Current Research Journal of Biological Sciences 3(2): 88-94, 2011
ISSN: 2041-0778

Received: September 27, 2010 Accepted: October 23, 2010 Published: March 05, 2011

Changes in Lipids Utilisation During Moisture and Temperature Stress of Infective (L3) and its Implication on the Epidemiology of *Haemonchus contortus* in Arid and Semi Arid Lands

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Abstract: A study was undertaken to establish the pattern and level of lipid utilization in third stage infective larvae (L_3) of *Haemonchus contortus* under temperature and moisture stress conditions. The stress factors were representative of an arid and semi arid site in Kenya. The L_3 were subjected to gradual increase and reduction in temperature and moisture, respectively, using a programmable cold/heat testing chamber. Optical density per area (Corrected average pixels) derived from image analysis of individual stained larvae using an image analysis software (UN SCAN IT gel$^{(r)}$), were used to estimate the lipid content of larvae subjected to different stress treatments. It was observed that lipid content of the L_3 declined and was negatively correlated with duration of exposure with significantly ($p = 0.012$) lower rates in moisture compared to temperature stressed L_3. It was also observed that the decline occurred in phases signifying possible adaptive physiological process aimed at preserving lipid reserves and viability. During revival, there was a drastic decline in lipid reserves probably as a result of increased lipid utilisation by the reviving larvae. The epidemiological significance of these findings in field larvae is discussed.

Key word: Lipid reserves, *Haemonchus*, temperature, moisture, stress

INTRODUCTION

In arid and semi arid environments in the tropics, the third stage infective larvae of trichostrongyles often encounter very extreme weather conditions such as desiccation and high temperature. Consequently, the nematodes have developed strategies to survive these adverse environmental conditions. They are, for example, known to undergo phases of behavioural and metabolic adaptations, characterized by extended periods of reversible inactivity termed as cryptobiosis (Womersley, 1987; Clegg, 2001). They revive when conditions become favourable. The infective, non-feeding L_3 stage, lives on stored substrates such as neutral lipids or glycogen (Barret *et al*., 1976; Lee and Atkinson, 1996). These limited energy stores are normally used during host finding and for infection (Selvan *et al*., 1993; Medica and Sukhdeo, 1997). Therefore, the lifespan of the L_3 will relate to its ability to mobilise the energy reserves at a rate sufficient to meet non-growth (survival) demands. Excessive mobilisation in environments not conducive to infection of the target host by L_3 would constitute wasteful energy consumption and impact negatively on survival of L_3 and subsequent chance of infection (Wharton, 2002). Therefore, behavioural adaptations and phases of anhydrobiosis (Womersley and Ching, 1989; Siamba *et al.*, 2009) would most likely influence the pattern of lipid utilization that favours survival. The purpose of the present study was to quantitatively evaluate and outline the pattern of the lipid reserves of *H. contortus* under adverse conditions characteristic of arid and semi arid environments in the tropics.

MATERIALS AND METHODS

Study site: The study was conducted at the National Animal Husbandry Research Centre- Naivasha, Kenya from May 2007 to February 2008. The centre is situated at an elevation of approximately 1700 m above sea level and has a semi-arid climate with strong desiccating winds (upto 13 m/sec) during the dry season. The area with weather characteristics as described by Waruiriu *et al*. (1998) and Gatongi *et al*. (1998) is classified as semi arid.

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 (L_3) larvae of

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Table I: Experimental design and treatments

	Temperature	
Moisture	Low	High
Moist	T_1 (LM)	T_2 (HM)
Dry	T_3 (LD)	T_4 (HD)

T_1: Low temperature, high moisture (LM); T_2: High temperature, high moisture (HM); T_3: Low temperature, low moisture (LD); T_4: High temperature, low moisture (HD)

H. contortus for the experiments were obtained by culturing faecal material from donor goats artificially infected with parasite monoculture. Faeces were cultured at 27ºC for 10 days. The third stage larvae (L_3) 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) 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 representative of the study site, as a substrate for the larvae. About 5000 *H. contortus* L_3 larvae aliquots in 4 mL of distilled water (dH_2O) 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 dH_2O was dispensed in each of the remaining 48 dishes.

The dishes were randomly assigned to four equal treatments (25 seeded dishes each) groups (T_1, T_2, T_3 and T_4). 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*. (2010) (Table 1).

