COMPARATIVE PHYSIOLOGICAL, BIOCHEMICAL AND BEHAVIOURAL RESPONSES TO HEAT STRESS AND LOW DIETARY ENERGY IN SELECTED TANZANIANLOCAL CHICKEN ECOTYPES

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A DISSERTATION SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

2018

EXTENDED ABSTRACT

As an important source of income and protein, local chickens are widely reared by a majority of rural and peri-urban households in many developing countries including Tanzania.However, these birds are seasonally exposed to challenging environments that includehigh temperatures and decreased scavengeable food materials.It was hypothesized that local chickens bred from different regions of Tanzania might have selected ecotypes with stronger tolerance to high temperatures andsuboptimal nutrition.Two groups of studies were conducted to compare effects of heat stressand low dietary energy in three Tanzanian chicken ecotypes: Kuchi (KU), Ching'wekwe (CH) and Morogoro medium (MM). In the first study conducted at prevailing cyclic ambient temperatures, 4 weeks old hens wereeither fed a control diet containing 2864 Kcal/kgMEor diets containing 40 or 55% less energy than the control over a period of 7 weeks. Results showed ecotype-specific responses through differences in growth performance, feed conversion ratios (FCRs),behavioural responses, blood indices,and liver hsp70 and iNOS gene expressions.MM showed better performance at 55% restriction level whereas Kuchi exhibited better performance at 40% restriction and control energy levels. In the second study, the first batch of chickens was exposed to a constant temperature of 32±1°C for 7 days and thereafter raised and maintained at 37±1°C (8hrs per day) for 10 days, whereas the second batch was subjected to similar conditions but fed 55% less dietary energy than the control. Results showed ecotype-based differences in responses to both heat stress and a combination of heat stress with low dietary energy. MM had greater tolerance to heat stress and its combination with low dietary energy than KU and CH but similar to CH when only heat stress was applied, with respect to liver hsp70 gene expression and serum corticosterone.Collectively, the results show that growth performance and responses to heat stress and low dietary energy in the three local chicken ecotypes are different andhave

provided starting points for future research to devise programs that include physiological, biochemical and behavioral traits that would enhance selection for heat and low dietary energy tolerance among the local chicken stocks.

Key words: *behaviour,ecotype, gene expression, restriction, stress,temperature, tolerance*

DECLARATION

I, Paul Khondowe, do hereby declare to the Senate of Sokoine University of Agriculture that, this dissertation is my own work done within the period of registration and that it has neither been submitted nor being concurrently submitted to any other institution.

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ACKNOWLEDGEMENTS

First and foremost I thank the Almighty God for His Grace, Faithfulness, Providence and Mercy throughout the period of my study. My supervisors, Prof. E. C. Phiri, Prof. B. M. Mutayoba, and Prof. A. P. Muhairwa are greatly appreciated for the key roles they played during the course of my studies and stay at SUA. A special thanks to Prof. B. M. Mutayoba 'for dedicating so much of your valuable time ensuring that this work was a success'.

I thank my sponsors 'Intra-ACP Mobility Scholarship Program through the Education, Audiovisual and Culture Agency of the European Union for the generous financial and moral support throughout the period of my studies. Many thanks to the local coordinators of the scholarship program Prof. D. Kambarage and Prof. E. Karimuribo for the timely advice and support during the entire period.In the same way, I would like to thank USAID Feed the Future Innovation Lab for Genomics to Improve Poultry Project through Prof. A. P. Muhairwa for the partial scholarship support rendered to me. 'Indeed, without your huge material, financial and moral input it could have been difficult to start and finish this work'. I also extend thanks to the staff and workers at the GIP project, SUA, including Dr. Esther Mollel, Mr. Muga, Mr. Mwangalile, Dr. James Mushi, Mr. George Makingi, Ms. Prisca Oscar and others for the great help rendered to me during the time of my study at the farm, including collection and incubation of eggs, care of chicks, and help in biosecurity. Special thanks go to all the members of the Department of Physiology, Biochemistry and Pharmacology, College of Veterinary Medicine and Biomedical Sciences whose company I enjoyed. Finally, I thank my lovely wife Mercy and sons Dalitso, Ephraim, Chimwemwe and Yamiko David for the great support, love, and patience even when it meant staying far away from each other.

DEDICATION

By the Grace of God, I dedicate this work to my lovely wife Mercy and sons, Dalitso, Ephraim, Chimwemwe and Yamiko David.

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ACTH	Adrenocorticotropic Hormone
AME	Apparent Metabolisable Energy
AVP	Vasopressin
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary Deoxyribonucleic Acid
CH	Ching'wekwe
COX-2	Cyclooxygenase
CRF	Corticotropin Releasing Factor
CRFR1	Corticotropin Releasing Factor Receptor 1
DAG	Diacylglycerol
DEFRA	Department for Environment, Food and Rural Affairs
EDTA	EthyleneDiamine Tetracetic Acid
ERK	Extracellular signal-Regulated Kinase
FAO	Food and Agricultural Organisation
FCR	Feed Conversion Ratio
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GSH	Glutathione
GSH-Px	Glutathione Peroxidase
H/L	Heterophil/Lymphocyte
Hb	Hemoglobin
Hct	Hematocrit
HPA	Hypothalamic-Pituitary-Adrenal
HSE	Heat Shock Binding Element
HSF	Heat Shock Factor

HSP	Heat Shock Protein
I/R	Ischemia/reperfusion
IFN-γ	Interferon gamma
IL-1β	Interleukin – 1 beta
iNOS	Inducible Nitric Oxide Synthase
IP_3	Inositol
JNK	Jun N (amino) – terminal Kinase
Kcal	Kilo Calories
KU	Kuchi
MAPK	Mitogen-activated Protein Kinase
MAPKK	Mitogen-activated Protein Kinase Kinase
MAPKKK	Mitogen-activated Protein Kinase Kinase Kinase
MC2-R	Melanocortin type 2 receptor
MM	Morogoro Medium
mRNA	Messenger Ribonucleic Acid
NF_kB	Nuclear Factor kappa-light-chain enhancer of activated B cells
NO	Nitric Oxide
NRC	National Research Council
PCR	Polymerase Chain Reaction
PVN	Para-Ventricular Nucleus
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SAPK	Stress Activated Protein Kinase
SOD	Superoxide Dismutase
SPSS	Statistical Package for the Social Sciences

TGF-β	Transforming Growth Factor Beta
Thr-X-Tyr	Threonine-X- Tyrosine (X: any amino acid)
TNF-α	Tumor Necrosis Factor alpha
URT	United Republic of Tanzania
V1B	Vasopressin receptor 1 B

CHAPTER ONE

INTRODUCTION

1.0 GENERAL INTRODUCTION

Local or indigenous chickens still constitute a major component of the chicken population in many developing countries. At least 80% of rural households of Sub- Saharan Africa keep local chickens, and this greatly contributes to their nutrition and income even though expansion is limited by low productivity (Lwelamira, 2012). In Tanzania, Msoffe *et al.* (2001) identified five local chicken ecotypes based on their geographical origin and phenotypic characteristics. These include: Kuchi (KU), Singamagazi, Ching'wekwe (CH), Morogoro medium (MM) and Mbeya ecotypes. The other local chicken ecotypes identified in Tanzania over the years based on geographical origin and locations include Pemba, Tanga, Unguja, and N'zenzegere (Msoffe *et al.*, 2005). Generally, all the identified ecotypes show variations in adult body weight, body size, egg weight, production capacity, plumage characteristics and some indications of resistance to disease (Msoffe *et al.,* 2002). Genetic uniqueness between local chicken ecotypes was reported and limited interbreeding between them was highlighted due to their geographical separation and possible preferential mate selection (Msoffe *et al*., 2005). The current studiesfocused on the CH, KUand MM local chicken ecotypes because of their productive and disease resistance potential as reported in previous studies, and also because they representsome groupsof unique ecotypes found across the country(Msoffe *et al.,* 2002).

Since local chickens are generally left to scavenge for feed on their own,they are not spared from exposure to a myriad of environmental stressors. Just like in other tropical regions of Africa, seasonal higher temperatures and suboptimal nutrition are among the

major environmental stressors faced by scavenging local chickens in some parts of Tanzania (Mwalusanya *et al.,* 2001). This in part contributes to the low production capacity generally associated with these chickens. Currently, there is a high demand but inconsistent supply of local chickens at the market place (Queenan *et al.,* 2016).The current drive is to increase poultry production by encouraging improved husdandly practices for the majority of rural and peri-urban small scale farmers whose nutrition and income may partly depend on this sector (Alders*et al*., 2014; Queenan *et al*., 2016). In order to achieve this, chicken ecotypes with traits that better enhance their adaptability to environmental stressors, including high ambient temperatures, disease and suboptimal nutrition need to be earmarked for selection and improvement.

Chickens exposed to high ambient temperature frequently experience heat stress, and stressed birdsusually elicit behavioral changes associated with difficulty in achieving a balance between body heat production and body heat loss. This occurs at all ages and in all types of poultry (DEFRA, 2005).Stress can be detrimental to gene expression,leading to posttranscriptional changes to signaling genes and disruption of the health of an animal at the genetic level (Allen and Tresini, 2000; Fleming *et al.,* 2016).The mechanism of stress regulation begins from stimulation of the hypothalamus and release of corticosterone which is one of the main glucocorticoidsor stress hormone in chickens (Ognik and Sembratowicz, 2012). As the bird attempts to maintain its homeostasis, increased production levels of reactive oxygen species (ROS) occurs and as a consequence, the body enters a stage of oxidative stress, and starts producing and releasing heat shock proteins (HSPs) to protect itself from deleterious cellular effects of ROS(Xie *et al.,* 2014). Among the HSPs, HSP70 and 90 have received the most interest in poultry species and are the most conserved and best studied families, each with several inducible and constitutively expressed members exhibiting different functions (Xie *et al.,* 2014).

The HSP70 is the most temperature sensitive HSP whilst HSP90 is essential for survival, and it makes up to 1–2% of total cytosolic proteins found in eukaryotic cells. Acquisition of thermotolerance related to induction of HSP70 and HSP90 has been demonstrated in both chickens and turkeys (Lowman *et al*., 2014). Vertebrates have both enzymatic (e.g. superoxide dismutase, SOD) and non-enzymatic (e.g. glutathione, GSH) antioxidative defence systems against ROS-related damage. At cellular level, mitogen-activated protein kinase (MAPK) pathways are activated by a variety of environmental stressors; and these elicit adaptive responses that require the coordinated expression of stress-response genes, which affect cell survival and apoptosis. Chronic exposure to unpredictable stress can cause nuclear factor kappa B (NF_kB) activation and mRNA expression of pro-inflammatory genes (e.g. inducible nitric oxide synthase - iNOS and cyclooxygenase - COX-2) (Takekawa *et al*., 2011).

Local chickens commonly bred and reared in different ecological/geographic regions of Tanzania, and exposed over time to existing stress factors might have possibly resulted in the natural selectionof chickens with stronger resilience to different forms of stress. Comparative studies on physiological, behavioural and molecular mechanisms underlying heat stress responses in different local chicken ecotypes is envisaged to helpuncover these natural adaptive processes between them.These then could be exploited inbreeding programs involving selection for chickens that are more adapted to high ambient temperatures. Thisultimately will help in conservation of indigenous genetic resources with better heat tolerance, survivability and productivity traits.Appropriate application of such information has the potential of improving the production of local chickens by households and small scale farmers, and thereby improving food security, nutrition and livelihoods in Tanzania.

1.1 Objectives

The general objective of this study wasto compare physiological, biochemical and behavioural responses to heat stress and low dietary energy among CH,KUand MM local chicken ecotypes of Tanzania.

The specific objectives were as follows:

1. To determine and compare growth performance and behavioural responses to stress induced by low dietary energy among CH,KU and MM local chicken ecotypes.
2. To determine and compare the effects of low dietary energy-induced stress on serum corticosterone, hemoglobin, hematocrit, biochemical parameters and liver hsp70 and iNOS gene expressionsamong CH,KU and MM local chicken ecotypes.
3. To investigate and compare effects of heat stress and a combination of heat stress and low dietary energy on growth performance and hematological parametersamong CH,KU and MM local chicken ecotypes.
4. To investigate and compare the effects of heat stress and a combination of heat stress and low dietary energy on behavior, biochemical parameters,serum corticosterone, liver hsp70 and iNOS gene expressionsamong CH,KU and MM local chicken ecotypes.

1.2Hypotheses

1.Changes and shifts in liver hsp70, iNOS, serum corticosterone, behavioural, biochemical, haematological, and growth parameters are ecotype-dependant under heat stress, low dietary energy and/or a combination of heat stress with low dietary energy.

2. Across all stressing factors, as stress intensity increases, shifts and changes in the targeted behavioural, biochemical and physiological parameters arelinear and ecotype-specific.

