of stressed third stage larvae (L_3) of H. contortus

An estimated 3000 larvae as a 2 ml suspension in 70 (v/v)%

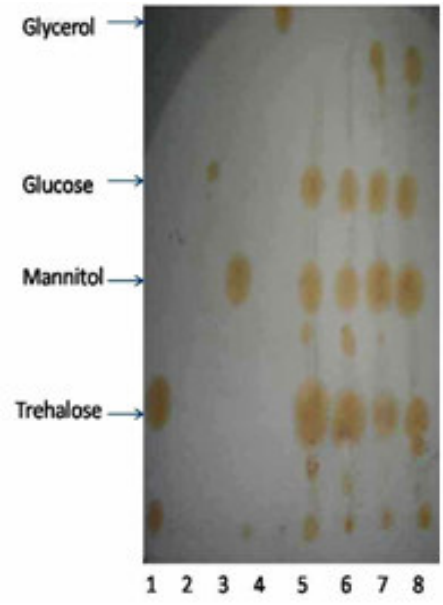

Figure 1. Thin layer chromatogram of standards – trehalose (lane1), glucose (lane 2), mannitol (lane 3) and glycerol (lane 4) spotted alongside *H. contortus* L_3 subjected to combined high temperature and low moisture stress (T_4-lane 5), low moisture stress (T_3-lane 6), high temperature stress (T_2-lane 7) and control (T_1-lane 8).

ethanol (to stop further trehalose metabolism) from each treatment in microtubes were used. The larvae were ultrasonically homogenised (sonicated) using a Sonoplus HD 2070 ultrasonic Homogenizer (Bandelin, Germany) for 1 min at amplitude of 10 microns at maximum power (70 W). The process was repeated twice with intervals of 15 min in a boiling water bath. After cooling to room temperature, the residues were removed by centrifugation and the resultant supernatant stored at -20°C until used.

Processing of hypobiotic (L_4) and mature H. contortus

H. contortus, recovered from experimentally infected goats as described above, were separately pooled per treatment as inhibited and mature stages. They were washed twice by centrifugation with distilled water. Ten (10) mature clean parasites from each treatment were suspended in 2 ml of 70% ethanol before drying at 80°C to constant weight which was recorded. The process was repeated for the arrested larvae only that 100 larvae were used to compensate for small size. After cooling to room temperature, equal weights of the dried parasites from each developmental stage and treatment were re-suspended in 5 ml 70% ethanol and ultrasonically homogenised as described above. The process was repeated for each of the four treatments.

Thin layer chromatography and estimation of trehalose content in samples

Stress induced sugars namely; trehalose, mannitol and glycerol in stressed L_3, were determined by thin-layer chromatography (TLC) as described by Managbanag and Torzilli (2002). Digital images of the developed thin layer chromatogram were analyzed by UN SCAN IT gel computer software (Silk Scientific, USA) to estimate relative quantities of individual spots by peak integration analysis. Three images of each chromatogram were analysed to obtain the average relative quantities.

Statistics and data analysis

Worm counts (mature and immature) after $\log_{10}$ X transformation and trehalose content (Av. pixels) were subjected to two way analysis of variance (ANOVA) using Genstat (2007) with a level of significance set at 5%. Means were separated by the least square difference (LSD) procedure (Steal and Torrie, 1987). In each case, the mean percentage composition of arrested larvae recorded in various treatments was compared to those recovered from the unstressed control group by Chi-square test. Pearson product moment correlation was computed to establish the association of trehalose content and hypobiosis (Genstat, 2007).

RESULTS

Temperature was gradually raised from 27 - 34.5°C in T_2 and T_4. On the other hand the stress procedure resulted in a moisture reduction of at least 93% in treatment T_3 and T_4. These two factors constituted the stress either singly or in combination.

