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Effects of heat stress and a low energy diet on blood parameters and liver hsp70 and iNOS gene expressions in local chickens

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ABSTRACT

Two experiments were conducted to compare effects of heat stress and its combination with low dietary energy on blood indices, liver hsp70 and iNOS gene expressions in three Tanzanian local chicken ecotypes. In experiment one, five weeks old Kuchi (K), Ching'wekwe (C) and Morogoro medium (M) were randomly allocated to separate pens in a 3 × 2 factorial design in two adjacent rooms with controlled temperature. The study had three replicates consisting of 39 chickens per room, 13 per ecotype per pen making a total of 234 chickens. In one room, temperature was maintained at 26.5 ± 0.5 °C while in another it was maintained at 32±1 °C for 7 days and thereafter 37±1 °C for 10 days. A similar design was used in experiment two except that chickens were fed 55% less energy. In experiment one, serum corticosterone levels increased ($p<0.05$) in C and K. Gene expressions for hsp70 and iNOS were unchanged though hsp70 levels for K were higher ($p<0.05$). In experiment two, corticosterone levels were significantly elevated ($p<0.05$) in all ecotypes. Heterophil/lymphocyte ratios were markedly increased and changes in Hb and Hct at higher temperatures showed ecotype differences. Serum triglycerides were significantly reduced in all ecotypes. Hsp70 and iNOS levels were up-regulated in all ecotypes with levels in K higher ($p<0.05$) than in M. In both experiments, there were marked reductions in serum total protein. These results suggest that ecotype-based differences exist in local chickens' responses to heat stress and its combination with low energy diets. M and C demonstrated better tolerance than K when only heat stress was applied but a synergistic effect of heat stress and low dietary energy suggested M is more tolerant.

Abbreviations

C	Ching'wekwe
K	Kuchi
M	Morogoro medium
Hb	Hemoglobin
Hct	Hematocrit
H/L	Heterophyl/lymphocyte ratio
GIP	Genomics to improve poultry

1. Introduction

Local chickens, as an important source of income and protein, are widely reared by a majority of rural and peri-urban households in many developing countries like Tanzania (Queenan et al., 2016). The chickens are seasonally faced with stressors, mainly in the form of elevated temperatures, low quality nutrition and disease (Ayo, Obidi & Rekwot, 2011; Mwalusanya et al., 2001). Stressors contribute to the low production capacity in this sector. Although local chickens are better adapted to the harsh environments in areas where they are reared and can produce under conditions where exotic breeds may not survive, ecotype-differences in production performance, low dietary energy stress tolerance (Khondowe, Mutayoba, Muhairwa & Phiri, 2018) and disease resistance have been reported (Lwelamira, 2012; Msoffe et al., 2002). With current efforts aimed at improving local chicken production systems to foster income generation and improve food security of households (Queenan et al., 2016), studies aimed at identification of local chicken ecotypes which show better performance traits when exposed to various common stressors are highly recommended. These will fill the missing gaps useful in selection.

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When chickens are exposed to stressors, such as temperatures above the thermal comfort zone and/or low dietary energy, there is a deviation from physiological homeostasis, leading to impairment of the bird's well-being (Cheng & Jefferson, 2008) and marked reduction in production capabilities (IUCN, 2010). Similarly, biochemical parameters in the blood may reflect the physiological state of the birds (Hrabcakova et al., 2014; Lin, Du, Gu, Li & Zhang, 2000). Heat stress has been shown to increase plasma glucose levels (Garriga et al., 2006; Lin et al., 2000), body core temperature (Soleimani, Zulkifli, Omar & Raha, 2011), and alter the electrolyte balance and blood pH (Van Goor et al., 2016) in commercial broilers and layers. The hypothalamus-pituitary-adrenocortical axis is usually activated, leading to a rapid increase in circulatory corticosterone levels (Akbarian et al., 2016; Quinteiro-Filho et al., 2010; Rimoldi et al., 2015). The release of corticosterone and other glucocorticoids causes dissolution of lymphocytes in lymphoid tissues leading to lymphopenia and eventual increase in heterophil release by the bone marrow (Borges, Fischer da Silva, Majorka, Hooge & Cummings, 2004; Zulkifli & Siegel, 1995). In addition, several blood parameters, including hemoglobin (Hb), CO_2 levels and saturated O_2 are varied under heat stress and may be good candidates for predicting response to heat stress and for use as biomarkers of heat tolerance (Lamont et al., 2015).

Stress can lead to posttranscriptional changes to signaling genes and disruption of the health of an animal at the genetic level (Allen & Tresini, 2000; Fleming et al., 2016; Khondowe et al., 2018). The vulnerability of poultry to stress varies according to genetic potential, life stage and nutritional status (Felver-Gant, Mack, Dennis, Eicher & Cheng, 2012; IUCN, 2010; Soleimani & Zulkifli, 2010; Tamzil, Noor, Hardjosworo, Manalu & Sumantri, 2014). Heat stress retards synthesis of most proteins but heat shock proteins (Hsps) are rapidly synthesized (Al-Aqil & Zulkifli, 2009). It has been observed in previous studies that liver hsp70 concentrations are increased in hens exposed to heat stress (Felver-Gant et al., 2012). In broiler chickens for example, hsp70 is highly induced after acute heat exposure (Lowman, Edens, Ashwell & Nolin, 2014; Xie et al., 2014; Yu & Bao, 2008). On the other hand, iNOS expression has also been shown to increase after exposure to stress in broiler chickens (Zhao et al., 2013) and ducks (Zeng et al., 2014). In a recent study in selected Ugandan and Rwandan local chickens, Fleming et al. (2016) showed that these birds have alleles which may aid in adaptation to harsh environments, including elevated ambient temperatures. Meanwhile, Indonesian village or native chicken lines were found to have an interaction with hsp70 genotypes in heat resistance (Tamzil et al., 2014). However, research information is scarce and lacking on the effects of heat stress on liver hsp70 and iNOS expression and the extent of high temperature tolerance levels in local chickens.

As a viable strategy to improve production, selective breeding of local chickens for genetic or phenotypic features associated with specific behavioural and physiological characteristics is encouraged (Cheng & Jefferson, 2008). Information on the relationships between biochemical and hormonal homeostasis in local chickens when responding to stressful stimulations caused by high temperatures and low energy diets is needed. In the present study, three local chicken ecotypes, Ching'wekwe (C), Kuchi (K), and Morogoro medium (M), which represent unique local dual-purpose chickens raised under free range in the mid and northwest Tanzania were recruited. The hens have average adult body weights ranging from 1746 g to1910 g for the K ecotype, 1048 g to 1168 g for the M ecotype, and 1371 g to 1442 g for the C ecotype (Msoffe et al., 2001). The age at first lay in all the ecotypes ranges from 6 to 8 months (Mwalusanya et al., 2001). The assumption is that the differences in resistance to disease between ecotypes as shown by previous studies may be reflected in their responses to heat stress and in combination with a low energy diet. Thus, the objective of the current study was to compare and investigate the effects of heat stress and a combination of heat stress and low dietary energy on blood parameters, liver hsp70 and iNOS gene expressions in Tanzanian local chicken ecotypes. Low dietary energy was included as a stressor in order to mimic the natural conditions as these chickens are faced with a seasonal combination of stressors in areas where they are bred. Identification of physiological traits that have ecotype-specific differences in response to stress will provide additional information needed for selection of local chickens that perform better under high ambient temperatures.

2. Materials and methods

2.1. Birds and management

Day-old chicks from the M, C and K local ecotypes were obtained from the parent flock kept by the Feed the Future GIP Project at Sokoine University of Agriculture. The chicks were brooded and reared using similar production techniques under hygienic conditions before being subjected to treatment groups. Feed and water were supplied *ad libitum*. Initially, all chicks were fed the same diet consisting of 18% crude protein and 2864 kcal ME/kg up to when they were 5 weeks old. All chickens were vaccinated against Newcastle disease, Infectious Bursal Disease (Gumboro), and Fowl pox. Only female chicks were used to avoid sex-induced response differences.

2.2. Feed formulation

Two types of feeds were formulated the first contained 2864 kcal/kg ME and served as control diet while the second feed contained about 55% less energy than the control (that is, 1319 kcal/kg ME) and served as energy restriction diet. The basis to use 55% energy restriction as an additional stressor was from earlier studies (Khondowe et al., 2018) that evaluated the responses of the same local chicken ecotypes to low dietary energy and findings showed that this level of restriction was low enough to induce stress. Both diets were formulated using locally available feedstuffs as described previously (Khondowe et al., 2018) and chemical (proximate) analyses of different feed ingredients were carried out using standard methods (FAO, 1994).

2.3. Study design

Two studies were conducted (experiment one and experiment two) and all procedures used were in compliance with the Sokoine University of Agriculture's guidelines for care and use of animals in research and special care was taken to avoid unnecessary suffering of the chickens.