1.3 Literature Review

1.3.1 Poultry production in Tanzania

Poultry production is an important sector that developing countries can embrace and spearhead in the quest to alleviate rural poverty. In Tanzania, the poultry production system is of two types, that is, traditional (mainly consisting of local free scavenging chickens, ducks, pigeons, guinea fowls and turkeys) and commercial production (consisting of broilers and layer chickens) (URT, 2011). In the traditional scavenging sector the production of eggs is a major component or sometimes equal with meat in terms of food production and income for the primary producer (Wilson, 2015). Almost 99% of indigenous poultry is kept by small-scale traditional producers whose birds mostly scavenge for their food (URT, 2011; Wilson, 2015). While there is no proper management, insignificant feed provision, and usually limited and sporadic use of health care in the scavenging system, the commercial system has good level of management and health care (Wilson, 2015).

Production and productivity in the poultry industry have have shown little growth due to several factors, including inadequate nutrition and high prevalence of diseases like Newcastle Disease, Gumboro (or Infectious Bursal Disease), Fowl Pox and Fowl Typhoid (Wilson, 2015). By 1st October, 2008, Tanzania had about 43.7 million chickens of which 41.9 million (96%) were local, 1.3 million (2.7%) were layers and 0.6 million (1.3%) were broilers (URT, 2012). The commercial sector mainly uses hybrids such as Hybro, Boyan Brown, Boyan Gold Line, Arbo Acres, Ross 208, Nera and Hubbard, and the parent stock has been imported either as eggs or day-old chicks (Wilson, 2015).Generally, country-wide improvement of poultry production will contribute to better family nutritional outcomes by supplying high quality protein and micronutrients (Alders *et al*., 2014).

1.3.2 Local chicken production in Tanzania

In Tanzania just like in other African countries, demand from consumers for local or indigenous chickens is high and they are subsequently pegged at higher prices than broilers (Mlozi *et al*., 2016;Queenan *et al.*, 2016). Local chickens are reared under scavenging, semi-intensive and to a lesser extent, intensive production systems (Sanka and Mbaga, 2014). Scavenging and semi-scavenging are characterized by low plane of nutrition that varies with season (Mwalusanya *et al*., 2001; Goromela *et al.*, 2007), while by design in intensive system optimal feed is readily and obligatorily provided.Free scavenging accounts for the majority of chickens reared but because of a myriad of challenges including, disease, seasonal higher temperatures and a diet that does not consistently meet chickens' nutritional demands in some regions of the country, production remains very low (Mwalusanya *et al.*, 2001). Tanzania has diverse geographical and climatic zones and this contributes to the great diversity in local chicken varieties. The country has four main climatic zones, namely, the tropical, which includes coastal area and immediate hinterland, with temperatures averaging 27^{o}C, and the central plateau, which is hot and dry, with temperatures averaging 32^{o}C. The rest are semi-temperate highland areas, which include south and southwest regions, with temperatures between 10 and 20^{o}C; and the high moist lake regions in the northwest, with annual average temperatures ofabout 27^{o}C (Nations Encyclopedia, 2017). Tanzania is however, divided into about seven agro-ecological zones, namely, coastal, arid lands (Dodoma), semi-arid lands (Shinyanga), plateau (Mbeya), southwestern highlands (Mbeya), northern highlands (kilimanjaro) and alluvial (Morogoro). Althoughthe recognized local chicken ecotypes originate from different regions of the country, they are currently bred countrywide (Fig. 1.1)(Msoffe *et al*.,2002,2005). For example,KU and Singamagazi are considered to originate from the northwest, CH,MM and N'zenzegere from central

regions, Mbeya from Mbeya region in the southwest, and Pemba,Tanga, and Ungujafrom Zanzibar Islands and the coastal regions (Msoffe *et al*., 2002;2005;Lyimo *et al.*, 2013). In 2007/2008 Shinyanga, Mbeya, Mwanza, Tabora and Morogoro regions had the highest indigenous chickens(Fig. 1.1) than any other region while Dar-es- salaam, Kigoma and Arusha regions had the least number (URT, 2012).

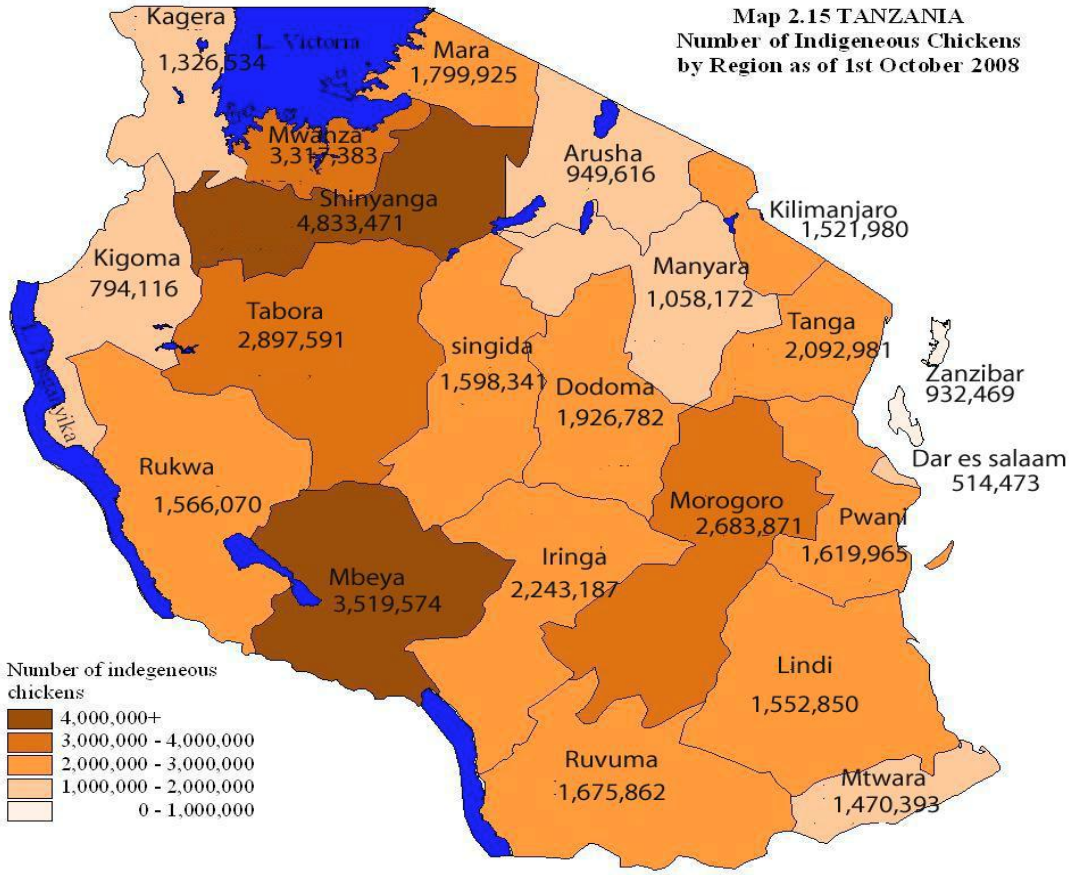

Figure 1.1: Map of Tanzania depicting the number of indigenous chickens by region as of 1st October, 2008. Source: URT (2012).

Research work has been done on local chicken ecotypes over a number of years, however, it has been restricted to themes such as: genotype-environment interaction (Lwelamira, 2012), cross breeding, disease resistance and prevalence (Msoffe *et al.*, 2002), microsatellite DNA typing and genetic structure or diversity (Msoffe *et al*., 2005), and immunocompetence (Msoffe *et al*., 2001). Genetic relatedness within than between

indigenous chicken ecotypes has been elucidated though the sharing of genetic materials between ecotypes is equally evident (Msoffe *et al*., 2005; Mayardit*et al*., 2016). Microsatellite analysis studies suggest that KU might have originated from a different ancestral population from CH and MM (Lyimo *et al*., 2013).KU, MM and CH have been previously shown to have a better disease resistance potential, with respect to *S. gallinarun*and fowl typhoid (Msoffe *et al*., 2002). Studies are underway to compare and elucidate innate resistance to New castle disease in these local chicken ecotypes. However, studies on genetic improvements on native indigenous chicken genetic resources arerare, with a few currently underway. Past studies focused on improving the productivity of local birds by crossing with exotic cockerels but failed due to putting more emphasis on rapid genetic improvement while neglecting managemental aspects, such as feed-intake improvement needed for the crossbreds adopted by the rural people (Goromela, 2007). Although crossbreeding programmes have shown to markedly improve productivity,current global initiatives are on conservation of indigenous genetic resources (Lweramila, 2012; Chesoo *et al*., 2016).

Local chickens will certainly be rearedin more temperature extreme conditions in the future due to both expansion into naturally hotter environments and global warming(Lamont *et al.,* 2015). In the natural localities where these chickens are reared, seasonal summer high temperatures are generally accompanied by a lack of adequate feed resources to meet their energy or nutritional requirements(Sonaiya, 2007; Mwalusanya*etal.,* 2010;Mutayoba *et al.,* 2012). Further, it is already well established that local chickens are supremely adapted to the harsh environments in areas where they are bred and can produce under conditions where exotic breeds may not survive. However, there are some ecotype-differences in some aspects of productive performance and disease resistance between them as shown by previous studies (Msoffe *et al.,* 2002;Lwelamira,

2012).These differences between ecotypes shown by other studies on the same ecotypes may also differently affect their responses to heat stress, low dietary energy or even a combination of these stressors. Therefore, selection of chickens for resilience to heat stress could be a valid strategy to reduce the negative impact of elevated ambient temperaturesand thereby improve productivity (Lan*et al.*, 2016). Apart from understanding molecular mechanisms underlying heat stress response in local chickens,the focus of such a selection should be to identify from among the local chicken ecotypes and strains those that are more adapted to high ambient temperatures. An assessment of physiological stress indices under high ambient temperatures and suboptimal nutrition would provide a correct reflection of the tolerance levels of these chickens. Selection can greatly help in thedrive to increase and improve production for the majority of rural and peri-urban small scale farmers whose nutrition and income may partly depend on this sector.

1.3.3The stress response

Stressors are any internal or external stimuli or threats that disrupt homeostasis, whilst stress is a state in which homeostasis is threatened and it is stimulated by a stressor that affects the body through activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (Burdick, 2010). Stress response is activated in response to the altered state in order to help the body deal with the threat and return to or maintain homeostasis (Burdick, 2010). Stressors may be acute, sequential, episodic, chronically intermittent, sustained, or anticipated (Greenberg *et al.*, 2002). Exposure of cells to stress elicits adaptive responses that require the coordinated expression of stress-response genes, which affect cell survival, apoptosis, cell cycle progression and differentiation (Tort and Teles, 2011). Most animal species have differentially evolved constitutive and inducible mechanisms for coping with stressors(Martin *et al.*, 2011). Although the importance of

animal responses to environmental challenges applies to all species, poultry seem to be particularly sensitive to temperature-associated environmental challenges, especially heat stress (Lara and Rostagno, 2013).

Corticotropin releasing factor (CRF) plays a central role in the stress response by regulating the HPA axis (Fig. 1.2), leading to a cascade of events that culminate in the release of glucocorticoids from the adrenal cortex(Smith and Vale, 2006).The principal effectors of the stress response are localized in the paraventricular nucleus (PVN) of the hypothalamus, the anterior lobe ofthe pituitary gland, and the adrenal gland, and in addition, the brain stem, noradrenergic neurons, sympathetic andrenomedullary circuits, and parasympathetic play important roles in the regulation of adaptive responses to stress(Habib *et al*., 2001;Smith and Vale, 2006).In response to stress, the hypophysiotropic neurons localized in the medial parvocellular subdivision of the PVN synthesize and secrete CRF, which is released into hypophysial portal vessels that access the anterior pituitary gland (Rivier and Vale, 1983). Binding of CRF to its receptor on pituitary corticotropes induces the release ofadrenocorticotropic hormone (ACTH) into the systemic circulation (Smith and Vale, 2006). The principal target for circulating ACTH is the adrenal cortex, where it stimulates glucocorticoid synthesisand secretion from the zona fasciculate, and these glucocorticoids are the downstream effectors of theHPA axis (Fig. 1.2) (Smith and Vale, 2006). Thebiological effects of glucocorticoids are usually adaptive;however, inadequate or excessive activation of the HPAaxis may contribute to the development of pathologies (Muncke*t al.,* 1984).