Differences in sugar profile of L_3 of *H. contortus* were evaluated by means of TLC and densitometric analysis. Although no qualitative differences in the sugar profile could be detected between stressed and non stressed larval extracts (Figure 1), quantitative differences were

Table 2. Relative quantities (Average pixels) and percentage change in different sugars in infective (L_3) larvae of *H. contortus* subjected to different combination of temperature and moisture stress

	Sugars and percent change					
Treatment	**Trehalose (Av pixels)**	**% change**	**Mannitol (Av pixels)**	**% change**	**Glucose (Av pixels)**	**% change**
T_1	7.8[a]	-	21.1[a]	-	25.2[a]	-
T_2	10.2[a]	30.8	23.3[a]	10.4	24.1[a]	-4.4
T_3	23.4[b]	200	22.8[a]	8.0	23.4[a]	-7.1
T_4	27.2[b]	248	24.0[a]	13.8	25.4[a]	0.8

[ab]Means with the same superscript in the same column are not significantly different at p = 0.05.

Table 3. Number of *H. contortus* recovered from goats 24 days after trickle infection with a total of 12,000 live infective larvae either stressed or unstressed.

	Worm counts			% arrested (Hypobiosis)
Treatment	**Total**	**Mature**	**Arrested**	
T_1	4,376[a]	3,640[a]	735[a]	16.8[a]
T_2	3,640[a]	2,600[a]	1,064[b]	29.6[b]
T_3	1364[b]	800[b]	586[c]	43.6[c]
T_4	794[c]	460[c]	394[c]	49.6[c]

[abc]Means with the same superscript in the same column are not significantly different at p = 0.05.
** derived as the proportion of the immature to the total established (percentage).

identified in trehalose levels of the stressed larvae, where trehalose levels (Table 2) increased by 30, 200 and 248% in T_2, T_3, T_4,respectively. This increase in trehalose levels was found to be statistically highly significant (p = 0.004) especially in T_3 and T_4. In contrast, there was no significant (p = 0.453) differences in either mannitol or glucoselevels among treatments.

Following infection of the experimental animals, eggs were first detected in the control group 19 days post infection and by the 21st day, all the groups were positive for nematode eggs in their faeces. Animals especially from T_1 and T_2 exhibited signs of acute haemonchosis characterised by anaemia and diarrhoea. Post mortem recovery of worms from the abomasa of the infected animals 24 days post infection revealed that animals infected with stressed L_3 had significantly lower parasite load, than the control. Examination of individual treatment means (Table 3) showed that stress involving desiccation (T_3 and T_4) had significantly (p = 0.007) lower establishment than the control (T_1) and temperature stressed larvae (T_2). This trend was also observed in the number of arrested and mature parasites recovered from the abomasa of the infected animals. This resulted in proportionally (% hypobiosis) high levels of hypobiotic larvae from goats infected with stressed L_3 compared to controls. Separation of means by LSD technique revealed that temperature stress in presence of adequate moisture did not significantly (p = 0.126) lead to a higher % Hypobiosis than the controls.

Figure 2 shows the results of the evaluation of pooled hypobiotic and pooled mature *H. contortus* extract for trehalose by TLC. As indicated by the arrow, trehalose was detectable in both the hypobiotic and mature worm extracts. However, hypobiotic larvae had x4 relative trehalose content (Table 4) compared to the mature parasites (average pixel of 48.3 and 11.6 in immature and mature parasite, respectively). Individual treatment evaluation for trehalose content in mature and hypobiotic *H. contortus* was also evaluated. As shown in Table 4, it was generally found that relatively higher content of trehalose was observed in hypobiotic larvae derived from stressed L_3 compared to those from the control group. Significant (p = 0.0024) differences in relative trehalose content between treatments was also detected with the highest amount accumulated in (hypobiotic) larvae subjected to combined temperature and moisture stress (T_4). In contrast, there were no significant (p = 0.854) differences in trehalose content of mature parasites between treatments. Figure 3 shows the regression analysis of trehalose content of L_3 against the observed hypobiosis in goats was conducted to detect the relationship between the two parameters. It was revealed that hypobiosis and accumulated trehalose in L_3 were strongly positively correlated (r = 0.939).