2.3.1. Experiment one

Five-week-old hens belonging to K, C and M ecotypes were weighed and randomly allocated into separate pens in two adjacent rooms with controlled temperature. Each room had three pens, each having an average area of 2.5 m^2 floor space per 13 birds and rice husks were used as litter material. A 3 (3 ecotypes) x 2 (2 different ambient conditions) factorial design was used and the study had three replicates consisting of 39 chickens per room, 13 per ecotype per pen making a total of 234 chickens. The rooms were artificially lit with a 10 Light: 14 Dark cycle. To acclimatize to their new environment, all chickens had ad libitum access to water and feed consisting of 18% crude protein and 2864 kcal ME/kg, and were maintained at normal ambient temperature of 26.5 ± 0.5 °C for 5 days. At the start of the study, the same ambient temperature was maintained in one room (control) during the whole period of study, which consisted of 17 days. In the adjacent room, temperature was raised gradually to reach 32±1 °C within 4 h and was maintained at this temperature for 7 days. After 7 days temperature was raised again and was maintained for 10 days at 37±1 °C for 8 hrs per day starting at 08:00 hrs to 16:00 hrs; all the other times the temperature was reduced to 32±1 °C. The relative humidity in the control room was maintained in the range of 60±5% whilst in the adjacent high temperature room was 50±7%.

2.3.2. Experiment two

A similar design and chicken number (234) were used except that

chickens in the high temperature group were fed with a diet formulated to contain 55% less dietary energy than the control. Low dietary energy was included as a stressor in order to mimic natural conditions whereby these chickens are faced with a seasonal combination of stressors in areas where they are bred.

2.4. Blood sampling and analysis

Whole blood was collected as described previously (Khondowe et al., 2018) via the wing vein at similar times of the day (between 10:00 and 12:00 hrs) using syringes and was immediately transferred into ethylene diamine tetracetic acid (EDTA)-containing vacutainers and/or plain vacutainer tubes (for serum preparation). During the period when birds were reared at 32±1 °C, blood was collected 6 hrs, 24 hrs, and 7 days after exposure to this temperature and when the temperature was raised to 37±1 °C blood was collected 4 hrs, 24 hrs, 7 days and 10 days after exposure. The sampling procedure lasted for about less than 1 min per bird. For serum preparation, blood samples were allowed to clot, serum separated, and stored at −20 °C until analysis. The corticosterone levels were assayed using ELISA commercial kits (Sunlong Biotech. Co. Ltd., Hangzhou, China) and measurements were done using Multiskan EX Primary EIA V. 2.3 Reader (Applied Biosystems, USA). Serum levels of uric acid, total protein, triglycerides and glucose were determined using commercial kits (Erba Diagnostics Mannheim, Germany).

2.5. Liver sampling, RNA extraction and quantitative real-time PCR

At the end of the study (17 days), 5 chickens from each pen were randomly selected, weighed and then humanly sacrificed and decapitated. Liver samples were quickly collected, weighed on a kitchen scale and placed on ice before storage at −80 °C. Total RNA was extracted from liver samples (50 mg) using the Quick-RNA™ MiniPrep Plus kit (Zymo Research, USA) and first-strand complementary DNA was synthesized from about 5 μg of total RNA according to manufacturer's instructions as described previously (Khondowe et al., 2018). Predesigned primers for hsp70, iNOS (Zhao et al., 2013) and GAPDH (Xie et al., 2014) were used and the quantitative real-time PCR (qPCR) was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA) on an ABI 7500 (Applied Biosystems USA). Reactions and the cycling protocol were performed as reported previously (Khondowe et al., 2018). The relative expression levels of the genes tested were calculated using the $2^{-\Delta\Delta Ct}$ method and were normalized to the mean expression of GAPDH, where ΔΔCt corresponds to the difference between the ΔCt measured for the mRNA level of each tissue.

2.6. Statistical analysis

The Independent Sample *t*-test was used to compare means between treatment and control groups and One-way ANOVA (SPSS 20, IBM SPSS Statistics USA) was used to analyze differences among the ecotypes. In case of detection of differences in treatment means by ANOVA, LSD and Tukey's tests for post hoc multiple comparisons were used to separate means, with significance statements based on $p < 0.05$. Results are presented as Means ± SE.

3. RESULTS

3.1. Experiment one

3.1.1. Liver hsp70 and iNOS relative gene expression

To determine the effect of heat stress on hsp70 and iNOS relative gene expression, the chickens were exposed to 32±1 °C for 7 days and thereafter to 37±1 °C (8 hrs per day) for 10 days, and the results are depicted in Fig. 1 and Fig. 2. At the end of the study (17 days), the relative gene expression of hsp70 for K was up-regulated, though not significantly whereas the expression for C and M clearly remained unchanged. The levels of expression of hsp70 for the K ecotype were markedly higher ($p<0.05$) than in C and M (Fig. 1). There was no change in the relative gene expression levels of iNOS in all ecotypes and no between-ecotype differences were observed (Fig 2).

3.1.2. Corticosterone

The results for serum corticosterone concentration of the hens at control (26.5 ± 0.5 °C) conditions for 17 days and after exposure to 32±1 °C for 7 days and 37±1 °C for 24 hrs and 10 days are shown in Fig. 3. Exposure of the chickens to 32±1 °C for 7 days caused a significant rise ($p<0.05$) in serum corticosterone for K but not C and M. Within 24 hrs of raising the temperature to 37±1 °C, K and C but not M showed a marked increase ($p<0.05$) in corticosterone levels. After a 10 day-exposure to 37±1 °C, no significant increases in serum corticosterone level were observed in all the chicken ecotypes.

3.1.3. Uric acid

The results for serum uric acid concentration of the hens at control (26.5 ± 0.5 °C) conditions and after exposure to 32±1 °C for 7 days and 37±1 °C for 24 hrs and 10 days are shown in Fig. 4. Exposure of the chickens to 32±1 °C for 7 days caused a significant reduction ($p<0.05$) in serum uric acid levels for C but not for K and M. Serum uric levels were further reduced equivocally ($p<0.05$) in all ecotypes when exposed to 37±1 °C for 24 hrs. Exposure of the chickens to 37±1 °C for 10 days

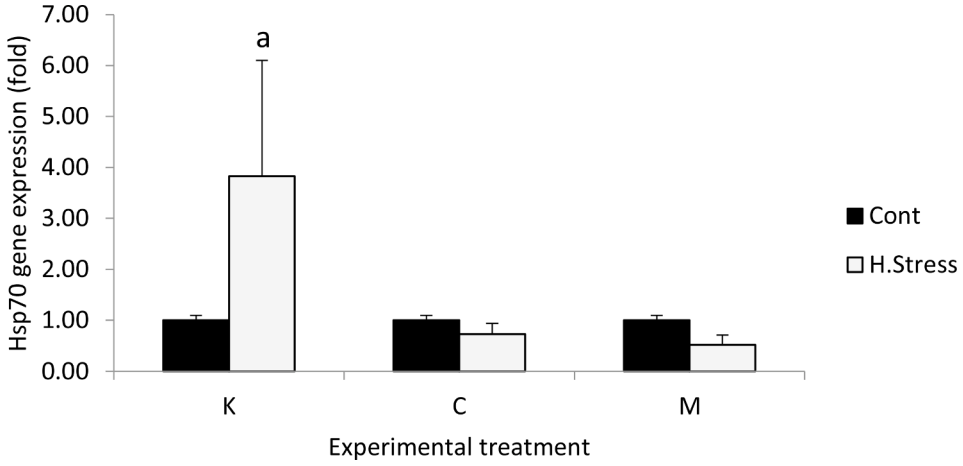

Fig. 1. Liver hsp70 gene expression at control conditions (26.5 ± 0.5 °C) and after exposure to 32±1 °C for 7 days and thereafter to 37±1 °C (8 hrs per day) for 10 days; [a]Significantly higher than C and M; K: Kuchi, C: Ching'wekwe, M: Morogoro medium; Cont: control, H.Stress: heat stress.