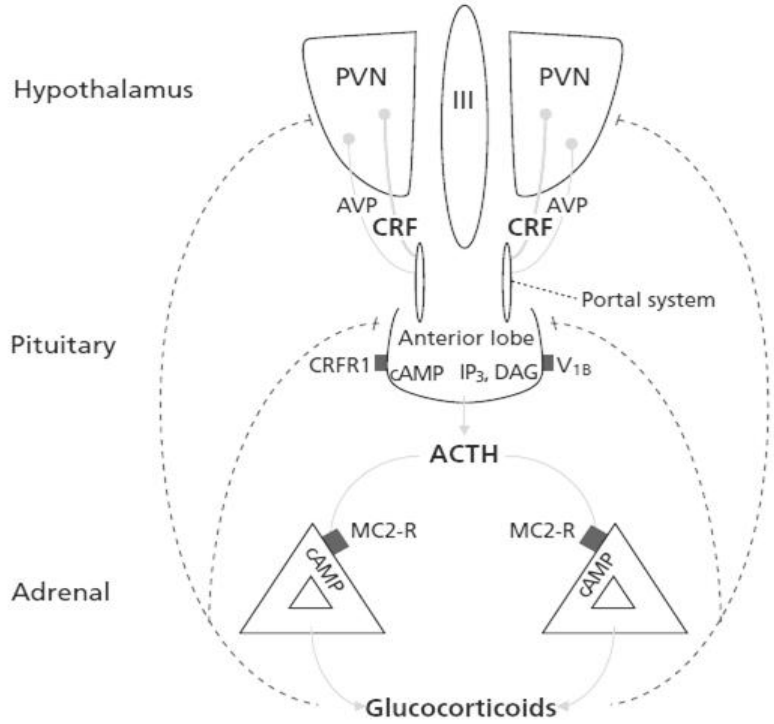

Figure1.2:Schematic representation of the hypothalamic-pituitary-adrenal (HPA) axis depicting sources and targets of the main hormones. ***ACTH: adrenocorticotropic hormone; AVP: vasopressin; cAMP: cyclic adenosine monophosphate; CRF: corticotropin releasing factor; CRFR1: corticotropin releasing factor receptor 1; DAG: diacylglyceral; IP3: inositol; MC2-R: melanocortin type 2 receptor; PVN: paraventricular nucleus; V1B: vasopressin receptor.*** **Source: Smith and Vale (2006).**

A variety of extracellular stimuli in eukaryotic cells generate intracellular signals that converge on MAPK pathways and the core of any MAPK pathway is composed of three tiers of sequentially activated protein kinases, namely, MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK (Avruch, 2007;Takekawa *et al*., 2011). Activation of MAPKs is achieved by phosphorylation of a threonine and a tyrosine residue within a conservedThr-X-Tyr motif in the activation loop (also called the T-loop), which is catalyzed by MAPKKs; then MAPKKs are activated by any of several MAPKKKs, via

phosphorylation of serine and/or threonine residues within their activation loop (Takekawa *et al*., 2011). At least four different subfamilies of MAPKs are present, namely,extracellular signal regulated kinase1/2 (ERK1/2), Jun amino terminal kinase 1/2/3 (JNK1/2/3), p38a/b/g/d, and ERK5. The p38 and JNK, are more potently activated by a variety of environmental stresses and are thus collectively called stress-activated protein kinase (SAPK) pathways, which play pivotal roles in cellular stress responses such as cell cycle arrest and apoptotic cell death(Takekawa *et al*., 2011). Persistent activation of p38 and JNK, especially in the absence of mitogenic stimuli, has been shown to induce apoptotic cell death; in contrast, inhibition ofJNK and/or p38 activation, either by genetic inactivation or by the use of a dominant inhibitorymutant, confers resistance to cell death induced by stress stimuli including DNA damage (Kyriakis and Avruch, 2001).

Stress can lead to ROS formation and oxidative injury;andinflammation is an important indicator of animal tissue damage due to stress conditions and one of the most pivotal enzymes involved in maintaining it is inducible nitric oxide synthase (iNOS), which is responsible for the catalysis of nitric oxide (NO) (Surh *et al.*, 2001;Zhao *et al.*, 2013). The iNOS gene is expressed by hepatocytes in a number of physiologic and pathophysiologic conditions affecting the liver but the molecular regulation of its expression is complex and occurs at multiple levels in the gene expression pathway (Taylor *et al*., 1998).The cytokines, tumor necrosis factor alpha(TNF-α),interleukin-1 beta(IL-1β)and interferon gamma (IFN-γ) are known to synergistically activate iNOS expression in the liver and this expression requires transcription factor NF_kB, and the expression is down-regulated by steroids, transforming growth factor beta(TGF-β), heat shock responseand NO (Taylor *et al*., 1998).

Generally iNOS is absent in normal liver but is markedly increased in response to inflammation and a variety of oxidative stresses, and is responsible for the synthesis and catalysis of NO, which has anti-inflammatory activities (Clemens, 1999). NO acts as an inhibitor or agonist of cell signaling events (Chen *et al*., 2003).In the liver, constitutively generated NO maintains the hepatic microcirculation and endothelial integrity whereas iNOS governed production can either be beneficial or detrimental depending on the type of stress stimulus, abundance of ROS, source and amount of NO production and the cellular redox status of the liver (Chen *et al*., 2003). For example, NO potentiates the hepatic oxidative injury in ischemia/reperfusion while iNOS expression may protect against hepatic apoptotic cell death; and these anti-apoptotic actions are either cyclic nucleotide dependent or independent, including the expression of HSPs and prevention of mitochondrial dysfunction (Chen *et al*., 2003). NO exerts a protective effect through its ability to prevent intravascular thrombosis by inhibiting platelet adhesion and neutralizing toxic oxygen radicals (Taylor *et al*., 1998). Both *in vivo* an *in vitro*, NO also exerts protective effects by blocking TNF-α-induced apoptosis and hepatoxixcity, in part by a thiol-depenent inhibition of caspase-3-like protease activity (Taylor *et al*., 1998). Liver iNOS gene expression has been shown to increase after exposure to stress in broiler chickens (Zhao *et al*., 2013) and ducks (Zeng *et al*., 2014). The liver is more susceptible to oxidative stress and injury than other body organs and plays an important role in energy metabolism (Xie *et al.*, 2014;Lan *et al*., 2016).Some studies (Zhao *et al*., 2013; Zeng *et al.*, 2014) have also shown anincrease of other inflammatory factors such ascyclooxygenase-2 (COX-2), NF-κB, and TNF-αafter poultry were exposed to stressing conditions.

The antioxidant system is responsible for the protection of cells from the actions of free radicals and the system includes natural fat-soluble antioxidants (e.g. vitamins A, E, and carotenoids); water-soluble antioxidants (e.g. ascorbic acid and uric acid); antioxidant

enzymes(e.g. GSH-Px and SOD); and the thiol redox system consisting of the glutathione (Surai, 2016). The first level of defense consists of antioxidant enzymes and is responsible for prevention of free radical formation by removing precursors of free radicals, the second level consists of chainbreaking antioxidants (e.g. carotenoids, uric acid, vitamins A, C and E)and the third level is based on systems that eliminate damaged molecules or repair them and includesenzymes and chaperones such as HSPs (Surai, 2016).Previous studies in chickens have shown contradictory results, with some indicating elevated production (Azad *et al.*, 2010;Yang *et al.*, 2010; Ghazi *et al.*, 2012), while some decreased production (Liu *et al.*, 2014;Huang *et al.*, 2015) of antioxidants under stress conditions.However, information is lacking on oxidative, inflammatory, and antioxidative responses in local chickens.

1.3.4 Heat shock response

Chickens exposed to high ambient temperature can experience heat stress or evenoxidative-stress, and stressed birds have difficulty achieving a balance between body heat production and body heat loss at all ages and in all types of poultry (DEFRA, 2005).Stress can be detrimental to gene expression,leading to posttranscriptional changes to signaling genes and disruption of the health of an animal at the genetic level (Allen and Tresini, 2000; Fleming *et al*., 2016). Exposure of cells to conditions of environmental stress including heat stress, retards synthesis of most proteinsbutresults in the inducible expression of HSPs that function as molecular chaperones or proteases(Jolly and Morimoto, 2000;Al-Aqil and Zulkifli, 2009). Heat shock proteins as molecular chaperones, interact with diverse protein substrates to assist in their folding and recovery from stress either by repairing damaged proteins or by degrading them, thus restoring protein homeostasis and promoting cell survival (Jolly and Morimoto, 2000).Acute heat stress elicits rapid Hsp synthesis and causes dramatic changes in gene expression (Purdue

et al., 1992;Xie *et al.*, 2014). In contrast, long-term heat exposure may induce adaptations andaffects nuclear processes like RNA splicing (Richter *et al.*, 2010; Xie *et al.*, 2014).If heat stress is not lethal, it may lead to the tolerance of more severe and fatal stresses and the increased levels of Hsps synthesized in response to moderate stress conditions are the basis for this resistance (Richter *et al*., 2010). In addition, it is suggested that Hsps induced by one type of stress may provide protection against other stresses (Richter *et al*., 2010).

Among the HSP families, HSP70 is the most extensively studied because of its prominent response to diverse stressors (Zhao *et al*., 2013). HSP70 is highly inducible and plays a protective role under stressful conditions, and in addition to hyperthermia, a number of stimuli are known to induce its transcription, including energy depletion (Gabriel *et al*., 2002;Kregel, 2002;Zhao *et al.*, 2013). Under physiological conditions, HSP70 is involved in the *de novo* folding of proteins, and under stress they prevent the aggregation of unfolding proteins and can even refold aggregated proteins (Richter *et al*., 2010).A suggested mechanism for increased hsp70 expression within a cell involves an interaction of heat shock transcription factors (HSFs) and HSPs (Fig. 1.3) through MAPK/SAPK signaling cascades activating HSFs (Morimoto, 1993;Juhasz *et al*., 2014). HSF3, a uniqueavian HSF, has been shown to function as a heatresponsive transcription factor, though roles of distinct HSFshave been proposed to overlap depending on stimulatory signals (Pirkkala *et al*., 2001). The HSFs in the cytosol are bound by HSPs and are maintained in an inactivestate (Kregel, 2002). Heat stress and/or energy depletion would activate HSFs, causing them to separate from HSPs, andthe HSFs are then phosphorylated by protein kinases to form trimers in the cytosol and these HSF-trimer complexes enter the nucleus andbind to heat shock elements in the promoterregion of the hsp70 gene (Fig. 1.3);the hsp70 mRNA is thentranscribed and leaves the nucleus for the cytosol, where new hsp70 is synthesized (Kregel, 2002).

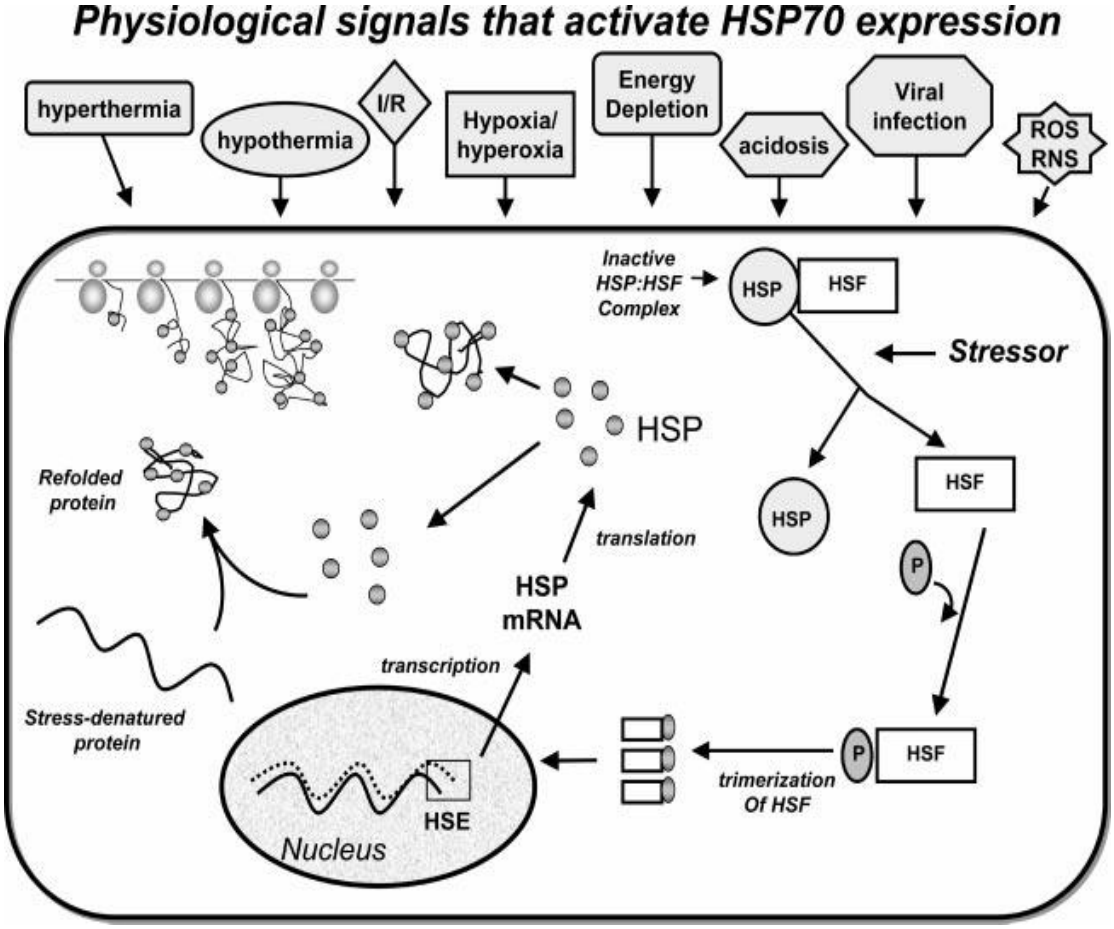

Figure1.3: A summary of some major physiological signals that activate the inducible form of HSP70 synthesis (top) and a proposed mechanism for increased HSP70 expression within a cell.*HSP70: heat shock protein 70; HSF: heat shock transcription factor; P: phosphate; HSE: heat shock binding element; I/R: ischemia/reperfusion; ROS RNS: reactive oxygen species reactions.* Source: Kregel (2002).