Recovery from substrate and staining of larvae: 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 staining as follows: the larvae suspension in the test tubes was vigorously agitated to ensure even suspension. Two (2) separate 1 mL aliquots were transferred to a 25 mL measuring cylinder by means of a graduated 1 mL pipette with a wide delivery aperture. Distilled water was added to the 20 mL mark and the suspension was mixed by bubbling air through it from the pipette. Once adequately mixed, 1 mL was taken out and 0.1 mL delivered on to a microscopic slide. This process was repeated five times so that the total suspension dispensed was 0.5 mL. Each drop was spread out and covered by a cover slip. The total larvae counted in 0.5 mL were multiplied by 20 (the final volume in which the original number of L_3 were now diluted) and the results expressed as the number of infective larvae per mL.

Suspensions of larvae from each treatment groups (approximately 3000 L_3/ mL) were dispensed into clean test tubes and two (2) mL of saturated solution of Oil Red O stain (No 0-0625, Sigma Chemical Co., St Louis, MO. USA) in ethanol was added. The stain was made by adding 3 g of Oil Red O powder to100 mL of 95% ethanol as described by Stamps and Linit (1995) and stirred for 20 min. The solution was then filtered through a vacuum filter apparatus using a Whatman no. 2 paper filter.

The flooded larvae were held at 60ºC for 20 min in a shaking water bath. After cooling, the excess stain was pippated off. The stained larvae were washed through a Whatman no. 4 paper filter to retain the larvae on the paper.

Estimation of lipid reserves: The lipid content of larvae subjected to different treatments was estimated by densitometry following modification of the method of Croll (1972) and recorded as average pixels (measure of optical density) of individual stained worms as follows: clean stained larvae were washed off the filter paper into a test tube and a sample transferred to clean standard multi-cavity microscope slides. Digital photographic images of individual stained larvae (n = 10) were obtained using a Sonny DSC-W100 digital still camera mounted on a Wild M11 light microscope. The digital images were transferred to a computer and thereafter, analyzed by UN SCAN IT gel® software (Silk Scientific USA). Relative lipid content of each individual stained larva was estimated as average pixels after adjustments for larval size and background as illustrated in Table 2.

Statistical analysis: Data analysis was carried out using Genstat (2007). Analysis of variance (ANOVA) was used to analyze lipid content during stress and revival cycles, overall lipid depletion and overall % viability. Means were separated by the Least Square Means method (Steel and Torrie, 1980). Trend analysis was carried out by the moving average method as described by (Gupta, 2002). Correlations between lipid utilization and larval activity was evaluated by regression analysis.

RESULTS

Oil Red O stained *H. contortus* larva is presented alongside unstained larva in Fig. 1. Stained lipids appeared as pinkish droplets distributed under the cuticle and around the intestinal tract of the larvae stretching from the tail to the bucal cavity.

Lipid content estimated during the experimental period for different stress treatments during the experimental period is presented in Fig. 2. The lipid

Table 2: Illustration of image analysis and computation of the average pixel as a measure of lipid content

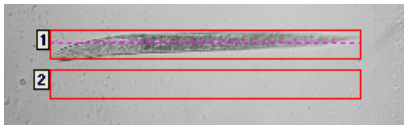
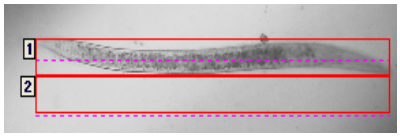
	Segment	Pixel totals	Segment size	Average
Larva No 1	1- Larva and background	3883431	108682	35.73
	2- Background	615446	108682	5.66
	Relative lipid content larvae No. 1			30.07
Larva No 2	1- Larva and background	5064667	101520	49.89
	2- Background	1606948	101520	15.83
	Relative lipid content larvae No. 2			34.06

Fig. 1: Comparison of Oil Red Ostained (a-left) and unstained (b-right) *Haemonchus* larvae

content of the L_3 declined over the experimental period. The lipid content was negatively correlated with the exposure time for all the treatments. The models describing the change in lipid content with time fitted the data closely as indicated by the coefficients of determination (r^2 = 0.996, 0.937, 0.812 and 0.714 for treatments T_1, T_2, T_3 and T_4, respectively). There were also significant ($p = 0.012$) differences in the rates of decline (lipid utilization) between treatments, with the slowest rate recorded for T_4 followed by T_3.