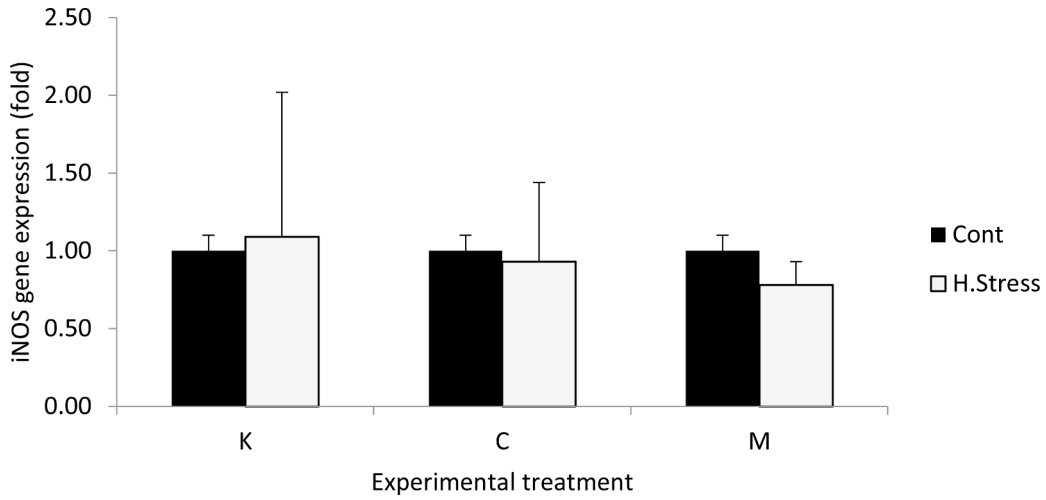

Fig. 2. Liver iNOS gene expression at control conditions (26.5 ± 0.5 °C), exposure to 32±1 °C for 7 days and to 37±1 °C (8 hrs per day) for 10 days; K: Kuchi, C: Ching'wekwe, M: Morogoro medium; Cont: control, H.Stress: heat stress.

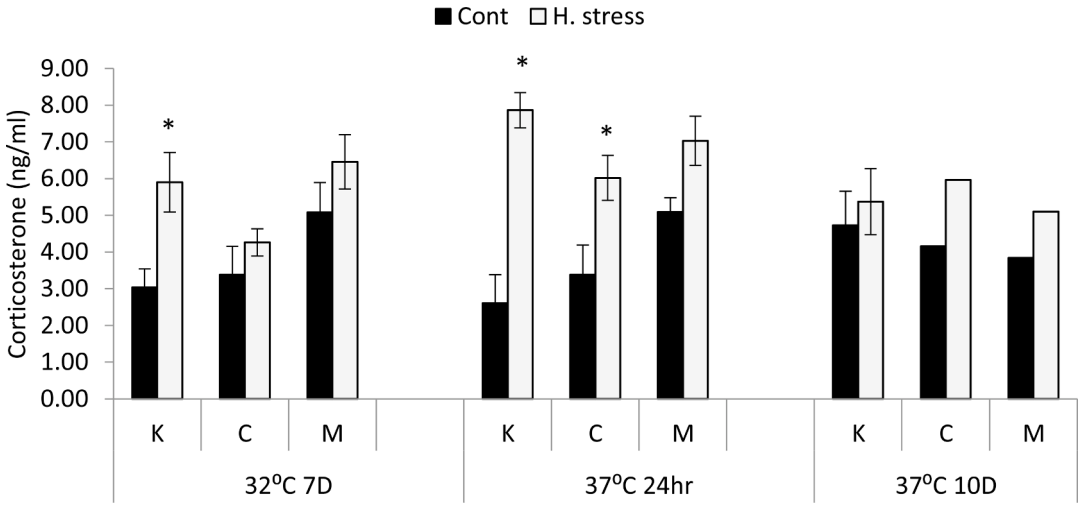

Fig. 3. Serum corticosterone concentration of the hens at control (26.5 ± 1 °C) conditions and after exposure to 32±1 °C for 7 days and 37±1 °C for 24 hrs and 10 days; *Significantly higher than the control; K: Kuchi, C: Ching'wekwe, M: Morogoro medium; D: day, Cont: control, H.Stress: heat stress.

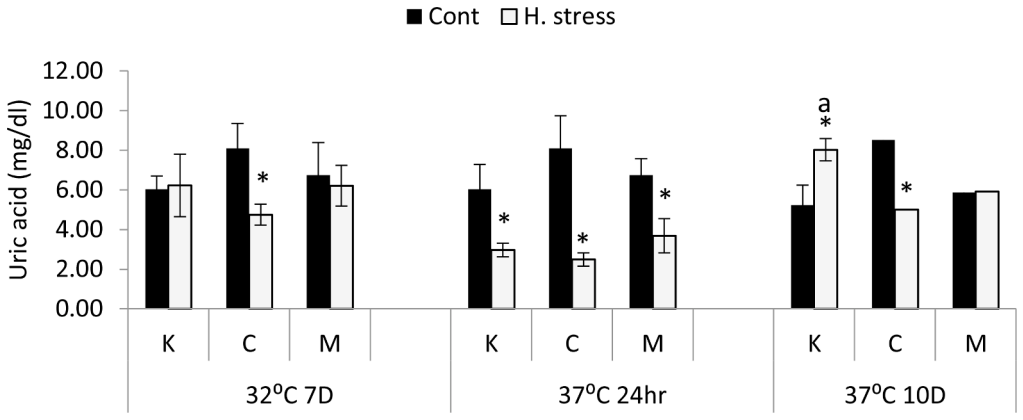

Fig. 4. Serum uric acid concentration of the hens at control (26.5 ± 1 °C) conditions and after exposure to 32±1 °C for 7 days and 37±1 °C for 24 hrs and 10 days; *Significantly different from the control; [a]Significantly higher than C; K: Kuchi, C: Ching'wekwe, M: Morogoro medium; D: day, Cont: control, H.Stress: heat stress.

led to an increase ($p<0.05$) in serum uric acid levels for K only but caused a marked decline for C and had no effect for M. Tables 1 and 2

3.1.4. Total protein, glucose and triglycerides

Results for serum total protein, glucose and triglyceride concentrations are depicted in Table 3. While serum levels for glucose and triglyceride were not changed in all ecotypes, exposure of chickens to 32±1 °C for 7 days caused a marked decrease ($p<0.05$) in total protein levels for C but not for K and M. Moreover, the baseline total protein serum levels for C were significantly higher than for K and M. While

Table 1
Composition and nutrient levels of experimental diets.

Ingredients	Control diet **2864** kcal/kg ME (%)	55% Energy **1319** kcal/kg ME (%)
Maize meal	37.8	10
Maize bran	26	2
Sun flower meal	20.5	21
Fish meal	11	22.3
Ground charcoal	0	40
Limestone	2	2
Premix[n]	0.3	0.3
Methionine	0.3	0.3
Lysine	0.3	0.3
Dicalcium phospate	1.3	1.3
Salt	0.5	0.5

[n]Vitamin-mineral premix provided the following per kg of diet: vitamin A: 8000IU, vitamin D3: 3000IU, vitamin E: 10 mg, vitamin K3: 200 mg, vitamin B12: 2.5 mg, niacin: 6 mg, pantothenic acid: 5 mg, selenium: 0.2 mg, Fe: 80 mg, Cu: 80 mg, Zn: 100 mg, and Mn: 120 mg.

Table 2
Target gene primers used in determining effects of heat stress and a low energy diet on liver HSP70 and iNOS gene expressions.

Gene	Primer set	Product (bp)	Tm (°C)
HSP70	F 5′-CGGGCAAGTTTGACCTAA-3′	250	58
	R 5′-TTGGCTCCCACCCTATCTCT-3′		62
iNOS	F 5′-CCTGGAGGTCCTGGAAGAGT-3′	82	64
	R 5′-CCTGGGTTTCAGAAGTGGC-3′		62
GAPDH	F 5′-CTTTGGCATTGTGGAGGGTC-3′	128	60
	R 5′-ACGCTGGGATGATGTTCTGG-3′		60

glucose levels were not significantly altered, there was a marked reduction ($p<0.05$) in serum total protein for C and M and a significant increase ($p<0.05$) for K after 24 hr-exposure of the chickens to 37±1 °C. Between ecotypes, total protein concentration for K was over two times higher ($p<0.05$) than C and M. Exposure of the chickens to 37±1 °C for 10 days caused marked reductions ($p<0.05$) in glucose and triglyceride levels for K but not C and M. No notable differences in serum total protein levels were observed at this stage in all the chicken ecotypes.

3.1.5. Hematological indices

The changes in levels of Hb and Hct during the study period are presented in Fig. 5 and Fig. 6. Exposure of the birds to 32±1 °C for 24 hrs resulted in significant decline ($p<0.05$) in mean Hct and Hb levels for K and M (Fig. 5). The Hb levels for K returned to control levels after one week exposure while those for M remained significantly lower ($p<0.05$) during the same period. There was no notable change in mean Hb values for C at this temperature. However, the mean Hct levels for C and K but not M were significantly increased ($p<0.05$) after one week exposure to 32±1 °C. When the temperature was raised to 37±1 °C no significant changes in mean Hb and Hct values for K and M were observed for the entire 10-day period of exposure but C showed a significant increase in Hb and Hct values within 4 hrs of temperature rise (Fig. 6). In addition, the mean Hct for C was significantly lowered ($p<0.05$) after 24 hrs of exposure.