Research showing the effects of heat stress on HSP concentrations and gene expression levels in broilers and layer hens has been widely reported and findings suggest that genetic differences alter the type and degree of chickens' responses and their ability to adapt to a stressor (Mack*et al*., 2013).In a study to test the thermo-tolerance ability of five commercial chicken genotypes, Melesse *et al.* (2013) reported major differences in

thermoregulatory responses to heat stress in all five genotypes, possibly due to differences in their overall genetic background. Meanwhile, Tamzil *et al.* (2014) reported that Indonesian local chickens and Arabic chickens have better heat resistance than the commercial hybrid chickens. Liver HSP70 concentrations were greater and liver weights were reduced in layer hens exposed to heat stress when compared to those at thermo-neutral conditions (Felver-Gant *et al.,* 2012). Furthermore, a line of group-selected hens for high productivity and survivability had higher concentrations of HSP70 than a strain of White Leghorn hens selected for high egg production regardless of treatment (Felver-Gant *et al.*, 2012).In broiler chickens, HSP70 were highly induced after acute heat exposure (Yu and Bao, 2008;Lowman *et al.,* 2014; Xie *etal*., 2014).However, HSP70 mRNA levels were unaffected by heat stress (34^{o}C for 4 weeks) in slow growing and fast growing broilers, suggesting adaptation to maintenance of normal thermal homeostasis at elevated temperature (Felver-Gant *et al.,* 2012; Rimoldi *et al.,* 2015). Meanwhile, Indonesian village or native chicken lines were found to have an interaction with Hsp70 genotypes in heat resistance (Tamzil *et al*., 2014). It is generally considered that indigenous breeds of local chickens are better able to withstand high ambient temperatures (Soleimani and Zulkifli, 2010). However, research information is scarce and lacking on the effects of heat stress on HSP70 expression and the extent of high temperature tolerance levels in Tanzanian local chickens.

1.3.5 Physiological and behavioural effects of heat stress

Animals respond to heat stress by activating the stress response through a wide array of behavioral and physiological responses (Smith and Vale, 2006).In cases where the environmental temperatures exceedthe thermoneutral zone, the core body temperaturebecomes elevated and a number of responses are initiated, leading to the neutralisationof heat stress-induced metabolic changes (Melesse *et al*., 2013).The

optimum ambient temperature range for poultry is 12 to 26^{o}C (Ayo *et al.*, 2011).In poultry,heat stress alters the activity of the neuroendocrine system, resulting in activation of the hypothalamic-pituitary-adrenal axis, and elevated plasma corticosterone concentrations (Quinteiro-Filho*et al.,* 2012;Lara and Rostagno, 2013). Thereleaseofcorticosteronecauses thedissolutionoflymphocytes in lymphoid tissues leading to lymphopeniaandanincrease inheterophilrelease bythe bonemarrow (Borges *et al.*, 2004). In addition, several blood parameters, including haemoglobin (Hb), packed cell volume, CO_2 levels, saturated O_2 and pHare varied and these changes may be good candidates for predicting response to heat stress andfor use as biomarkers of heat tolerance (Lamont*et al.,* 2015). In the case of strong psychogenic or emotional stress, the sympathetic-spinal-adrenal axis is activated, which results in the release of catecholamines. Catecholamines induce immediate secretion of glucose to blood, degradation of glycogen accumulated in liver, stimulation of the activity of vasomotoric center, changes in the intensity of ventilation, and increased nervous sensitivity (Ognik and Sembratowicz, 2012). Too intensified and long-lasting stress induces disorders of a daily rhythm of hormones secretion, physiological and morphological changes, manifested mainly in changes of blood composition, changes in muscle tissue and formation of meat defects(Ognik and Sembratowicz, 2012).

The effects of heat stress on physiological parameters in broiler and commercial layer chickens have been well documented and include an increase in plasma glucose level (Lin *et al*., 2000;Garriga *et al*., 2006), increased body core temperature (Soleimani *et al.,* 2011), alteration of the electrolyte balance and blood pH (Van Goor, 2016), increasedplasma corticosterone levels associated with an alteration of the metabolic function (Quinteiro-Filho *et al.,* 2010;Rimoldi *et al.,* 2015;Akbarian *et al*., 2016),an increase in H/L ratiosafter both acute (Borges *etal*., 2004; Soleimani and Zulkifli, 2010; Felver-Gant *et al*., 2012;

Tamzil *et al.,* 2014) and chronic heat stress (Keambou *et al.*, 2014), and decrease in Hct and Hb(Lamont *et al.,* 2015). A major change in blood components is caused by cellular damage (Bogin *et al.,* 1996) and heat -induced increased respiration, which results in respiratory alkalosis (Van Goor *et al.,* 2016).However, research information is still scanty and scarce on the physiological effects of heat stress in local or indigenous chickens with constant or cyclic exposure to high ambient temperatures. Exposing Cameroonian local chickens to an average temperature of 35^{o}C over 8 weeksraised H/L ratios(Keambou *et al.*, 2014) but were not elevated in indigenous chickens of Malaysia after acute heat exposure (Soleimani and Zulkifli, 2010). An acute exposure to 40^{o}C of Indonesian native or local chickens caused an increase in H/L ratio (Tamzil *et al.,* 2014). Hct and Hblevels of Cameroonian local chickens were not significantly affected by the rise in breeding temperature from 25 to 35°C(Keambou *et al*., 2014).

The correct interpretation of behaviours expressed by poultry may be used to estimate their welfare (Costa *et al*., 2012). Under high temperature conditions, chickens exhibit behavioural changes such as panting and wing droop to aid thermoregulation and increase the flux of heat from the tissues to the environment thereby dissipating heat from the body leading to decreased body temperature (Syafwan *et al*., 2011;Mack *et al.,* 2013).Body temperature and metabolic activity are regulated by the thyroid hormones, triiodothyronine (T3) and thyroxine (T4), and their collective balance,involving complex metabolic pathways (Lara and Rostagno, 2013).Local activation of T4 to the active form T3, by 5'-deiodinase type 2 is a key mechanism of thyroid hormone regulation of metabolism (Muller *et al*., 2014). The enzyme 5'-deiodinase type 2, is expressed in the hypothalamus, white fat, brown adipose tissue and skeletal muscle, and is required for adaptive thermogenesis (Muller *et al.,* 2014).Dysregulation of concentrations of hormones such as corticosterone has been associated with induction of varied behavioural changes under

heat stress conditionsincluding, panting, sand bathing and standing with wings drooped and lifted slightly from the body to maximize heat loss (Cheng and Jefferson, 2008). They can also express their normal behaviour such as foraging, thereby ingesting those ingredients that avoid excessive heat loads while being ingested and metabolized (Syafwan *et al.,* 2011). In general, birds react similarly to heat stress but express individual variation in the intensity and duration of their responses (Mack *et al.,* 2013). Heat stress causes behavioural changes, including feeding, preening, feather ruffle and pecking among laying hens and broilers of different strains, with responses differing by genotype (Mack *et al.,* 2013; Li *et al*., 2015).

Research has not been done detailing the effects of heat stress on behavioural variations among the Tanzanian local chicken ecotypes.Previous research in broilers and layers has shown decreases in preening, locomotion, feeding, and increases in drinking behaviors (Mack *et al.,* 2013;Li *et al.,* 2015) under heat stress.High ambient temperatures and increasing stocking densities caused a decrease in percentage of birds that spent time walking, standing, sitting, preening, feeding and drinking in the feathered birdsunlike the featherless broilers (Lolli *et al.,* 2010).Alert behavior, feather ruffle and crowing were significantly frequent in White Leghorn than in Red Jungle fowl, while relaxed behavior and preening were more frequent in Red Jungle fowl in a study to compare behavioural response of two breeds of chickento acute stress(Ericsson *et al.,* 2014).Further, stressingconditions such astransportation (Cheng and Jefferson, 2008), stocking density (Lolli *et al.,* 2010) andsocial interactions (Dennis *et al.,* 2004)have also been shown to alter behaviour in commercial or exotic chickens.

1.3.6 Effects of heat stress on production parameters

Even though heat stress stands out as one of the most important environmental stressors,research information on its effects on productivity and metabolic viability of the local chickens still remains scanty. However, previous studies have shown significant variation between regions and ecotypes with respect to production attributes, including growth rate, adult body and egg weights(Msoffe *et al.*,2002;2005; Guni *et al.*, 2013). On the other hand, adverse effects of heat stress on broilers and laying hens have been extensively studied (Lin *et al.*, 2000; Deeb *et al.*, 2002;Deng *et al.*, 2012; Zhang *et al.*, 2012) and these include lowering of: cumulative feed consumption, feed utilization efficiencies, body weight, egg production, meat qualityand general production performance. In addition many researchers have reported breed and strain differences in tolerance to heat stress in chickens (Soleimani and Zulkifli, 2010; Felver-Gant *et al.*, 2012; Melesse *et al.*, 2013; Tamzil *et al.*, 2014).Heat stress conditions in poultry lead to increased panting that leads to increased carbon dioxide levels and higher blood pH (alkalosis), which in turn impedes blood bicarbonate availability for egg shell mineralization (Lara and Rostagno, 2013). Alkalosis also induces increased organic acid availability thereby decreasing free calcium levels in the blood (Lara and Rostagno, 2013).In the hens, heat stress can disrupt the normal status of reproductive hormones of the hypothalamus-pituitary-ovary axis and lead to decreased blood levels (Elnagar *et al.*, 2010). In the cocks, semen volume, sperm concentration, number of live sperm cells and motility decreased when males were subjected to heat stress (McDaniel *et al.*, 1995).

1.3.7 Effect of suboptimal nutrition on production parameters

Lack of access to quality feed and failure to balance between energy and protein requirements is still a huge challenge for local chickens' production sector (Sonaiya, 2007;Mutayoba *et al.*, 2012). Under natural environments scavenging local chickens are

exposed to suboptimal nutritionor limited feed availabilitydue to factors such as seasonal conditions, farming activities, land size available for scavenging and flock size (Goromela *et al.,* 2007). Previous research has shown that chemical composition of feeds eaten by rural scavenging chickens of Tanzania was below the nutritional requirements and varied with season, climate and age of birds (Goromela *et al.,* 2007; Mwalusanya *et al.,* 2010). The low levels of energy, protein and minerals in the crop and gizzard contents indicated that diets consumed by birds could not meet optimum requirements of scavenging birds for growth and egg production (Goromela *et al*., 2007). However, research information on the effect of dietary energy and protein levels on production variables in local Tanzanian chickens is limited. TheKU ecotype had a better body weight when compared to MM under both extensive and intensive management in a study to evaluate on-station and on-farm differences(Lwelamira *et al*., 2008). Live weight, breast, drumstick and head weight were markedly reduced when desi local or indigenous chickens of Bangladesh were fed a low energy diet of 2400 kcal/kg ME (Miah *et al.,* 2014). A study investigating the response of male Venda local chickens of South Africa to varying energy to protein ratios found that the dietary energy to protein ratio of 66 MJ ME/ kg protein supported optimum growth rate(Mbajiorgu*et al*., 2011). Meanwhile in Uganda, Magala *et al.* (2012) reported that a 2800 kcal/kg ME and 18% CP diet was sufficient for growing local chicken cockerels.The effects of dietary energy levels on productive performance have been extensively studied in commercial or exotic chickens (Chen *et al*., 2012; Perez-Bonilla *et al.,* 2012;Ribeiro *et al.,* 2014) and it is evident that in these chickens feed restriction has been commonly used to reduce metabolic disorders and control bodyweight (Fassbinder-orth and Karasov, 2006). Moreover changes in energy concentration of the diet have resulted in contrasting results with respect to productive performance and feed conversion ratios (FCR) of the laying hens. Ribeiro *et al.* (2014) reported that dietary apparent metabolisable energy (AMEn) levels did not influence body weight, egg weight, or

livability, and that increasing AMEn levels increased feed intake and feed conversion ratio whilst Perez-Bonilla *et al*. (2012) reported that an increase in energy concentration of the diet increased egg production, egg mass, energy efficiency and body weight gain but decreased feed conversion ratio per kilogram of eggs.

In summary, while it is clearthat suboptimal nutrition and heat stress adversely affect production and welfare, it is not yet clearly established what type of differences and variations occur, with regards to physiological, biochemical and behavioural responses among Tanzanian local chicken ecotypes. It is vital to devise programs that include physiological, biochemical and behavioral traits that would enhance selection for heat and low dietary energy tolerance among the local chicken stocks. It is also important to assess if different common ecotypes can show measurable differences in their heat stress tolerance. Further, there is need to determine whether heat stress effects are compounded by low quality feed sincein the natural localities where these chickens are reared seasonal summer high temperatures are generally accompanied by a lack of adequate feed resources to meet their energy or nutritional requirements. The apparent differences taken together may be beneficial in making informed recommendations for the future breeding programs.