Exploration of individual treatment data (Fig. 3a, b, c and d) revealed that whereas there was a steady decline in lipid content in unstressed larvae (T_1), the lipid utilization in stressed larvae (T_2, T_3, T_4) occurred in phases. The first phase was demonstrated in the first 48 h during which there was a more than twofold increase in lipid utilization in stressed larvae compared to the unstressed group. The rate of lipid depletion in this period was significantly ($p<0.05$) higher in stressed compared to unstressed larvae. This trend was however reversed during the second phase that occurred beyond 48 h. There

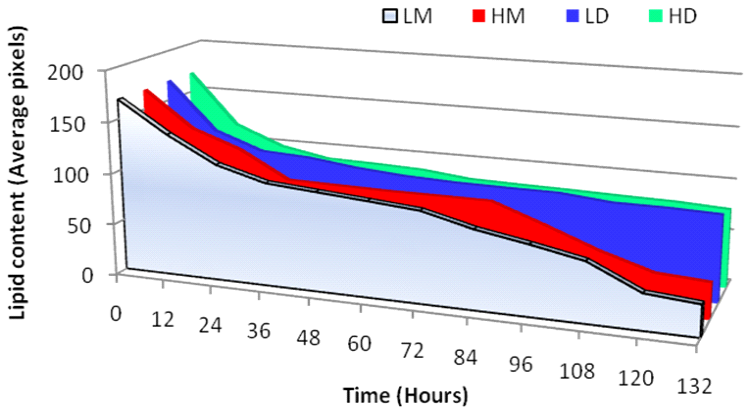


Fig. 2: The general trend of lipid content of *H. contortus* L_3 larvae subjected to different stress factors, T_1: Low temperature, high moisture (LM); T_2: High temperature, high moisture (HM); T_3: Low temperature, low moisture (LD); T_4: High temperature, low moisture (HD)

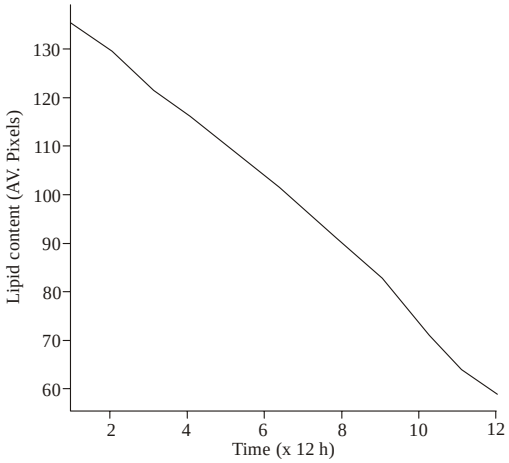


Fig. 3a: Pattern of lipid utilization in Unstressed (LM) *H. contortus* L3 larvae

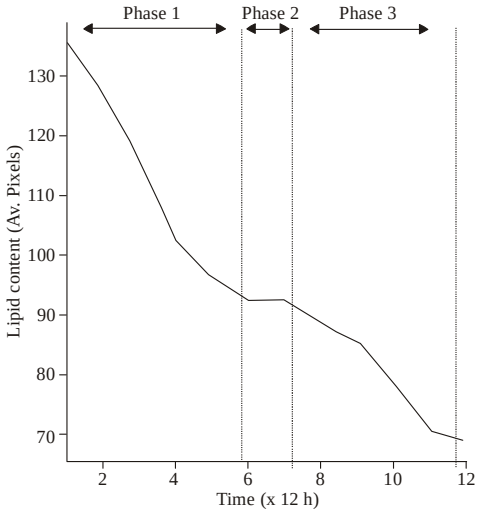


Fig. 3b: Pattern of lipid utilization in Temperature (HM) stressed *H. contortus* L3 larvae

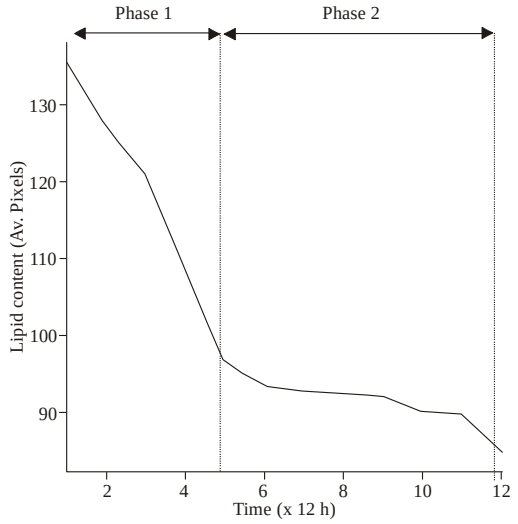


Fig. 3c: Pattern of lipid utilization in Moisture (LD) stressed *H. contortus* L_3 larvae