3.2. Experiment two

3.2.1. Liver hsp70 and iNOS relative gene expression

To determine the effect of heat stress and low dietary energy on hsp70 and iNOS relative gene expression, chickens fed 55% less dietary energy than the control were exposed to 32±1 °C for 7 days and thereafter to 37±1 °C (8 hrs per day) for 10 days, and the results are depicted in Fig. 7 and Fig. 8. At the end of Study (17 days), liver hsp70 relative gene expression was significantly ($p<0.05$) up-regulated in all the ecotypes, and the levels being markedly higher in K ($p<0.05$) than in M (Fig. 7). Similarly, iNOS relative gene expression levels were greatly increased ($p<0.05$) in all ecotypes but between-ecotype differences were absent (Fig. 8).

3.2.2. Corticosterone

Changes in serum corticosterone concentration of hens in experiment two are shown in Fig. 9. Exposure of chickens fed low energy diet to 32±1 °C for 7 days induced a marked rise ($p<0.05$) in serum corticosterone for K and C but not M. Between-ecotypes, the serum levels of corticosterone for K were significantly higher ($p<0.05$) than C and M. Within 24 hrs of raising the temperature to 37±1 °C, K and C but not M showed a marked increase ($p<0.05$) in corticosterone levels. A 10 day-exposure of the chickens to 37±1 °C induced a marked increase in serum corticosterone levels in all the chicken ecotypes. At this stage there were no between-ecotype differences in serum corticosterone levels

3.2.3. Uric acid

The results for serum uric acid concentration of the hens in experiment two are shown in Fig. 10. Exposure of chickens fed low energy diet to 32±1 °C for 7 days caused a significant increase ($p<0.05$) in uric acid levels for K but not for C and M. After 24 hrs of raising the temperature to 37±1 °C, uric acid levels were not markedly changed in all the chicken ecotypes. Exposure of the chickens to 37±1 °C for 10 days led to an increase ($p<0.05$) in serum uric acid levels for K and M but not for C.

Table 3
Serum total protein (T.P), glucose (Glu) and triglyceride (T.G) concentrations of the hens at control (26.5 ± 0.5 °C) conditions and after exposure to 32±1 °C for 7 days and 37±1 °C for 10 days.

		7days		24hrs		10days
	Cont	**32°C**	**Cont**	**37°C**	**Cont**	**37°C**
	T.P(g/dl)					
K	2.96±0.27	3.62±0.44	2.96±0.27	4.04±0.19[b]*	2.71±0.47	2.56±0.34
C	5.46±0.59[a]	3.30±0.9*	5.46±0.59	1.93±0.32*	3.70±0.39	3.37±0.24
M	2.51±0.36	2.81±0.21	2.84±0.19	1.94±0.19*	3.58±0.45	3.76±0.64
	Glu(mg/dl)					
K	117.4 ± 25.7	69.4 ± 19.7	142.1 ± 9.4	114.1 ± 11.9	96.9 ± 12.7	49.7 ± 7.5*
C	84.7 ± 18.8	67.8 ± 13.9	98.3 ± 16.6	122.6 ± 15.3	101.4 ± 26.9	99.2 ± 13.9
M	70.0 ± 27.0	51.0 ± 18.2	85.4 ± 28.7	107.9 ± 8.7	129.2 ± 19.4	82.9 ± 11.2
	TG(mg/dl)					
K	95.2 ± 19.0	154.3 ± 67.4			162.0 ± 46.0	47.3 ± 8.3*
C	66.9 ± 9.6	79.5 ± 5.8			72.8 ± 12.1	75.4 ± 8.4
M	87.1 ± 10.9	109.7 ± 14.7			68.1 ± 11.7	62.9 ± 13.3

*Significantly different from the control; [a]Significantly higher than K and M; [b]Signifiicantly higher than C and M; K: Kuchi, C: Ching'wekwe, M: Morogoro medium; Cont: control. Values are presented as Mean ± SE.

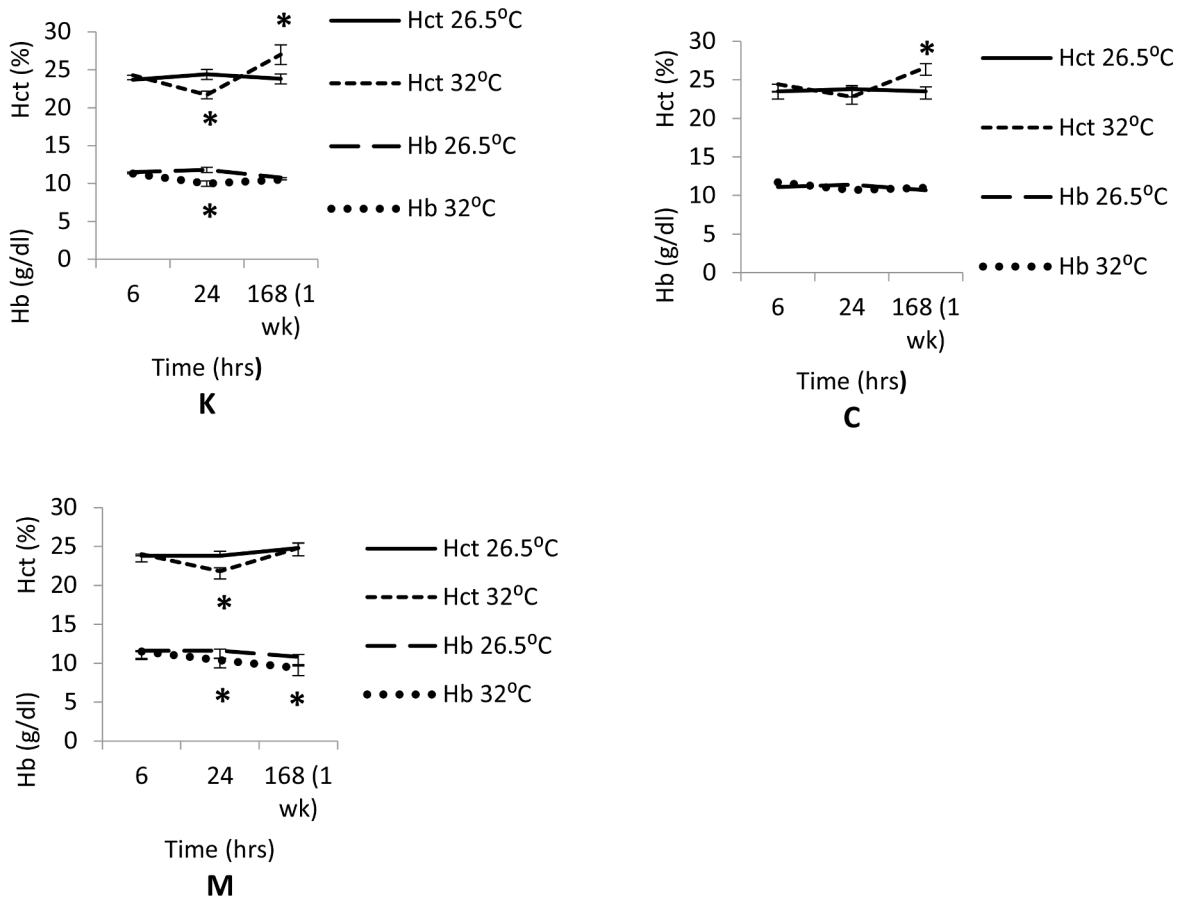

Fig. 5. Hct and Hb of 5 week-old K, C and M hens at control conditions (26.5 ± 0.5 °C) and 32±1°C after 6 hrs, 24 hrs and 1 week. *Significantly different ($p<0.05$) from the control. K: Kuchi; C: Ching'wekwe, M: Morogoro medium. Hct: hematocrit; Hb: hemoglobin.

3.2.3. *Total protein, glucose and triglycerides*

Results for serum total protein, glucose and triglyceride concentrations for experiment two are depicted in Table 4. Exposure of chickens fed low dietary energy to 32±1 °C for 7 days caused a significant decrease ($p<0.05$) in total protein levels for C but not for K and M, whilst glucose levels were not changed in all ecotypes. Between ecotypes, the serum glucose levels for K were notably higher ($p<0.05$) than for C and M after exposure. The triglyceride concentration levels were markedly decreased ($p<0.05$) for K and M but were not affected for C. While glucose levels were not significantly altered for C and M, there were marked reductions ($p<0.05$) in serum glucose levels for K and in total protein levels for all the ecotypes after 24 hr-exposure of the chickens to 37±1 °C. Exposure to 37±1 °C for 10 days caused marked reductions ($p<0.05$) in triglyceride levels in all chicken ecotypes, with M having the highest drop. Total protein levels were significantly reduced ($p<0.05$) for C and M but not for K. While a significant rise in serum glucose levels for K was noted, no notable differences were observed at this stage for C and M.

3.2.4. *Hematological indices*

H/L ratios for the chickens fed low energy diet during the study period are shown in Table 5.