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CHAPTER TWO

Ecotype Differences in Growth and Behavioural Responses to Stress Induced by Low Dietary Energy in Tanzanian Local Chickens

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ABSTRACT

A study was conducted to compare growth and behavioural responses to low dietary energy in three chicken ecotypes at 4 weeks old for 7 weeks. 351 hens belonging to Kuchi, Ching'wekwe and Morogoro medium ecotypes were allocated to 9 pens in a 3 x 3 factorial design, with 3 replicates.They were fed 3 diets containing 40, 55 or 0% less energy than prescribed for commercial layers. Low dietary energy increased feed intake but reduced growth rates of chickens in all study groups. At 40% and 0% restriction levels, Kuchi had significantly higher ($p<0.05$) weight gains, while Morogoro medium had significantly higher ($p<0.05$) weight gains and lower feed conversion ratio (FCR) at 55% restriction. Body lengths, shank lengths, chest circumferences and wing spans for Kuchi and Ching'wekwe but not Morogoro medium were significantly ($p<0.05$) reduced for both restricted groups. Foraging and feeding behaviours were higher in restricted groups of all ecotypes in the third week but not the seventh week of study. Morogoro medium had least mortality in both restricted groups and controls. Results of this study show ecotype-specific tolerance to low dietary energy through differences in growth performance, FCRs and behavioural responses. Morogoro medium showed better tolerance at the lowest energy levels whereas Kuchi exhibited better performance at 40% restriction and control energy levels.

Key words:*feeding, foraging, morphometric, restriction, stress, tolerance*

2.0 INTRODUCTION

Local chickens are prominent in many developing countries includingTanzania and they greatly contribute to the income and nutrition of households (Mwalusanya *et al*., 2001; Wilson, 2015; Padhi, 2016). Several local chicken ecotypes have been identified in Tanzania based on their geographical origin and phenotypic characteristics (Msoffe *et al*., 2001, 2005). These ecotypes show variations in adult body weight, egg weight, plumage characteristics and resistance to disease. Moreover genetic uniqueness and limited interbreeding among local chicken ecotypes have been previously reported and these have been attributed to their geographical separation and preferential mate selection (Msoffe *et al*., 2002, 2005; Mayardit *et al*., 2016). The current study focused on Kuchi (KU), (originally from north-west Tanzania), Morogoro medium (MM) (originating from central Tanzania), and Ching'wekwe (CH) (also originating from central Tanzania) local chicken ecotypes because of their productive and disease resistance potential as reported previously (Msoffe *et al*., 2002, 2005). Despite their distinct origins, these chickens are now reared almost throughout the country.

Dietary energy levels have an effect on feed intake, feed conversion ratio and growth of the chickens as they feed to satisfy their energy requirements (NRC, 1994). The effects of dietary energy levels on productive performance have been extensively studied in commercial chickens (Chen *et al*., 2012; Perez-Bonilla *et al*., 2012;Ribeiro *et al*., 2014) and it is evident that in these chickens feed restriction has been commonly used to reduce metabolic disorders and to control bodyweight (Fassbinder-orth and Karasov, 2006). Moreover, changes in energy concentration of the diet have resulted in contrasting results with respect to productive performance and feed conversion ratios (FCR) of the laying hens. Ribeiro *et al*. (2014) reported that dietary apparent metabolisable energy (AMEn) levels did not influence body weight, egg weight, or livability, and that increasing AMEn

levels increased feed intake and feed conversion ratio; whilst Perez-Bonilla *et al*. (2012) showed that an increase in energy concentration of the diet increased egg production, egg mass, energy efficiency and body weight gain but decreased feed conversion ratio per kilogram of eggs. However, information on the effect of dietary energy levels on production variables in local chickens is limited (Mbajiorgu, 2011;Nakkazi *et al*., 2015).

Despite their usefulness and contribution to the nutrition and income of rural communities, achieving increased productivity and sustainability of local chickens is still a huge challenge mainly because of lack of access to quality feed and failure to balance between energy and protein requirements (Sonaiya, 2007;Mutayoba *et al*., 2012). The availability of scavenging feed resources is crucial to appropriate rearing of local chickens (Sanka and Mbaga, 2014).Under natural environments scavenging local chickens are exposed to feed and dietary energy stress due to seasonal availability of feed. Thus, it is of interest to know how the local chicken ecotypes respond under different nutritional stresses that may appear in nature especially during the growing phase.A study by Mwalusanya *et al*. (2010) showed that chemical composition of feeds eaten by rural scavenging chickens of Tanzania was below the nutritional requirements and varied with season, climate and age of birds. This was ascertained after dissecting the crops of the local chickens from different climatic zones and analyzing their contents. Although the nutritional requirements for the local chickens have not been conclusively determined, requirements for slow growing commercial layer chickens may apply. A study investigating the response of male Venda local chickens of South Africa to varying energy to protein ratios (Mbajiorgu, 2011) found that the dietary energy to protein ratio of 66 MJ ME/ kg protein supported optimum growth. Meanwhile in Uganda, Magala *et al*. (2012) reported that a 2800 kcal/kg ME (11.73 MJ ME/kg) and 18% CP diet was sufficient for growing local chicken cockerels.

Expression of behaviour has been fundamental in understanding the welfare of chickens at a particular moment and its correct interpretation can be used to compare the effects of particular stressors on chickens of different strains (Costa *et al*., 2012;Ericsson *et al*., 2014). Generally, stress modifies the development of the hypothalamic-pituitary axis response thereby affecting growth and behaviour (Ognik and Sembratowicz, 2012). However, limited information is available on the relationship between stress and behaviour in chickens. Zulkifli *et al*. (2006) found that feed deprivation increased non-nutritive pecking among laying hens but did not have a significant effect on standing, drinking and preening activities. In a study to compare acute stress behavioural response of two breeds of chicken, Ericsson *et al*. (2014) reported that Red Jungle fowl had more frequent relaxed and preen behaviour but reacted stronger to acute restraint stress than White Leghorn. The present study was designed to compare the growth and behavioural responses to stress induced by low dietary energy in CH,KUand MM local chicken ecotypes. This was done with a hypothesis that local chickens commonly bred from different geographic regions of Tanzania might have selected ecotypes with stronger tolerance to stress induced by feed of lower energy levels. Selecting for ecotypes with better growth and behavior performance under restricted energy intake is beneficial in breeding programs aimed at conservation of indigenous genetic resources within local chicken stocks.

2.1 Materials and Methods

2.1.1 Experimental chickens

Day-old MM, CH and KU local chicken ecotypes were obtained from the parent flock kept by the Feed the Future Genomics to Improve Poultry Project at Sokoine University of Agriculture. The chicks were brooded and reared under similar environmental, managerial and 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 2,864 kcal ME/kg up to the 4th week. All chickens were vaccinated routinely against Newcastle disease, Infectious Bursal Disease (Gumboro) and Fowl pox.

2.1.2 Feed formulation

Three types of feeds were formulated, the first contained 2864 Kcal/kg ME and served as control diet, the second feed contained about 40% less energy than the control (i.e. 1696 Kcal/kg ME), while the third diet contained 55% less energy than the control (i.e. 1319 Kcal/kg ME). Research findings involving feed restriction as a stressor in local chickens have not been reported. However, previous studies have shown that 55% quantitative feed restriction significantly reduced the body weights of broiler breeder hens (Bruggeman *et al*., 2005). Diets were formulated using locally available feedstuffs and ground wood charcoal (Rezaei *et al*., 2006) was used to dilute the feed. The chemical (proximate) analyses of different feed ingredients were carried out using standard methods (FAO, 1994). Feed samples were analyzed for crude fiber, crude protein (Kjeldahl protein), moisture, ash, nitrogen-free extracts (digestible carbohydrates) and crude lipid; and then metabolisable energy levels were estimated (NRC, 1994; Janssen, 1989). The composition of specific ingredients in the feed is depicted in Table2.1.

Table 2.1: **Composition and nutrient levels of experimental diets**

	Control diet **2864** Kcal/kg ME	**40%Energy Restriction** **1696** Kcal/kg ME	**55%Energy Restriction** **1319** Kcal/kg ME
Ingredients	**(%)**	**(%)**	**(%)**
Maize meal	37.8	14.5	10
Maize bran	26	10.3	2
S.flr. meal	20.5	22.5	21
Fish meal	11	18	22.3
G. charcoal	0	30	40
Limestone	2	2	2
Premix[n]	0.3	0.3	0.3
Methionine	0.3	0.3	0.3
Lysine	0.3	0.3	0.3
DCP	1.3	1.3	1.3
Salt	0.5	0.5	0.5

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

2.1.3 Experimental design

A total of 351 four weeks old female chicks belonging to KU, CH and MM ecotypes were weighed and randomly allocated according to ecotype to 9 pens in a 3 x 3 (three ecotypes and 3 types of diets) factorial design, with three replicates. The birds were fed 3 types of iso-nitrogenous (18% crude protein) diets formulated to contain 40, 55, and 0% (control) less energy than prescribed by the NRC (1994) for commercial layer chickens for seven weeks. The birds were reared on littered (rice husks) floors in a well ventilated house. Feed and water were supplied *ad-libitum* throughout the experimental period (7 weeks). Each pen had on average an area of 2 m² floor space per 13 birds. The study was conducted at the prevailing cyclic ambient temperatures ranging from 21.6 to 34.3°C. The pens were artificially lit with a 12L: 12D cycle, corresponding to the natural conditions.

2.1.4 Data collection

Growth and behavioural responses were determined for 7 weeks; from 4 to 11 weeks of age. Feed consumption was recorded daily and morphometric parameters (body length, shank length, chest circumference, and wing span) were recorded at 2, 4 and 6 weeks of feed restriction using a measuring tape. Chicken body weights under all treatments and controls were recorded on a weekly basis and feed conversion ratios (conversion index = daily feed consumption/daily weight gain) and growth rates [(final weight – initial)/time interval] were subsequently calculated. Behaviour observations were measured using the direct observation method (Lolli *et al.*, 2013) and classified into 6 categories, namely: feeding (eating and drinking), foraging (scratching and litter pecking), aggression (intense feather pecking of another chicken), resting (sitting and standing), comfort (preening and sand bathing) and locomotory activities (moving around). The number of birds engaged in particular behaviour was counted (expressed as percentages) at 5 minute-intervals and mean values per week were computed for each pen. The observations were made between 11 and 14 hours everyday by a single observer (first author) within the chicken house and precaution was taken not to disturb the natural behaviour of the chickens. All procedures used in this study were in compliance with the Sokoine University of Agriculture's guidelines for care and use of animals in research.

2.1.5 Statistical analysis

One-way ANOVA (SPSS 20) was used to analyze differences among the treatments. In case of detection of differences in treatment means by ANOVA, Dunnett t- test and LSD test were used to separate means, with significance statements based on $P < 0.05$. Correlation analysis was performed by linear regressiontest using SPSS 20 softwareand the correlation coefficients wereconsidered significant at $P < 0.05$.Data for percent weight

gain, feed conversion ratios, morphometric parameters, and behavior are expressed as Mean±SD.

2.2 Results

2.2.1 Growth rate

The growth curves and mean growth rates of the chickens over the entire experimental period are presented in Fig.2.1 (A, B, C) and Table 2.2, respectively. For all ecotypes, there was a steady increase in body weight with age.The control groups had higher growth rates than the restricted groups (Table 2.2). Differences in growth rates within ecotypes were from two weeks (at 42 days of age) after the start of dietary energy restriction to the end of the experiment (Fig.2.1 A, B, and C). For the controls and 40% energy restriction groups, KU had the highest mean growth rate whilst CH had the least. However, for 55% energy restriction MM had the highest mean growth rate (Fig.2.1 C and Table 2.2), whilst CH had the least. Therefore, for all the feed-type groups CH had the lowest mean growth rate.

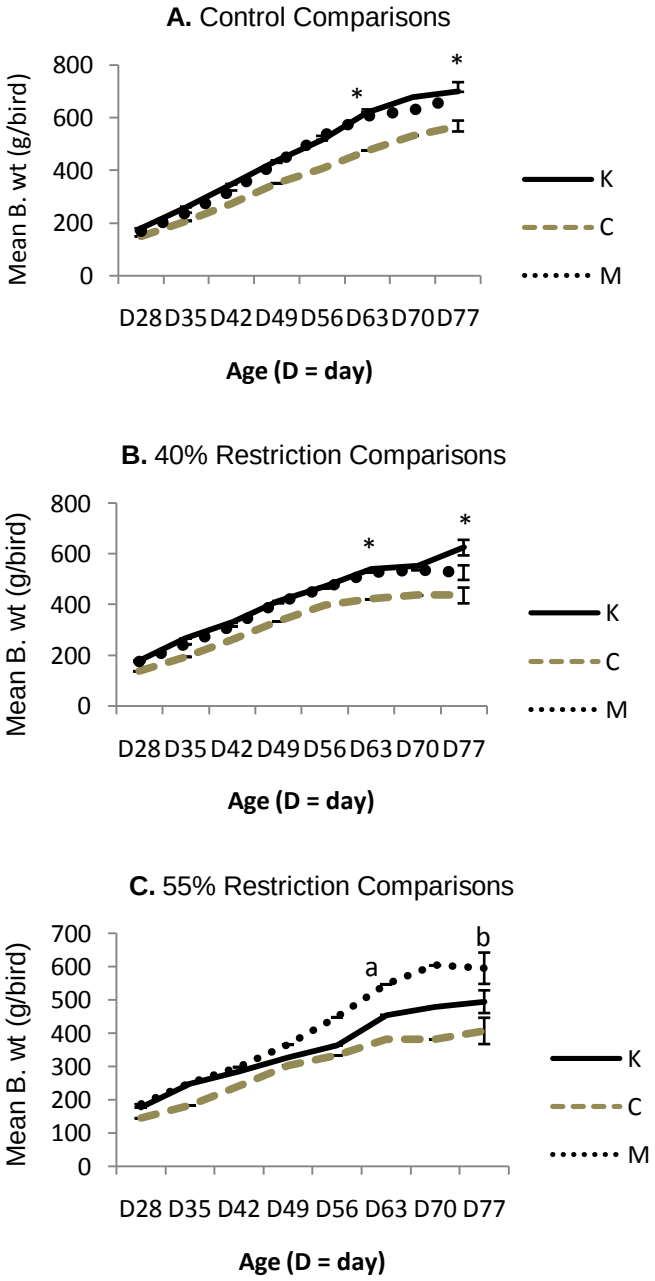

Figure 2.1: Growth curves of the three chicken ecotypes.