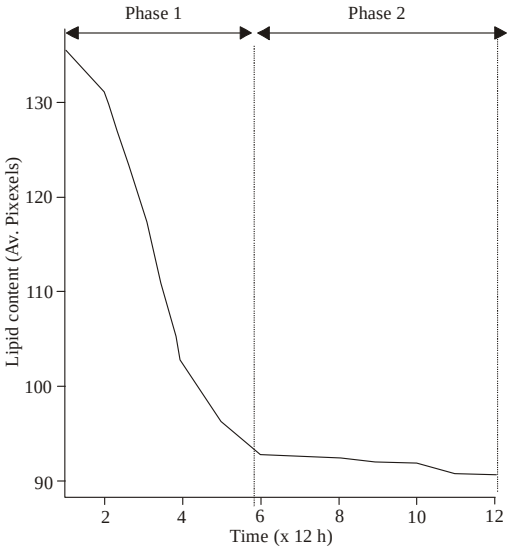


Fig. 3d: Pattern of lipid utilization in combined temperature and Moisture (HD) stressed *H. contortus* L_3

T_1: Low temperature, high moisture (LM); T_2: High temperature, high moisture (HM); T_3: Low temperature, low moisture (LD); T_4: High temperature, low moisture (HD)

was a sharp reduction in the rate of lipid utilization in stressed larvae to significantly ($p<0.05$) lower levels compared to the unstressed. This low levels persisted for the rest of the study period especially treatments involving moisture stress (T_3 and T_4). The heat stressed larvae (T_2) however recovered within 36 h to resume lipid utilization pattern similar to the unstressed larvae (T_1).

Mean lipid utilized during stress and revival cycles, rate of lipid utilization and the percentage of the original content utilized is presented in Table 3. It was observed that stress had a significant effect on overall lipid utilized as indicated by the percentage of the original lipid content per treatment. It was however noted that there were significant ($p = 0.0012$) differences in lipid utilization during stress and revival with more lipids used during revival. Ultimately, the unstressed (T_1) parasites had lower lipid content than the stressed parasites. This difference was however only significant ($p<0.05$) when control (T_1) was compared to treatments that involved moisture stress (T_3 and T_4).

Table 3: Mean total lipid units (pixels) utilized during stress and revival sessions, computed rate of lipid depletion and overall percentage of the original lipid utilized in 132 h

	Lipid utilization			
Treatment	Stress session (N = 10)	Revival session (N = 10)	Mean rate of use (Units/h)	Overall % lipid utilized
T_1 (LM)	4.86 ^{a1}	4.26 ^{a1}	0.583^{a}	56.04^{a}
T_2 (HM)	3.42 ^{a1}	4.48 ^{a1}	0.466^{a}	49.74^{a}
T_3 (LD)	1.43^{b1}	8.80 ^{b2}	0.349^{b}	37.74^{b}
T_4 (HD)	1.03^{b1}	9.23 ^{b2}	0.285^{b}	32.83^{b}

T_1: Low temperature, high moisture (LM); T_2: High temperature, high moisture (HM); T_3: Low temperature, low moisture (LD); T_4: High temperature, low moisture (HD)

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

[1]: Means with the same superscript in the same row are not significantly different at p = 0.05

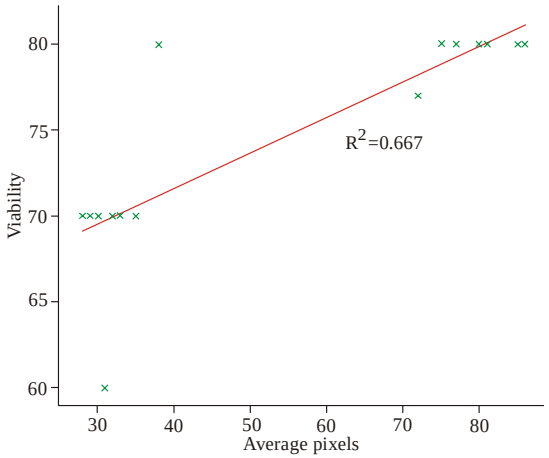


Fig. 4: Correlation between the lipid content and viability in stressed L_3 of *H. contortus*

Repeated cycles of stress and revival of L_3 resulted in decrease in percentage of larvae surviving each cycle (Table 3) especially for the desiccated groups. It was observed that after a few cycles, the percentage surviving each cycle remained constant. This resulted in significantly higher viability in desiccated larvae compared to either unstressed or temperature stressed larvae. Regressing viability of larvae against lipid content (average pixels, Fig. 4), revealed that viability was positively correlated (R^2 = 0.667) to the lipid content.