Raising the rearing temperature from 26±0.5 °C to 32±1 °C resulted in a marked increase ($p<0.05$) in H/L ratio within 6 hrs in all ecotypes, and levels remained high within 24 hrs but had returned to control levels after 1 week. However, raising the temperature to 37±1 °C did not alter the H/L ratios in all ecotypes within 4 hrs of exposure, but levels were increased to significant levels ($p<0.05$) after one week in all ecotypes.

The changes in levels of Hb and Hct during the study period are shown in Fig. 11 and Fig. 12. Exposure of chickens to 32±1 °C for 7 days did not alter Hb levels except after 24 hrs when C had markedly lower values ($p<0.05$) and after 1 week when K had significantly higher ($p<0.05$) values (Fig. 11). While the Hct levels for M were not altered during the entire 7-day period at 32±1 °C, the levels for K were markedly elevated ($p<0.05$) after 6 hrs of exposure and C had a decline ($p<0.05$) after 24 hrs. Raising the temperature to 37±1 °C did not significantly change Hb levels in K and M for the entire period of exposure but a marked reduction ($p<0.05$) was observed after 24 hrs in C (Fig. 12). Meanwhile, there was a reduction ($p<0.05$) in Hct levels at this temperature in all ecotypes.

4. DISCUSSION

In the present study, C and K produced stronger responses than M to both stressors as shown by increased serum corticosterone concentration levels after exposure to 32±1 °C for 7 days and 24 hrs after temperature was raised to 37±1 °C. On the other hand, both heat stress and its combination with low dietary energy did not significantly affect corticosterone levels for M after exposure to 32±1 °C for 7 days and also 24 hrs after the temperature was raised to 37±1 °C. The chickens' responses to combined heat stress and low dietary energy, with respect to serum corticosterone levels, were found to be different for acute and lower temperatures. However, they responded similarly at higher temperatures and longer exposures. In broiler chickens, previous studies have shown that acute heat stress elevates serum corticosterone levels (Quinteiro-Filho et al., 2010; Soleimani et al., 2011) in consistent with results for K and C. The findings of this study are also consistent with earlier studies (Khondowe et al., 2018) that demonstrated that M was the most tolerant ecotype to stress induced by low dietary energy under cyclic ambient temperatures of between 21.6 and 34.3 °C. However,

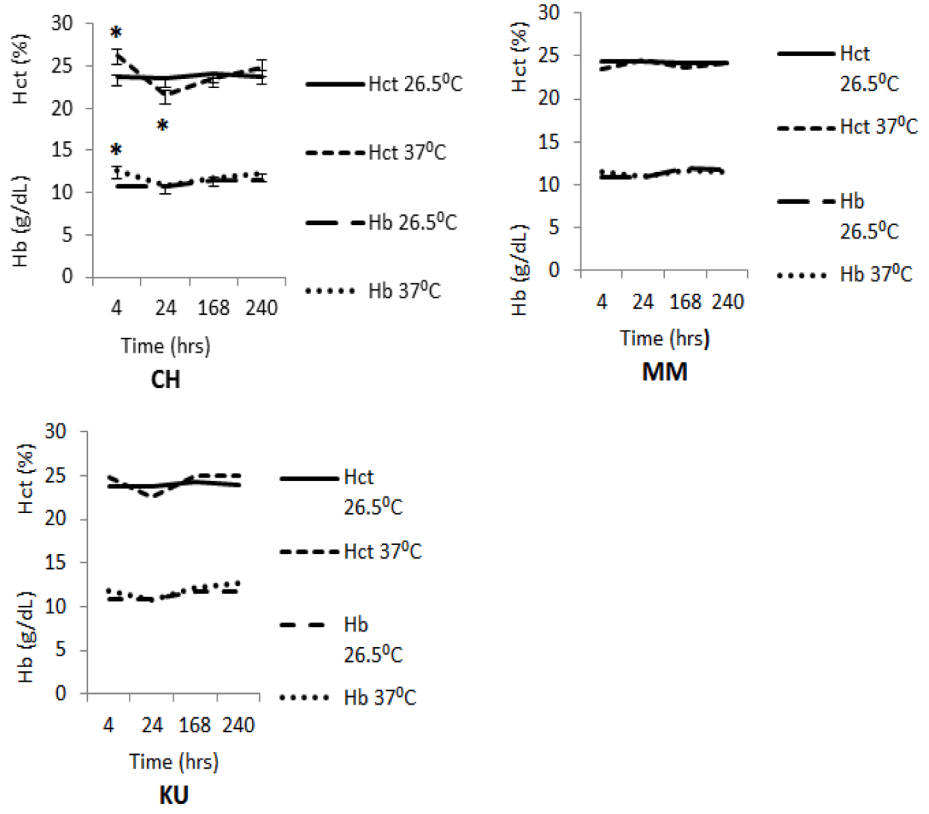

Fig. 6. Hct and Hb of 5 week-old KU, CH and MM hens at control conditions (26.5 ± 0.5 °C) and 37±1ºC after 4 hrs, 24 hrs 7 days and 10 days. *Significantly different ($p<0.05$) from control; *KU: Kuchi; CH: Ching'wekwe, MM: Morogoro medium. Hct: hematocrit; Hb: hemoglobin.*

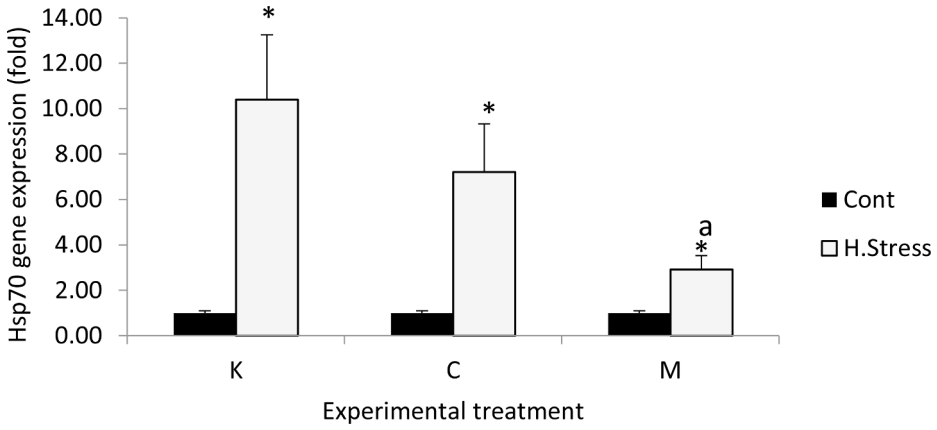

Fig. 7. Liver hsp70 gene expression at control conditions (26.5 ± 0.5 °C), exposure of chickens fed 55% of control energy to 32±1 °C for 7 days and to 37±1 °C (8 hrs per day) for 10 days; *Significantly higher than the control; [a]significantly lower than K; K: Kuchi, C: Ching'wekwe, M: Morogoro medium; Cont: control, H.Stress: heat stress.

chronic exposure for 9 days and also for 8 weeks to higher temperatures have previously been shown not to affect plasma corticosterone concentrations (Mack, Felver-Gant, Dennis & Cheng, 2013; Xie et al., 2014) in contrast to the current findings. Possible reasons for the differences might be that a higher temperature was used in the current study and chickens used in previous studies were of different genotypes.

Increases in corticosterone levels in the blood are linked to the hypothalamic-pituitary-adrenal (HPA) axis that controls animal adaptability in response to various stressors (Zulkifli & Siegel, 1995). Generally, despite the HPA axis activation under heat stress, plasma concentrations of corticosterone may decline within hours of the initial temperature increase (Mack et al., 2013). Short-term increases in corticosterone secretion might improve survival of adult animals during stressful conditions (Kitaysky, Piatt, Wingfield & Romano, 1999; Wingfield, Bruener & Jacobs, 1997) but chronic elevation of corticosterone may suppress immune systems (Kitaysky et al., 1999). Thus, plasma corticosterone may inhibit further HPA axis activation (Smith & Vale, 2006). In the current study, the response by M is probably an indication that by this time recovery had already ensued and therefore corticosterone levels had since been down-regulated to baseline levels. Based on these observations, it is likely that M showed greater acclimatisation to heat stress and its combination with low dietary energy than K and C but similar to C when only heat stress was applied. The ecotype differences could be the result of different genetically mediated