*significantly higher than C; [a] significantly higher than K and C; [b] significantly higher than C; *K = kuchi, C = ching'wekwe, M = Morogoro medium, B.wt = body weight.*

Table 2.2: **A summary of mean growth rates* for the entire experimental period**

Ecotype	Feed-type	Growth rate (g/day)	Drop in growth rate (%)
K	Control	10.6	-
	40R	9.11	14.0
	55R	6.49	38.7
C	Control	8.54	-
	40R	6.11	28.4
	55R	5.36	37.2
M	Control	10.39	-
	40R	7.18	30.9
	55R	8.35	19.6

* (final weight – initial)/time interval; *K = kuchi, C = ching'wekwe, M = Morogoro medium, 40R=40% energy restriction group, 55R=55% energy restriction group.*

2.2.2 Weight gain

The mean percent weight gains of the chickens on days 49, 63, and 77 of age (3, 5 and 7 weeks of experimental period) are presented in Fig.2.2. For all ecotypes, the control groups had higher mean percent weight gains than restricted groups. For the controls and 40% energy restriction groups, KU had markedly higher ($p < 0.05$) mean percent weight gain on days 49, 63 and 77 than MM and CH. At 55% energy restriction MM had higher ($p < 0.05$) mean percent weight gain than KU at 49, 63 and 77 days of age. KU had the lowest mean percent weight gain for the 55% restriction group.

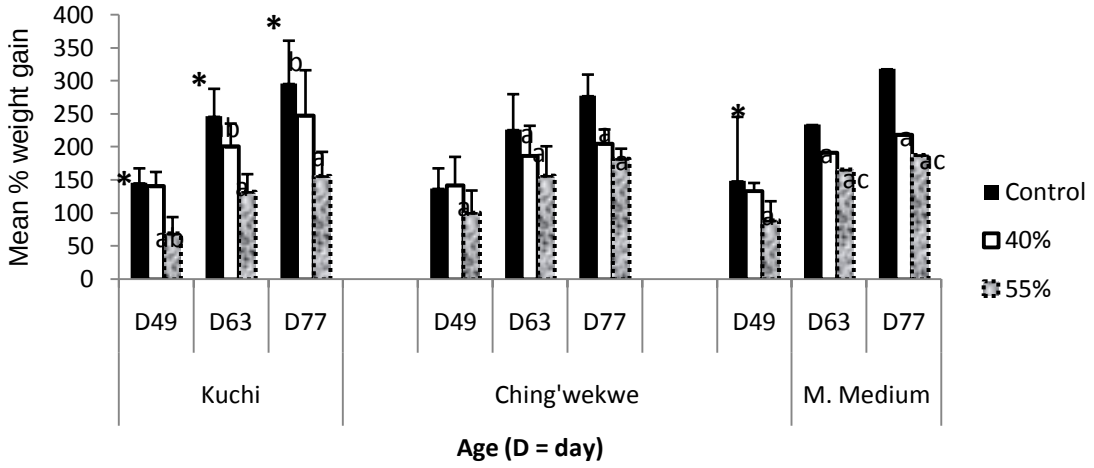

Figure 2.2:Mean % weight gain at 49, 63, and 77 days of age (3, 5, and 7 weeks of feed restriction) – feed-type and ecotype comparisons;

*a: significantly different from the control, *: significantly higher than in Ching'wekwe; b: significantly higher than in Ching'wekwe and M. Medium, ab: significantly different from the control and from that in Ching'wekwe and M. Medium; ac: significantly higher than in K (p<0.05)*

2.2.3Feed conversion ratios (FCRs)

The mean FCRs of the chickens are presented in Fig.2.3. The FCR tended to increase with reduced dietary energy in all groups. Controls had significantly lower ($p<0.5$) FCRs when compared with respective restricted groups for all ecotypes. MM had a markedly lower ($p<0.05$) mean FCR than CH among the controls. At 40% energy restriction no significant difference in mean FCRs was observed among the three ecotypes. At 55% energy restriction MM and CH had significantly lower mean FCRs than KU.

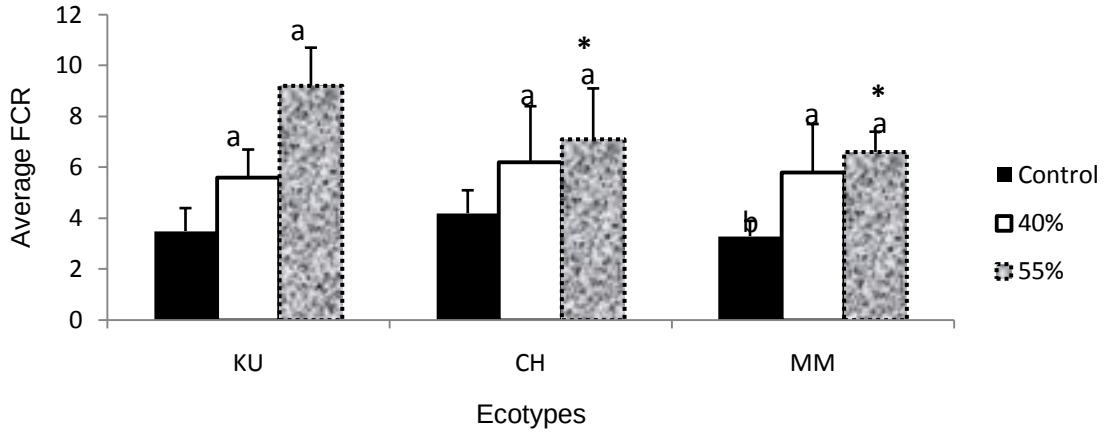

Figure 2.3: Mean feed conversion ratios (FCR) (conversion index = daily feed consumption/daily weight gain); *KU: Kuchi; CH: Ching'wekwe; MM: Morogoro medium; a: significantly different from the control; b: significantly lower than the control in CH; *: significantly lower than that of KU (p<0.05).*

2.2.4 Correlation analysis between energy restriction and growth parameters

Correlations between energy restriction and growth rate, FCR and mortality are presented in Table 2.3. Energy restriction was negatively correlated with growth rate but positively correlated with FCR in all chicken ecotypes. The energy restriction and growth rate correlation was only significant in CH ($p<0.05$) whilst the energy restriction and FCR correlation was significant in CH and MM ($p<0.05$). Energy restriction and mortality were positively correlated in all ecotypes but the correlation was only significant in CH ($p<0.05$).

Table 2.3: Correlations between energy restriction and growth rate, feed conversion ratio and motality

		Energy Restriction		
KU	**CH**	**MM**		
Growth rate	r	-0.915	-0.999* (p = 0.013)	-0.805
FCR	r	0.927	0.999* (p = 0.013)	0.999* (p = 0.010)
Mortality	r	0.688 0.259	0.998* (p = 0.021)	0.254 0.418

*Significantly different (p<0.05); *KU: kuchi; CH: ching'wekwe; MM: Morogoro medium.*

2.2.5Morphometric traits

The measured morphometric traits are presented in Table 2.4. There was no significant difference between controls and restricted groups for all morphometric traits at two and four weeks of energy restriction. KU and CH had their body lengths, shank lengths, chest circumferences, and wing spans significantly reduced ($p<0.05$) for the 40% and 55% restricted groups after 6 weeks of low dietary energy. MM had its body length and shank length significantly reduced ($p<0.05$) only for the 40% restricted group, with the chest circumference and wingspan not significantly different from the control for all the feed-types (Table 2.4). KU had higher whilst CH had the least values in morphometric measurements for all traits studied.

Table 2.4: Effect of low dietary energy on morphometric parameters after 6 weeks (data presented as Mean ± SD in cm)

	BL	SL	CC	WS
K Cont	31.5±0.8	7.10±0.3	24.4±1.0	39.8±1.6
K 40	28.6±1.5[n]	6.36±0.4[n]	22.3±1.3[n]	36.7±1.3[n]
K 55	28.3±1.2[n]	5.80±0.6[n]	21.5±1.8[n]	36.3±2.0[n]
C Cont	28.0±1.0[*]	5.68±0.4[*]	21.8±0.4[*]	35.5±1.4[*]
C 40	27.0±1.1	5.20±0.6	20.4±1.3[n]	32.6±2.1[n]
C 55	26.6±0.9[n]	5.00±0.5[n]	19.6±1.0[n]	32.9±1.8[n]
M Cont	30.3±1.3	6.77±0.5	23.8±1.7	39.0±2.1
M 40	28.7±1.2[n]	6.34±0.3[n]	22.7±1.1	37.0±1.9
M 55	30.0±1.3[i]	6.43±0.4[i]	22.7±1.3[o]	38.0±2.3[o]

K: kuchi; C: ching'wekwe; M: Morogoro medium; 40: 40% restriction; 55: 55% restriction; Cont: control; BL: body length; SL: shank length; CC: chest circumference; WS: wingspan; w: week. [n]*: significantly different (p<0.05) from the control of the respective ecotype;* [*]*:significantly lower (p<0.05) than the controls in K and M;* [i]*: significantly higher (p<0.05) than in K and C at 55% restriction;* [o]*: significantly higher (p<0.05) than in C at 55% restriction.*

2.2.6Behaviour

Behaviour results are presented in Fig.2.4 and 2.5. The mean percent of the number of chickens exhibiting feeding behaviour was significantly higher ($p<0.05$) in the 55% energy restriction group than controls for all ecotypes in the third week of energy restriction. Similarly, the mean percent of chickens involved in foraging was significantly higher ($p<0.05$) in both restricted groups than controls for all ecotypes. The mean percent of chickens exhibiting resting behavior per time interval was significantly lower ($p<0.05$) in both restricted groups than the controls, but MM showed no significant difference with controls (Fig.2.4). Fewer birds exhibited other behaviors such as locomotion, comfort and aggression in all the groups except for the controls. There were no ecotype-specific differences except among the control groups whereby MM exhibited significantly lower ($p<0.05$) level of resting behavior.

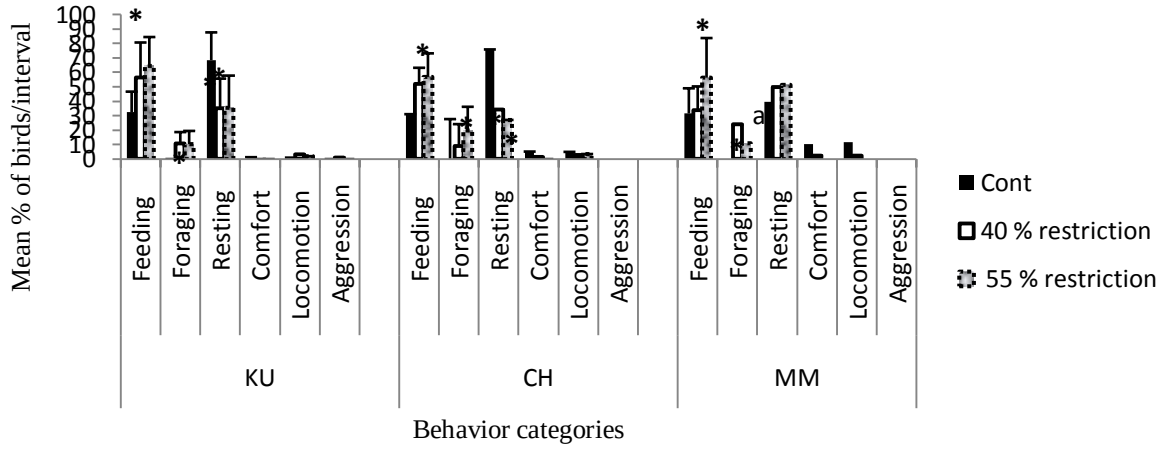

Figure 2.4: **Mean % of chickens exhibiting particular behavior per 5 minute interval – week 3**; **: significantly different from the control, a: significantly lower than in KU and CH (p<0.05). Cont: control; KU: Kuchi; CH: Ching'wekwe; MM: Morogoro medium.*

In the seventh week of energy restriction, there was no significant difference between the restricted groups and controls in the mean percent of chickens exhibiting feeding behavior in all ecotypes (Fig.2.5). Both restricted groups for KU showed a markedly higher ($p<0.05$) mean percent of chickens involved in foraging than the control. Mean percent of birds exhibiting resting behavior was significantly lower ($p<0.05$) than the controls for KU and CH in the 40 and 55% restricted groups. For both restricted groups, the mean percent of birds exhibiting all behavior types for MM was not significantly different from the controls (Fig. 2.5). There were no ecotype-specific differences except among the 55% restricted groups where KU exhibited significantly lower ($p<0.05$) mean percent resting behavior than CH and MM. Similarly, just like in the third week, fewer birds were involved in the other behaviors such as locomotion, comfort and aggression in all the groups at this stage.