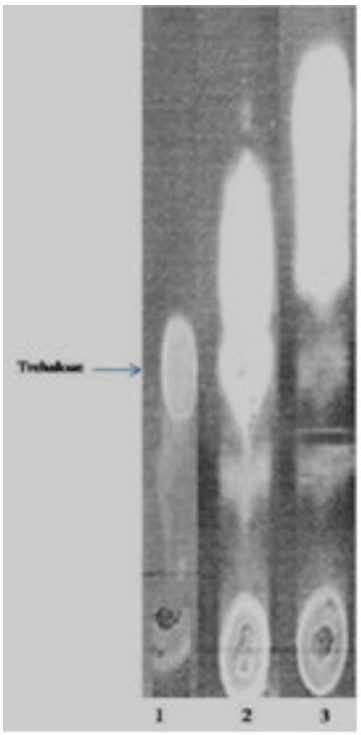

Figure 2. Thin layer chromatogram of standard –trehalose (lane1) spotted alongside homogenate of hypobiotic (lane 2) and mature (lane 3) *H. contortus*.

Table 4. Relative Trehalose content in arrested and mature *H. contortus* from goats infected with infective larvae subjected to different combination of temperature and moisture stress

	Stage of development	
Treatment	**Arrested (L_4) larvae (n = 5)**	**Mature(n = 5)**
T_1	25.9[a]	9.8[a]
T_2	27.6[a]	11.1[a]
T_3	61.3[b]	12.2[a]
T_4	67.8[b]	11.9[a]
Pooled sample	47.3	11.6

[abc]Means with the same superscript in the same column are not significantly different at p = 0.05

DISCUSSIONS

The objective of this study was to artificially induce hypobiosis by exposure of L_3 to simulated environmental factors and to investigate the possible association of concurrently accumulated trehalose with hypobiosis of *H. contortus* in goats. The results obtained from the study showed L_3 subjected to temperature and moisture stress accumulates trehalose. As a eukaryotic organism, the result is consistent with findings from previous observations in plants (Ingram and Bartels, 1996) and in plants and animal nematodes (Madin and Crowe, 1975; Crowe, 2002; Womersley, 1981; Watanabe et al., 2002, 2003; Leopold, 1986; Storey and Storey, 1991) who found that when stressed these organisms converted much of their dry weight into trehalose. It has been proposed that trehalose prevents phase transitions of the drylipids thus protecting the cellular membranes (Crowe and Crowe, 1990; Crowe and Crowe, 1988), stabilize proteins (DeVirgilio et al., 1994) and suppresses aggregation of proteins that have already been denatured (Singer and Lindquist, 1998) allowing renaturing of the

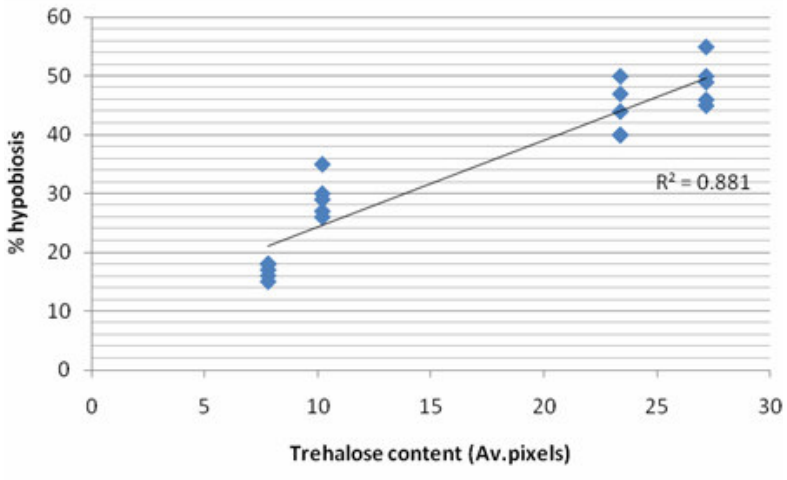

Figure 3. Relationship of trehalose content of infective larvae (L_3) and level of hypobiosis (%) of *H. contortus* in goat

proteins following withdrawal of the stressful factors. Thus it is presumed that the accumulation of the sugar in the present study was primary a response to protect the integrity of the larvae during stress.