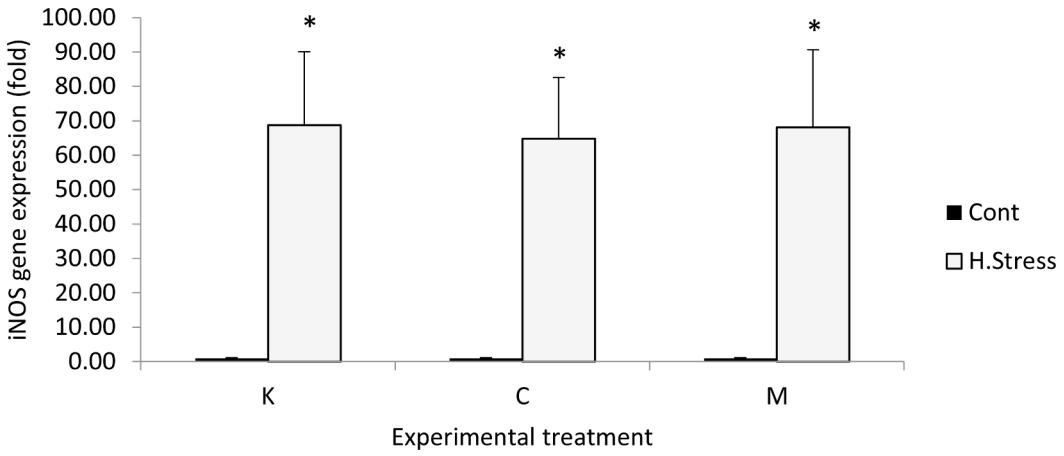

Fig. 8. Liver iNOS gene expression at control conditions (26.5 ± 0.5 °C), exposure of chickens fed 55% less energy than the control to 32±1 °C for 7 days and to 37±1 °C (8 hrs per day) for 10 days; *Significantly higher than the control; K: Kuchi, C: Ching'wekwe, M: Morogoro medium; Cont: control, H.Stress: heat stress.

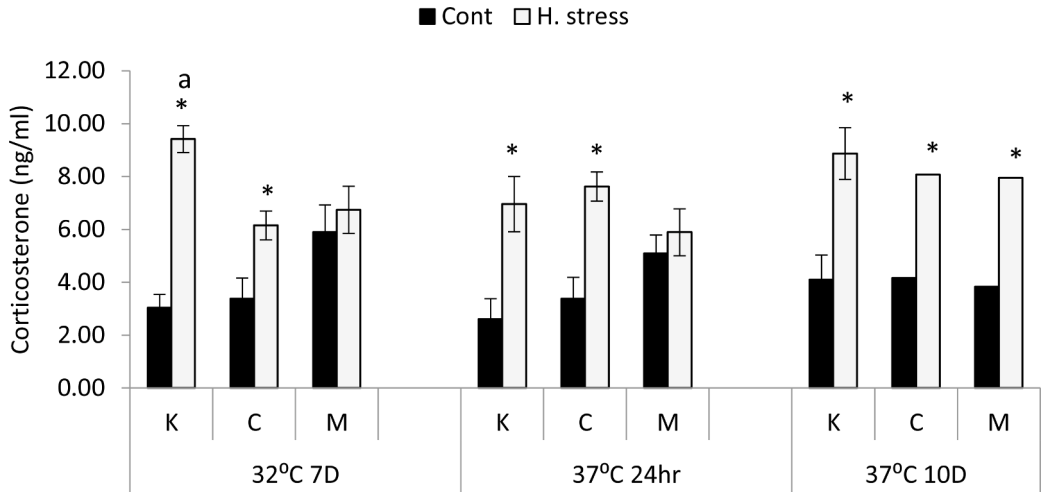

Fig. 9. Serum corticosterone concentration of hens at control (26.5 ± 1 °C and control diet) conditions and of hens fed 55% less dietary energy than the control, after exposure to 32±1 °C for 7 days and 37±1 °C for 24 hrs and 10 days. *Significantly higher than the control; [a]Significantly higher than C and M; K: Kuchi, C: Ching'wekwe, M: Morogoro medium; D: day, Cont: control, H.Stress: heat stress.

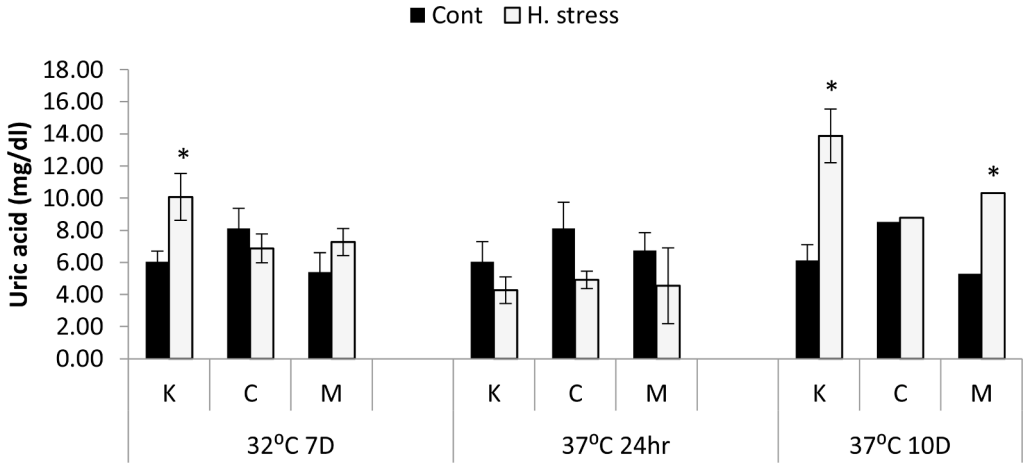

Fig. 10. Serum uric acid concentration of hens at control (26.5 ± 0.5 °C and control diet) conditions and of hens fed 55% less dietary energy than control, after exposure to 32±1 °C for 7 days and 37±1 °C for 24 hrs and 10 days. *Significantly higher than the control; K: Kuchi, C: Ching'wekwe, M: Morogoro medium; D: day, Cont: control, H.Stress: heat stress.

Table 4
Serum total protein (T.P), glucose (Glu) and triglyceride (TG) concentrations of hens fed 55% less dietary energy and exposed to 32±1 °C for 7 days and 37±1 °C for 10 days.

		7days		24hrs		10days
	Cont	**32°C**	**Cont**	**37°C**	**Cont**	**37°C**
	T.P(g/dl)					
K	2.96±0.27	3.04±0.19	2.96±0.27	1.56±0.17*	3.03±0.43	2.13±0.28
C	5.46±0.59[a]	2.49±0.11*	5.46±0.59[a]	1.85±0.23*	3.70±0.39	2.60±0.12*
M	2.84±0.19	2.81±0.15	2.84±0.19	1.32±0.23*	3.91±0.38	2.80±0.20*
	Glu(mg/dl)					
K	142.1 ± 9.4	120.6 ± 14.5[b]	142.1 ± 9.4	72.6 ± 12.5*	96.9 ± 12.7	152.2 ± 14.3*
C	84.7 ± 18.8	71.6 ± 7.0	98.3 ± 16.6	94.3 ± 9.8	101.4 ± 26.9	139.6 ± 8.5
M	85.4 ± 22.3	54.1 ± 4.0	85.4 ± 28.7	61.7 ± 7.2	110.5 ± 6.0	133.6 ± 14.5
	TG(mg/dl)					
K	95.2 ± 19.0	15.3 ± 2.3*			142.3 ± 42.4	39.5 ± 7.7*
C	90.0 ± 24.2	52.6 ± 13.4[d]			72.8 ± 12.1	27.8 ± 8.6*
M	87.1 ± 10.9	13.9 ± 1.9*			68.1 ± 11.7	12.3 ± 2.7[e]*

*Significantly different from the control; [a]Significantly higher than K and M; [b]Signifiicantly higher than C and M; [d]Significantly higher than K and M; [e]Significantly lower than K and C; K: Kuchi, C: Ching'wekwe, M: Morogoro medium; Cont: control. Values are presented as Mean±SE.

Table 5
H/L ratios for chickens at control (26.5 ± 0.5°C) and heat stress (32±1 and 37±1°C) with low dietary energy (55%) conditions.

	[5wk old]	6hrs	24hrs	1 week	[6wk old]	4hrs	1 week
K	**Control**	0.17±0.02	0.18±0.02	0.16±0.03	**Control**	0.20±0.03	0.20±0.03
	32±1°C	1.63±0.99*	1.49±0.86*	0.14±0.01	**37±1°C**	0.15±0.03	0.53±0.02*
C	**Control**	0.15±0.01	0.17±0.02	0.15±0.01	**Control**	0.10±0.04	0.10±0.02
	32±1°C	1.24±0.58*	1.50±0.69*	0.10±0.01	**37±1°C**	0.16±0.03	0.60±0.03*
M	**Control**	0.18±0.01	0.19±0.01	0.18±0.02	**Control**	0.12±0.02	0.11±0.02
	32±1°C	0.77±0.27*	1.62±0.72*	0.16±0.03	**37±1°C**	0.11±0.01	0.57±0.03*

*Significantly different ($p<0.05$) from the control; K: Kuchi, C: Ching'wekwe, M: Morogoro medium. H/L: heterophyl/lymphocyte ratio. Values are presented as Mean±SE.