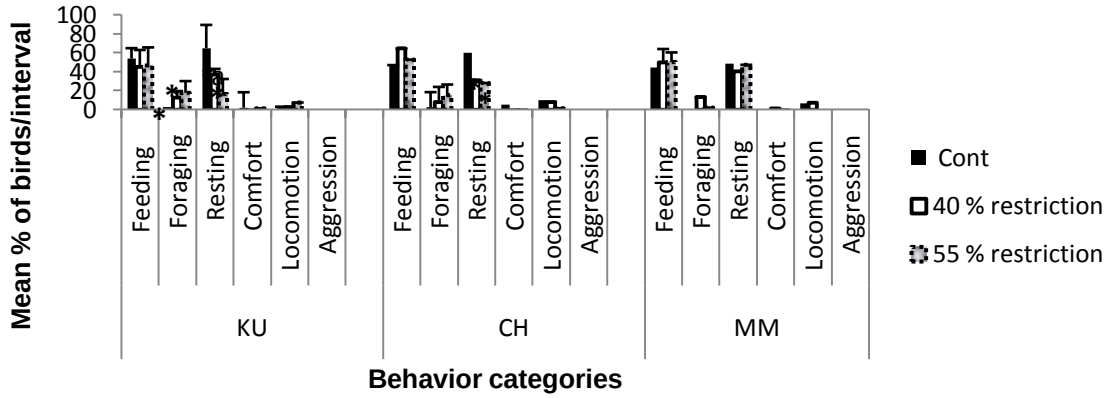

Figure 2.5:**Mean % of chickens exhibiting particular behavior per 5 minute interval – week 7***; **: significantly different from the control, a: significantly lower than in CH and MM (p<0.05). Cont: control; KU: Kuchi; CH: Ching'wekwe; MM: Morogoro medium*.

2.2.7 Correlation analysis of energy restriction and behavioural responses

Correlations between energy restriction and behavioural responses are presented in Table 2.5. Energy restriction was strongly positively correlated with feeding behaviour and foraging in the third week for all chicken ecotypes. The correlation with feeding behaviour was significant ($p<0.05$) for CH and KU. While energy restriction and resting behaviour were negatively correlated in CH ($p<0.05$) and KU, the correlation was positive for MM. In the seventh week, energy restriction and resting behaviour were negatively correlated while the correlation between energy restriction and foraging was positive in all chicken ecotypes. Energy restriction and feeding behaviour were positively correlated in CH and MM but negatively correlated in KU.

Table 2.5: Correlations between energy restriction and behavioural responses

Energy Restriction

	KU	CH	MM
Week 3			
Feeding	0.999* (p = 0.006)	0.997* (p = 0.025)	0.762
Resting	-0.960	-0.992* (p =0.041)	0.988 (p = 0.05)
Foraging	0.962	0.954	0.672
Week 7			
Feeding	-0.914	0.556	0.979
Resting	-0.985 (p = 0.05)	-0.983 (p = 0.05)	-0.363
Foraging	0.999* (p = 0.013)	0.939	0.408

*Significantly different ($p<0.05$); *KU: kuchi; CH: ching'wekwe; MM: Morogoro medium.*

2.3 Discussion

The present study compared the growth and behavioral responses to stress induced by low dietary energy in local chickens commonly bred from different geographic regions of Tanzania. The relationship between energy restriction and growth rate was highly linear and negatively correlated in all the chicken ecotypes. It was observed that dietary energy restriction at both 40 and 55 % (1696 and 1319 kcal/kg ME, respectively) levels reduced growth rates and feed utilization efficiencies as evidenced by the negative (growth rate) and positive (FCR) correlations. The birds were able to similarly tolerate and adapt to this stress but in some cases differently and in an ecotype-specific manner. Dietary energy restriction increased feed consumption in all the ecotypes during the study period. This was probably an adaptive measure to meet the deficit in the daily energy requirement. Birds usually eat to satisfy their energy needs and adjust their feed intake according to

their metabolisable energy requirements (NRC, 1994; Nakkazi *et al*., 2015). The MM ecotype performed better when compared to other groups at 55% dietary energy restriction throughout the experimental period with respect to growth rates, FCRs and percent average weight gains. This was the lowest dietary energy restriction level used in the present study. Although CH ecotype also had significantly lower average FCR than KU at 55% energy restriction, it had an inferior growth rate and percent weight gain.

KU ecotype, however, showed a better performance under less stressing energy levels as exemplified by a significantly higher percent weight gain and higher growth rates up to the end of the experiment for the controls and at 40% energy restriction. The growth performance of KU at control conditions in this study is in line with Lwelamira *et al.* (2008) who reported a better body weight for KU when compared to MM under both extensive and intensive management in a study to evaluate on-station and on-farm differences. However, the current study is the first to compare various Tanzanian local chicken ecotypes under dietary energy restriction conditions.The results of the current study are also in agreement with other previous studies on commercial lines with respect to decreasing feed utilization efficiency, growth rate and weight gain as dietary energy density is decreased (Leeson *et al*., 1996; Bruggeman *et al*., 2005; Rosa *et al.*, 2007; Chen *et al*., 2012). In contrast to the findings of the current study, Chen *et al*. (2012) reported that energy restriction significantly increased the feed efficiency of female broiler chickens from 40 to 48 days old. The differences can be due to less stressful restriction regimes (30% energy restriction) used in their study and also because of differences in the chicken strain and phase of growth with the current study. Furthermore, Magala *et al*. (2012) found that an increase in dietary energy from 2800 kcal/kg to 3000kcal/kg did not affect weight gain and FCR in Ugandan local chicken cockerels. It can be said that the

restriction dietary energy levels used in the present study are much lower and this explains the differences.

Feed efficiency, expressed as the amount of feed intake per body weight gain, is reflected in the FCR, and lower FCR means a better performanceas the birds were more efficient in using the feed supplied (Aggrey *et al*., 2010). Dietary energy, as a priority, is directed towards basal metabolism and maintenance, with the remaining energy used for growth and tissue accretion; and therefore, any limitation in dietary energy intake results in reduced growth and tissue accretion (Veldkamp *et al*., 2005). In the present study, 1696 Kcal/kg ME (40% restriction) only led to 14% drop in growth rate for KU as compared to 28.4% and 30.9% drop for CH and MM, respectively. This shows that at this restriction level KU was better tolerant to low dietary energy levels than both CH and MM. Nonetheless, reducing the energy level to 1319 Kcal/kg ME (55% restriction) led to 38.7, 37.2 and 19.6 % drop in growth rate for KU, CH, and MM, respectively, indicating a better tolerance at this dietary energy restriction level for MM.TheMM ecotype's better performance at very low energy levels could be an evolutionary adaptation to how these chickens have been bred in localities they originate from. This might be the reason why MM is the most widespread ecotype in Tanzania (Minga *et al*., 2003) just as the present findings imply that it can better withstand periods and seasons of the year when feed supply is limiting or scarce.On the other hand the current findings suggest that KU thrives better only when dietary energy levels in the feed are optimum.Nutritionally stressed individuals rely on catabolism of proteins to fuel their activities thereby leading to loss of skeletal muscle proteins and hence loss of body weight (Axelrod and Reisine, 1984;Kitaysky *et al*., 2001). In the current study it is evident that after two weeks of restriction (42 days of age) the birds in the restricted groups could no longer compensate

energy deficiency in the feed through increased feed intake as shown by a genesis of their reduced growth rate lasting up to the end of the experiment.

Comparisons of morphometric measurements show that KU and CH had their body lengths, shank lengths, chest circumferences, and wing spans significantly reduced for both restricted groups after 6 weeks of energy restriction. However, with MM having all of the morphometric parameters not significantly different from the control for the 55% restriction (1319 kcal/kg ME) group is again an indication of better performance under very low dietary energy (stress) conditions. The ecotype-specific differences in body weights and morphometric traits of Tanzanian local chickens have been reported in previous studies (Msoffe *et al*., 2001, 2002), and it is in agreement with the current study. Nonetheless, this is the first study to compare these chickens under dietary energy restriction conditions. Meanwhile Prieto and Campo (2011), reported that quantitative feed restriction (60% of *ad libitum*) effect was significant for the fluctuating asymmetry of wing length, being greater in feed restricted white leghorn chicks than the controls (2800 kcal/kg) in an experiment conducted from 1 to 42 days of age. Generally, linear or morphometric body measurements could serve as predictors of body weight; therefore, their variability in poultry arises due to genotypic and environmental effects, and the magnitude of variability may differ under different environmental conditions (Assan, 2015).

In the current study, as evidenced by positive correlations with energy restriction,feeding and foraging behaviours were dominant in all ecotypes in the restricted groups through to the third week. At this stage, metabolic hunger may be linked to such increased activity in the energy-restricted birds (Webster, 2003).Fewer birds exhibited the other behaviors such as locomotory activities, comfort and aggression. The energy restricted chickens appeared

to have experienced metabolic hunger andthe reduction in other behaviours such as locomotory activity was vital as a way of energy conservation. All the chicken ecotypes in the current study showed a similar trend without between-ecotype differences. However, by the seventh week there were no significant differences between restricted groups and controls in the number of chickens exhibiting feeding behavior in all ecotypes. This observation may entail that by this time the hypothalamic hunger stimulation was minimised in the restricted groups and adaptation had since ensued.

KU restricted groups, unlike the other ecotypes had a higher percentage of chickens exhibiting foraging behaviour, showing between-ecotype differences at this stage (seventh week) and as also evidenced by a significant positive correlation with energy restriction. This is in agreement with other findings of the current study that have shown that KU 55% restricted group was the most negatively affected in terms of growth parameters. In addition, resting behaviour in KU and CH ecotypes was significantly negatively correlated with energy restriction, entailing that as the level of restriction increased (reduced dietary energy), the birds became more restless. On the other hand, for both restricted groups, the number of birds exhibiting particular behaviour types for MM was not significantly different from the control in the seventh week. This may signify that the restricted groups for MM were less impacted (or were able to adapt faster) by low energy levels than CH and KU. Moreover resting behavior was significantly lower than controls in CH and KU restricted groups. Research on behaviour in Tanzanian local chicken ecotypes as affected by dietary energy restriction stress has not been done. However, between-breed differences in behavioural stress response have been reported in other studies elsewhere. For instance, Ericsson *et al*. (2014) reported a significant between-breed difference in relaxed and preen behaviors between Red jungle fowl and White leghorn breeds; that is, they were more frequent in Red jungle fowl after acute stress. Cheng and Jefferson (2008)

also showed that transportation stress-induced behavioral changes in feeding and preening in the commercial chickens from two strains, with a strain selected for high group productivity and survivability showing a greater increase.

Low dietary energy had an effect on mortality of the chickens in this study, though the observation may not be entirely conclusive as postmortem on the affected chickens was not done. Mortality was the lowest in MM at both levels of dietary energy restriction. Almost all mortality cases recorded were presented with severe muscle wasting and general body weakness. It can be inferred therefore, that MM was the most tolerant to low energy levels with respect to mortality, and this is also in agreement with other parameters assed in this study. However, Miah *et al*. (2014) reported that energy levels of diet reduced up to 2400 kcal/kg ME had no effect on the survivability of indigenous chickens of Bangladesh. In the current study, the energy levels were reduced to very low levels of 1696 and 1319 Kcal/kg ME and the experiment was for a longer period of time, hence the effect. In cases in which the stressor changes from acute to chronic, individuals may experience the negative effects that may include muscle wasting, impaired immune function, depressed growth, inhibition of reproduction and in extreme cases, death (Walker *et al*., 2005).

2.4 Conclusion

This study has shown that feed containing lower energy levels led to decreased growth rates and feed utilization efficiencies. Ecotype-specific tolerance to decreased dietary energy levels through differences in growth and behavioural stress responses was evident. At control energy levels (2864 Kcal/kg ME) and when energy levels were reduced to 1696 Kcal/kg ME, the KU ecotype had a better performance than MM and CH with respect to growth rate, percent weight gain and feed utilization efficiency. On the other hand, MM

was better tolerant than KU and CH at lowest energy levels used in this study (1319 Kcal/kg ME) with respect to growth rate, mean percent weight gain, feed efficient utilization, behavioral and mortality indicators. These findings therefore, suggest that MM can thrive better even under conditions of feed insufficiency, making it a recommended ecotype in regions of the country facing seasonal decreased availability of scavengeable feed stuff.