As observed in the current study, accumulation of trehalose is not uniform across treatments. The results tend to suggest that high temperature in presence of adequate moisture may be less stressful factor than desiccation and when both factors are combined. It may simply be a reflection of a concurrent hydrolysis of trehalose resulting in low levels compared to desiccated larvae in which hydrolysis could cease completely.

Upon ingestion, the L_3 moults to the L_4 within the host and under normal circumstances, the L_4 emerges from the abomasal mucosa to undergo further moults to maturity in the abomasal lumen. It takes on average, 21 days for development to proceed to maturity from the time of ingestion. During hypobiosis, development beyond L_4 is delayed and a large proportion of the parasites are recovered still in the abomasal mucosa at L_4 stage.

The results from the present study therefore supports the supposition that exposure of infective larvae to stressful environmental factors such as reduced moisture and elevated temperatures prior to infection, influences the proportion of worms that become inhibited. Combination of the factors investigation tended to have a greater influence on the arrested larvae. When considering both factors independently, it can be noted that decreasing moisture had a stronger influence on hypobiosis than elevated temperature. This observation corroborates the finding from this study on accumulation of trehalose. Thus temperature attained during this study as an adversity on L_3 may not be an important trigger for hypobiosis on its own unless in conjunction with moisture reduction. Other studies have pointed out the same and concluded that in the tropics, hypobiosis is related to dry conditions rather than elevated temperatures (Pandaey, 1990; Eysker and Pandey, 1989; Ndao et al., 1995).

Following the above arguments, the presence of the sugar in arrested larvae observed in the current study may indicate persistent stress stimuli provided by the animal environment. Although elevated temperature may constitute the persistent stress, additional insults to the exsheathed L_4 such as host immune response could contribute to further accumulation of trehalose.

As hypothesized in the current study, the level of trehalose is positive correlated with hypobiosis. The differences in the levels of this sugar between the arrested and the mature stages of *H. contortus* suggests that trehalose metabolism is linked to hypobiosis. Biochemically, past research has focused more on the protein profiles in induced and non induced larvae (Kooyman and Eysker, 1995; Dopchiz et al., 2000; Langrova et al., 2004) have provided the first description of enzymatic activity of *Trichostrongylus colubriformis* induced for hypobiosis. It is assumed that the proteins including the enzymes are involved in a process that leads to hypobiosis and possibly influences the progression from L_4 to maturity. It can be speculated that trehalose in stressed larvae has to be hydrolysed to levels compatible with development to maturity. This means that, it may be important to have active trehalase (or any other enzyme) present once the stress is alleviated to reverse the state for development to continue. Indeed, this preposition was advanced by Singer and Lindquist (1998) who suggested that accumulated trehalose in stressed organisms must be degraded rapidly after the stress has ended to allow refolding of the denatured proteins by molecular chaperones. It is likely that the delayed development to maturity observed in the current and previous studies is

linked to trehalose concentration in such way that the refolding process is slowed thus affecting the resumption of development to maturity within the normal period of 21 days. If this concept is true, then the rate of enzymatic trehalose degradation would most likely determine the rate of resumption of development to maturity especially if the enzymes themselves are affected. Since the worms are known to spontaneously resume development, it is likely that the trigger for further development is the reduction of trehalose to levels compatible with normal development.

Conclusion

From the observations made in this study it can be concluded that there is a strong association between trehalose metabolism in stressed L_3 of *H. contortus* and hypobiosis of this parasite in goats. The differences between the trehalose levels in stressed L_3 as well as in hypobiotic larvae and fully devoloped *H. contortus* tends to suggest that degradation of accumulated trehalose may be the link to resumption of development. It is suggested that further research be conducted focusing on trehalase activity at all levels of parasite development to further describe the molecular factors underlying hypobiosis.

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