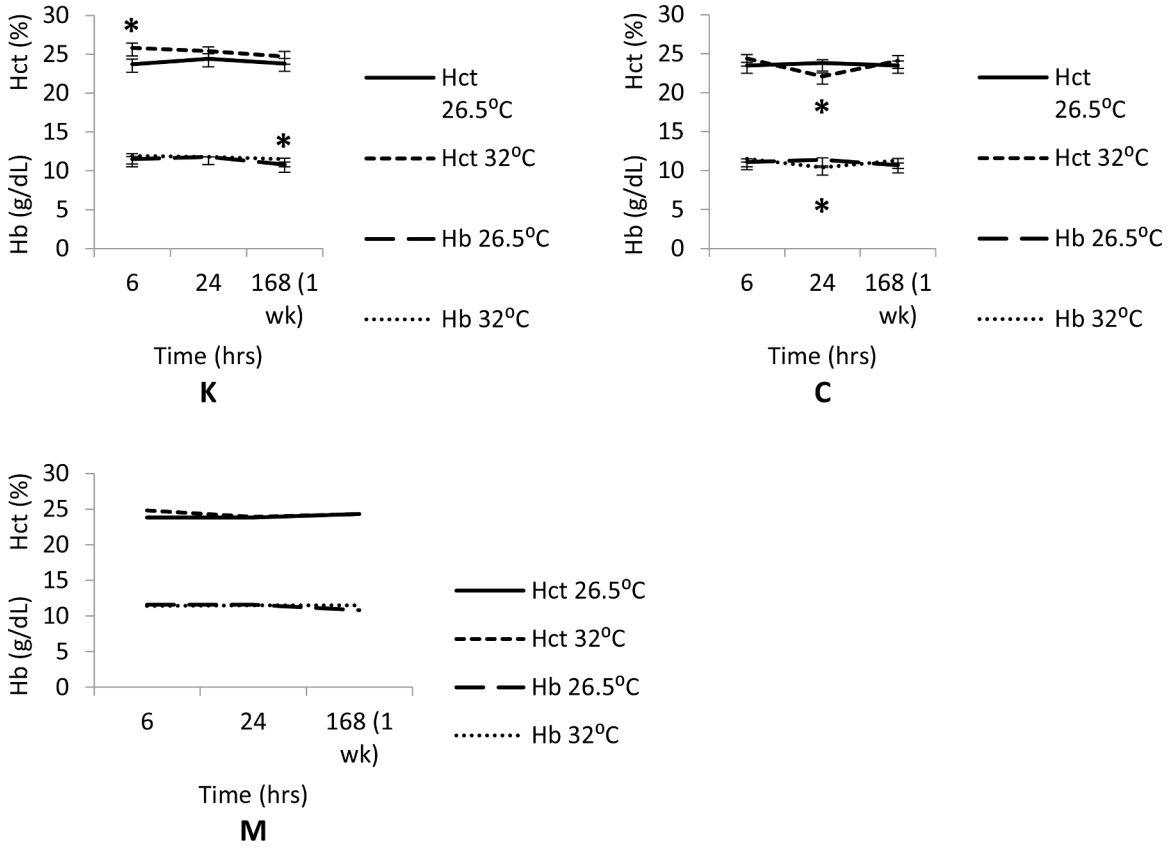

Fig. 11. Hct and Hb of 5 week-old K, C and M hens at control conditions (26.5 ± 0.05 °C) and at 32±1 °C with 55% dietary energy restriction for a 7-day period. *Significantly different ($p<0.05$) from the control; K: Kuchi, C: Ching'wekwe, M: Morogoro medium.

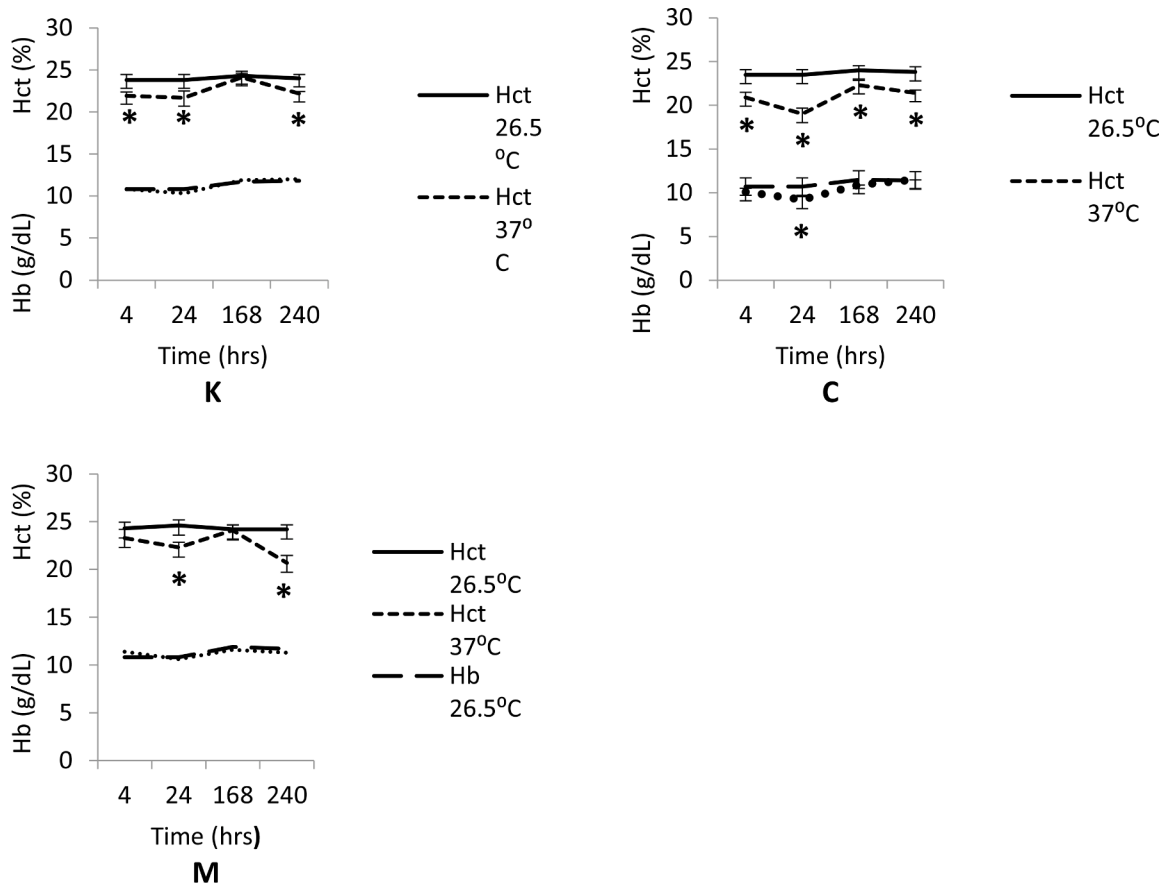

Fig. 12. Hct and Hb of 5 week-old K, C and M hens at control conditions (26.5 ± 0.5 °C) and at 37±1 °C with 55% dietary energy restriction for a 10-day period. *Significantly lower ($p<0.05$) than the control; K: Kuchi, C: Ching'wekwe, M: Morogoro medium.

stress responses of the adrenal system (Cheng & Jefferson, 2008).

Heat stress did not significantly alter relative gene expression levels of both liver hsp70 and iNOS in all ecotypes after exposure of chickens fed control dietary energy to 37±1 °C for 10 days. In broilers and layers, previous studies have shown that acute heat challenge increased hsp70 expression levels (Felver-Gant et al., 2012; Lowman et al., 2014; Yu & Bao, 2008) in the liver but not the chronic heat stress treatment (Xie et al., 2014) partly in contrast to the current findings. In the current study, it is very likely that acclimatisation in all the chicken groups had since ensued thereby stimulating reduced secretion and expressions of serum corticosterone, liver iNOS and hsp70. However, the observation that the fold increase in hsp70 for K was markedly higher ($p<0.05$) than levels in C and M highlights the suggestion that acclimatisation to heat stress was taking place at different rates and that tolerance levels may not be the same in these chickens. This finding implies that selection for better tolerance to heat stress is possible among local chicken ecotypes. Short-term heat stress may provoke heat shock response, resulting in rapid initiation of hsp70 synthesis and rapid changes in gene expression, whereas long-term heat exposure induces larger scale adaptations by altering thermoregulatory activity (Purdue, Ballinger & Hogan, 1992; Xie et al., 2014). The heat stress applied in the current study was for a longer period of time, which appeared to have allowed some acclimatisation activity to take place by the end of 17 days of study. The C and M appeared to have coped and tolerated heat stress more efficiently than K as shown by the clearly unchanged hsp70 levels.