DISCUSSION

The study was undertaken to establish the pattern and level of lipid utilization in L_3 of *H. contortus* under various types and intensities of stress conditions. The stress factors included temperature, moisture and combined temperature/moisture modelled around weather characteristics of a typical arid and semi arid area. The larvae were subjected to gradual increase and reduction in temperature and moisture, respectively, using a programmable cold/heat testing chamber.

Results from the study demonstrated that lipid content of the larvae declined with time and with increased intensity of stress. The pattern was however different in stressed compared to unstressed larvae. The decline (depletion) reflected the lipids utilized by the larvae during the experiment. The lipid utilization in stressed parasites occurred in distinct phases of enhanced depletion followed by depressed lipid metabolism. This could be an indication that under stress, larval activity occurred with a similar pattern. According to Rossanigo and Grunner (1994), under natural and optimal conditions, the lipid is utilized for mobility away from the faecal materials and in search of a host animal and as such, the lipid content will decline in tandem with the larval activity. In the present study, as the conditions become increasingly unfavourable, additional energy was mobilized for initial short term survival mechanisms such as migration to shaded, cool and moist locations. This move increased the rate of lipid utilization and may account for the first phase demonstrated in the Fig. 3a, b and c.

H. contortus larvae subjected to temperature stress under moist environments, were able to recover from stress effects to resume normal lipid metabolism. This behaviour is probably due to reprogramming and expression of genes coding for stress proteins and energy metabolism enzymes to cushion the parasites from deleterious effects of stress. Thus, acclimation described in *Ostertagia circumcincta*, (Walker *et al*., 2007) most likely also operates in *H. contortus*. 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 45ºC). These glycolytic enzymes, including hexokinase, pyruvate kinase and malate dehydrogenase may similarly be expressed in *H. contortus* for the same purpose (Watanabe *et al.*, 2007). This behaviour may partially explain the ability of the parasites to survive in the host animal despite the drastic change in temperature around 22ºC in the environment to 37ºC in the host.

Unlike the distinct three phase response in heat stress in moist environment (T_2), lipid metabolism did not recover in desiccated larvae both at low and increasing (high) temperature (T_3 and T_4). The parasites tended to switch to long-term but seemingly reversible mechanisms aimed at conserving energy while preserving life. During this phase, lipid metabolism was negligible in stressed larvae. These results suggest that although desiccated parasites may attempt to undergo acclimation as stated above, recovery is only possible in the presence of adequate moisture. Otherwise the parasites drift into anhydrobiosis, a phenomenon characterized by severe but

reversible depression of larval metabolic activity in response to gradual desiccation (Watanabe *et al*., 2007; Womersley and Ching, 1989; Lettini and Sekhudeo, 2006)

As discussed in the preceding section, lipid utilization in stressed larvae occurred in distinct phases. The first phase, similar in all stress treatments, was characterized by an enhanced lipid depletion. The enhanced depletion was however, associated more with reviving than the stressed larvae. This finding was consistent with the results of Udonsi (1983) who showed that lipid depletion in *Necator americanus* was significantly higher in reviving larvae following desiccation. This, most likely, results from increased energy demand for increased activity during revival. The lipid contents of larvae (treatments) with low viability after repeated stress cycles would seem to support the preposition that lipid reserves are certainly an essential factor as energy substrate during revival.

It is thus clear that such repeated desiccation-revival cycles, occasioned by diurnal fluctuations in pasture moisture, would lower the viability of the larvae on account of continuous depletion of irreplaceable lipid reserves. This and other factors may account for larval susceptibility to drought and may partially contribute to low worm burdens especially under field conditions during the dry seasons.

The study has elucidated the pattern of lipid utilization in *H. contortus* larvae under various stress conditions and also demonstrated that this is a possible mechanism, stressed *H. contortus* larvae adopts to preserve lipid reserves and viability. This adaptation may have a significant influence on the epidemiology of this parasite in arid and semi arid environments and may partially explain the spread and persistence of haemonchosis in such environments

Besides the elucidation of the pattern of lipid utilization in *H. contortus* larvae under various stress conditions, the study has also demonstrated the possible mechanism, a stressed *H. contortus* larvae adopts to preserve lipid reserves and viability. This adaptation may have a significant influence on the epidemiology of this parasite in arid and semi arid environments and may partially explain the spread and persistence of haemonchosis in such environments.

ACKNOWLEDGEMENT

The authors wish to thank the Centre Director at the National Animal Husbandry Research Centre Naivasha for the logistical support. We are grateful to the Director KARI for availing funds for this work from the Kenya Agricultural Productivity Project (KAPP)

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