In the current study, there was no change in the relative gene expression levels of iNOS in all ecotypes, indicating lack of tissue damage expected under stress conditions. However, the significant increase in a similar pattern of iNOS and hsp70 relative gene expressions in all chicken ecotypes after exposure to combined stress could be an indication that heat stress and low dietary energy synergistically induced inflammation in the liver. These findings are similar to what was shown in White Leghorn laying hens where feed restriction increased liver iNOS gene expression (Kang, Ko, Moon, Sea-Hwan Sohn & Jang, 2011). Similarly, other previous studies have shown that iNOS expression levels increased after exposure to stress in broiler chickens (Zhao et al., 2013) and ducks (Zeng et al., 2014). Similar findings were reported in broiler chickens where feed restriction increased hsp70 gene expression levels in the liver (Al-Aqil & Zulkifli, 2009; Delezie, Swennen, Buyse & Decuypere, 2007). In the present study, it appears that there was liver tissue damage due to stress conditions and anti-inflammatory activities were at play by nitric oxide through the catalysis of iNOS. The responses with respect to iNOS did not show differences between the ecotypes entailing that the stress-induced tissue damage caused could be of similar degree in the chickens. The iNOS is involved in protecting the liver against hepatic apoptotic cell death during tissue damage by promoting the catalysis of nitric oxide (Clemens, 1999; Surh et al., 2001; Zhao et al., 2013). It is likely that the intensity of stress induced by these combined stressors led to intense activation of the HPA axis such that all the chickens were unable to completely recover by 17 days of the study. It therefore shows that the duration and severity of heat stress and low dietary energy could also influence the expression pattern of HSPs (Xie et al., 2014). The tolerance levels and adaptation patterns also seemed to differ among the chicken ecotypes as shown by the differences between K and M in the expression levels of hsp70 in the liver. The lower levels of hsp70 expression for M than for K may entail a better recovery as the lower the stress stimulation the lower the induction of hsp70. Therefore, the differences in hsp70 expression may also be linked to adaptive genetic variations in the hypothalamic-pituitary-adrenal axis and cellular activation.

Differential alterations in serum metabolites were evident among the chicken ecotypes and between heat treatments in the current study. The reduction of serum uric acid in C could be an indication that the chickens had not yet recovered and were sliding into increased inflammation and oxidative stress (Settle & Klandorf, 2014). Conversely, the marked increase in K may be a demonstration of a stronger antioxidant response aimed at countering the effects of heat stress and low dietary energy. The uric acid levels for M were only affected by a combination of heat stress and low dietary energy. Previous research in poultry portrayed contradictory observations, showing increases (Ozbey, Yildiz, Aysöndü & Ozmen, 2004), reductions (Bogin et al., 1996) and no alteration (Lin et al., 2000; Xie et al., 2014) in blood uric acid levels after heat challenge and/or feed restriction and these observations reflect genetic differences with chickens used in the current study. The differences in responses between the two treatment groups in the present study may also highlight the differences in metabolic rates and states, signifying protein catabolism for energy generation in energy restricted birds resulting from increased corticosterone levels (Virden et al., 2007). Uric acid is the metabolic product of purine metabolism and is an important plasma antioxidant in birds (Settle & Klandorf, 2014).

The observed reductions in serum total protein levels in the chicken ecotypes by a combination of heat stress and low dietary energy after increasing the temperature to 37±1 °C for 24 hrs, can be linked to elevated corticosterone levels as it can change metabolic pathways so that stressed individuals rely on catabolism of proteins to fuel their activities (Kitaysky et al., 1999). Previous research in commercial exotic poultry is consistent with the reductions (Ozbey et al., 2004) observed in the current studies after heat challenge. The energy restricted chickens probably relied more on protein catabolism for their energy needs as evidenced by marked decline in serum total protein levels for C and M after exposure to 37±1 °C for 10 days. The significant rise in K may highlight the apparent differences among these chicken ecotypes in metabolic adjustments under stressful conditions.

The results of the current study indicate that a combination of heat stress and low dietary energy significantly reduced serum triglyceride levels for K and M. However, only K had serum glucose levels markedly reduced by a combination of heat stress and low dietary energy after increasing temperature to 37±1 °C for 24 hrs, which is an indication of higher metabolic activities in this ecotype. It appears that carbohydrate and lipid metabolism was less affected for the chickens under less stressing conditions but the synergistic effect of heat stress and low dietary energy elicited changes differently among the chicken ecotypes. Conversely, exposure of chickens fed low energy diet to 37±1 °C for 10 days significantly increased glucose levels for K but markedly reduced triglyceride levels for all ecotypes, with M recording the highest drop. Generally, the present study shows that the alteration of serum metabolites was closely related to the intensity of heat challenge, in consistence with findings by Xie et al. (2014). It is likely that with an increase in stress intensity, metabolic alterations responses were applied as a coping strategy and as well as mobilization of body energy sources such as triglycerides (Cheng & Jefferson, 2008). The elevated glucose levels, such as those observed in K under heat stress and low dietary energy, might be an adaptation for survivability and tolerance just as Bogin et al. (1996) showed in their study that chickens that survived 40 °C heat shock had high blood glucose levels than the non-surviving.

The H/L ratios for the chickens fed low energy diet were increased when temperature was raised to 32±1 °C for 1 week and thereafter to 37±1 °C for 10 days and did not show inter-ecotype differences. The return of H/L ratios to control levels after 1 week exposure to 32±1 °C may signify that local chickens are more physiologically adapted to higher temperature than commercial breeds, which previously showed increased H/L ratios after both acute (Borges et al., 2004; Soleimani & Zulkifli, 2010; Tamzil et al., 2014) and chronic heat stress (Keambou et al., 2014). Soleimani and Zulkifli (2010) reported elevated H/L ratios in broiler chickens but not in village or indigenous chickens of Malaysia after acute heat exposure. In contrast, Tamzil et al. (2014) reported an increase H/L ratio after an acute exposure to 40 °C of Indonesian native or local chickens. In the current study, a combination of heat stress and low dietary energy may have induced a similar response as evidenced by similar increases in H/L ratios in all the ecotypes.

The changes in levels of mean Hb and Hct in chickens fed control diet and exposed to 32±1 °C show ecotype related differences. The significant reductions ($p<0.05$) in mean Hb and Hct for K and M but not for C after 24 hr-exposure may infer that there were more physiological adjustments in those ecotypes (Lamont et al., 2015), which may signify a stronger response to high temperature exposure. These adjustments might have triggered high water consumption in addition to behavioural responses. Previous studies have also shown that heat distress induced reductions in Hb and Hct, and this is apparently associated with hemo-dilution, which is an adaptive response enabling water loss by evaporation without compromising plasma volume (Borges et al., 2004). In the present study, significant changes in Hb and Hct for the chickens fed low energy diet after exposure to 32±1 °C were only observed in K and C. Reductions in Hb and Hct levels might be because of insufficient nutrients available for Hb production or even as a result of red blood cells lysis.

Exposure of chickens to 37±1 °C showed between-ecotype differences in Hb and Hct changes both for those fed control diet and low dietary energy. Marked changes in mean Hb for C but not K and M after 4 hrs and 24 hrs for chickens fed control diet and low energy diet, respectively indicate physiological adjustments and a struggle to tolerate stress conditions. However, a similar pattern of Hct reduction in all chicken ecotypes fed low energy diet may imply that low dietary energy compounded stress levels at high temperature, which triggered significant changes in physiological components. Lamont et al. (2015) reported decreased Hb and Hct in Fayoumi chickens under heat stress consistent with current results for CH. Oladele, Ogundipc, Ayo and Esievo (2001) linked low values of Hb and Hct during the hot-dry season in Northern Nigeria to heat and nutritional stress, which impair the synthesis of blood cells in birds. In the present study, energy intake may have been inadequate for energy costs of blood cells synthesis and the general effect of reduced Hct is a decrease in circulating concentration of oxygen. However, Keambou et al. (2014) reported that Hct and Hb were not significantly affected by the rise in breeding temperature from 25 to 35 °C of Cameroonian local chickens. When compared to the current study, this finding is only consistent with results relating to K and M but not C at 37±1 °C with control diet where an increased temperature did not affect Hb and Hct. The duration of exposure and genetic or ecotype-related variations in adaptation and tolerance levels could be a reason for the differences. Decreases of Hct and Hb could also be potentially important parameters, contributing to chicken's heat stress resistance (Lamont et al., 2015).

5. CONCLUSION

Ecotype-based differences exist in local chickens' adaptive responses to heat stress and low energy diets. The current findings indicate that the chickens' physiological responses to heat stress and low dietary energy are different for acute and lower temperatures but similar as the stress intensity is increased and prolonged. Metabolic adjustments were closely related to the intensity of stress challenge, with effects minimal and similar under less stressing conditions but the synergistic effect of heat stress and low dietary energy elicited changes differently among the chicken ecotypes. This study has therefore provided possible avenues for future research to devise programs that include physiological and biochemical traits that would enhance selection for heat and low dietary energy tolerance among the local chicken stocks.

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