2.5 Acknowledgements

The authors acknowledge financial support from Intra-ACP Mobility Scholarship Program through the Education, Audiovisual and Culture Agency of the EU. Chicken flocks utilized in this study were generated through the support by the US Agency for International Development on Feed the Future Innovation Lab for Genomics to Improve Poultry.

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CHAPTER THREE

Effects of Stress Induced by Low Energy Diets on Liver hsp70 and iNOS Gene Expression and Blood Parameters in Tanzanian Local Chickens

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ABSTRACT

A study comparing the effects of low dietary energy-induced stress on liver hsp70 and iNOS gene expression and blood indices of three Tanzanian chicken ecotypes was conducted using hens at 4 weeks old for 7 weeks. A total of 351 four weeks old hens belonging to Kuchi (KU), Ching'wekwe (CH) and Morogoro medium (MM) ecotypes were weighed and randomly allocated to 9 pens in a 3 x 3 (three ecotypes and 3 types of diets) factorial design, with three replicates. The birds were fed 3 types of diets formulated to contain 40, 55 and 0% less energy than prescribed for commercial layer chickens. As assessed by the $2^{-\Delta\Delta Ct}$ method after realtime PCR, low dietary energy caused a markedup-regulation ($p<0.05$)of liver hsp70 relative gene expression for the KU 55% restriction group after 3 weeks. After 7 weeks, both restriction groups for KU and CH 55%restriction grouphad up-regulated levels of hsp70, but relative expression levels for MM restriction groups were not altered. While liver iNOS relative gene expression levels were notably up-regulated for the KU 55% dietary energy restriction group after 3 weeks, only CH 55% restriction group had iNOS expression levels markedly up-regulated($p<0.05$) after 7 weeks.Significant elevations ($p<0.05$) of serum corticosterone levels were only noted for KU restriction groups after 1 and 3 weeks. Low dietary energy at both 40 and 55% restriction levels significantly increased ($p<0.05$) serum uric acid levels of all ecotypes whilst levels of triglycerides were markedly reduced as determined after 1, 3 and 7 weeks. There were no significant differences between the controls and restricted groups in Hb and Hct levels except for the CH ecotype, which showed significantly lower ($p<0.05$) Hb and Hct levels after 5 and 7 weeks for both restricted groups. The results of this study show that feed containing lower energy levels induced stress in all the three chicken ecotypes studied. Ecotype-specific effects and tolerance of this stress were manifested in the liver iNOS and hsp70 up-regulations and changes in blood parameters, with MM showing better tolerance at lowest energy levels and KU the least tolerant.

Key words:*corticosterone, dietary energy, ecotypes, restriction, stress, tolerance*

3.0 INTRODUCTION

Local chickens are an important food resource and a source of income for rural households in many developing countries (Wilson, 2015). In Tanzania, like in many other tropical African countries, local chickens are kept under traditional free ranging management systems where they are left to scavenge for whatever is available(Sanka and Mbaga, 2014) and this exposes them to feed scarcities and disease attacks. The existing local chicken ecotypes have mostly evolved in part due to natural genetic selection compounded with human developmental need for affordable protein sources. Over the past few years, studies on Tanzanian local chickens have focussed on disease resistance, genotype variation and production potentials (Msoffe *et al*., 2001, 2005;Lwelamira *et al*., 2012;Guni *et al*., 2013;Mayardit *et al*., 2016). Findings from these studies have shown variations in disease resistance and production potential within local chicken populations, suggesting the possibility of improving the genetic potential through selective breeding within and between local chicken populations. According to Guni *et al*. (2013) Kuchi (KU) from Mwanza, Morogoro medium (MM) and Chingw'eke (CH) from Morogoro are some of the most prospective local chicken ecotypes under traditional production systems.

Dietary energy interferes with basal metabolic rate and plasma levels of different metabolic hormones (LeBlanc *et al*., 1986;Gabriel *et al*., 2000) in animals.Published research information on the extent of tolerance to low dietary energy in Tanzanian local chickens is scarce. A diet containing 18% CP: 2800 kcal ME/kg is sufficient for rearing Ugandan local chickens in early growth phase (Nakkazi *et al*., 2015), and similarly Miah *et al*. (2014) showed that 2800 kcal ME /kg would be required for Bangladesh desi local chickens to achieve a target weight of 950g at 14 weeks age. Stress, such as induced by low dietary energy, modifies the development of the hypothalamic-pituitary axis response thereby affecting the growth and behaviour of the chicken, and it may exert a negative

impact on physiological processes and pose many health problems, including disturbances of immune processes and antioxidative defenses (Ognik and Sembratowicz, 2012).

Commonly used physiological indices of stress following feed deprivation or restriction are plasma corticosterone, glucose and the heterophil to lymphocyte ratio (Zulkifli *et al*., 2006). In addition blood parameters, including hemoglobin (Hb), hematocrit (Hct), CO_2 levels, saturated O_2 and pH are potential biomarkers of stress tolerance as they closely depict physiological changes in a stressed animal (Lamont *et al*., 2015). Biochemical blood parameters such as uric acid and triglyceride levelsmay reflect the physiological state and metabolic changes due to stress in an animal. On the other hand, when living organisms are exposed to stressors such as energy depletion, the synthesis of most proteins is retarded, but heat shock proteins (Hsps) are rapidly synthesised (Kregel, 2002; Al-Aqil and Zulkifli, 2009;Zhao *et al*., 2013). Hsp70 is one the Hsp families and is highly inducible (Kregel, 2002) and the most extensively studied because of its prominent response to diverse stressors, and increased synthesis of these proteins is involved in the protection of stressed cells and organisms (Gabriel *et al*., 2002;Zhao *et al*., 2013). Inflammation is an important indicator of animal tissue damage due to stress and one of the most pivotal enzyme involved in maintaining inflammation is inducible nitric oxide synthase (iNOS), which is responsible for the catalysis of nitric oxide (NO) (Zhao *et al*., 2013; Surh *et al*., 2001).

Lack of access to quality feed and failure to balance between energy and protein requirements is still a huge challenge for local chickens' production sector (Sonaiya, 2007;Mutayoba *et al*., 2012). Under natural environments scavenging local chickens are exposed to feed and low dietary energy stress due to seasonal availability of feed. The chemical composition of feeds eaten by rural scavenging chickens of Tanzania is generally

below the nutritional requirements and varies with season, climate and age of birds (Mwalusanya *et al.*, 2010). Since it is already established that there are ecotype-differences in some aspects of productive performance and disease resistance among various local chickens (Msoffe *et al.,* 2002;Lwelamira, 2012), it could be of great interest to determine if the chickens' responses to low energy diets will show similar differences. The apparent differences would be beneficial in making informed recommendations for selection in the future breeding programs. The current study, therefore, was designed to compare the physiological responses of KU, MM and CH local chicken ecotypes to low dietary energy levels. It was hypothesized that low dietary energy would induce stress and affect the performance of the local chicken ecotypes differently, and this would be reflected in the blood physiological parameters and gene expressions of hsp70 and iNOS in the liver.

3.1 Materials and Methods

3.1.1 Experimental chickens

Day-old CH,KUand MMlocal chicken ecotypes were obtained from the parent flock kept by the Feed the Future Genomics to Improve Poultry Project at Sokoine University of Agriculture. The chicks were brooded and reared under similar environmental, managerial and 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 the 4^{th} week. All chickens were vaccinated routinely against Newcastle disease, Infectious Bursal Disease (Gumboro), and Fowl pox.

3.1.2 Feed formulation

Three types of feeds were formulated, the first contained 2864 Kcal/kg ME and served as control diet, the second feed contained about 40% less energy than the control (i.e. 1696

Kcal/kg ME), while the third diet contained 55% less energy than the control (i.e. 1319 Kcal/kg ME). Diets were formulated using locally available feedstuffs and ground wood charcoal (Rezaei *et al.*, 2006) was used to dilute the feed. The chemical (proximate) analyses of different feed ingredients were carried out using standard methods (FAO, 1994). Feed samples were analyzed for crude fiber, crude protein (Kjeldahl protein), moisture, ash, nitrogen-free extracts (digestible carbohydrates) and crude lipid; and then metabolisable energy levels were estimated (NRC, 1994; Janssen, 1989). The composition of specific ingredients in the feed is depicted in Table 3.1.

Table 3.1: Composition and Nutrient levels of the Experimental Diets

	Control diet	**40% Restriction**	**55% Restriction**
	2864 Kcal/kgME	**1696** Kcal/kg ME	**1319** Kcal/kg ME
Ingredients	(%)	(%)	(%)
Maize meal	37.8	14.5 10	
Maize bran	26	10.3	2
S. flower M	20.522.5 21		
Fish meal	11	18	22.3
Charcoal	0	30	40
Limestone	2	2	2
Premix[n]	0.3	0.3	0.3
Methionine	0.3	0.3	0.3
Lysine	0.3	0.3	0.3
DCP	1.3	1.3	1.3
Salt	0.5	0.5	0.5

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

3.1.3 Experimental design

A total of 351 (117 of each ecotype) four weeks old female chicks belonging to CH, KU and MM ecotypes were weighed and randomly allocated, according to ecotype, to 9 pens in a 3 x 3 (three ecotypes and 3 types of diets) factorial design, with three replicates. The birds were fed 3 types of iso-nitrogenous (18% crude protein) diets formulated to contain 40, 55, and 0% (control) less energy than prescribed by the NRC (1994) for commercial layer chickens for seven weeks. The birds were reared on littered (rice husks) floors in a well ventilated house. Feed and water were supplied *ad-libitum* throughout the experimental period (7 weeks). Each pen had on average an area of 2 m² floor space per 13 birds. The study was conducted at the prevailing cyclic ambient temperatures ranging from 21.6 to 34.3°C. The pens were artificially lit with a 12L: 12D cycle, corresponding to the natural conditions.

3.1.4 Tissue collection

After 3 and 7 weeks of dietary energy restriction, 5 chickens from each pen were randomly selected and humanely sacrificed by decapitation.Liver samples were quickly collected after chicken dissectionand were quickly put on ice before storage at -80^{o}C.

3.1.5 RNA Extraction and quantitative Real-time PCR

Total RNA was extracted from liver samples (50mg) using the Quick-RNA™ MiniPrep Plus kit (Zymo Research) following manufacturer's instructions of preparation and purification. The integrity of the isolated RNA was examined using 1.2 % agarose gels containing 0.1 % ethidium bromide. First-strand complementary DNA was synthesized from about 5µg of total RNA according to manufacturer's instructions in a 20µL reaction volume by using RevertAid First-Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's instructions. Predesigned primers for hsp70 and iNOS (Table

3.2) were used according to Xie *et al.* (2014) and Zhao *et al.* (2013). The quantitative real-time PCR (qPCR) was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) on an ABI 7500 (Applied Biosystems USA). Reactions were performed in a 25-µL reaction mixture. The cycling protocol included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 60sec. A dissociation curve was run for each plate to confirm the production of a single product. 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.

Table 3.2: Target gene Primers used

Gene	Primer set	Product (bp)	Tm (^{o}C)
HSP70	F 5′-CGGGCAAGTTTGACCTAA-3′	250	58
	R5′-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	

3.1.6 Blood sampling and analysis

Whole blood was collected via the wing vein at similar times of the day (between 10:00 and 12:00hrs) using syringes into ethylene diamine tetracetic acid (EDTA) containing and/or plain vacutainers at intervals of 1, 3, 5, and 7 weeks of energy restriction. The sampling procedure lasted for about less than 1 min per bird. For serum preparation, blood samples (in plain vacutainers) were allowed to clot, serum separated, and stored at −20°C

until analysis. The serum corticosterone levels were measured by ELISA using commercially available kits (Sunlong Biotech. Co. Ltd., Hangzhou, China) and measurements were calibrated by Multiskan EX Primary EIA V. 2.3 Reader (Applied Biosystems). Serum levels of uric acid, triglycerides and glucose were determined colorimetrically according to instructions provided with the commercial kits (Erba Diagnostics Mannheim, Germany). The differential white blood cell count and all other hematological indices were determined using the MS4S automated hematological analyser (Melet Schloesing Laboratories, Germany).

3.1.7 Statistical analysis

One-way ANOVA (SPSS 20) was used to analyze differences among the treatments. In case of detection of differences in treatment means by ANOVA, Dunnett t- test and LSD test were used to separate means, with significance statements based on $p< 0.05$. Correlation analysis was performed by linear regressiontest using SPSS 20 softwareand the correlation coefficients wereconsidered significant at $p< 0.05$.

3.2 Results

3.2.1 Thehsp70 and iNOS relative gene expression

Results for liver hsp70 and iNOS relative gene expression are presented in Fig.3.1 and 3.2. Low energy diets induced up-regulation in liver hsp70 relative gene expression (Fig.3.1 A) after 3 weeks in both restriction groups for all the chicken ecotypes but levels were only significant ($p<0.05$) for the KU 55% restriction group. After 7 weeks of the study, both groups of dietary energy restriction for KU and CH 55% restriction group had up-regulated levels of HSP70, and the levels in KU 40% restriction group were markedly higher ($p<0.05$) than in CH. On the other hand HSP70 gene expression levels for both

restriction groups of MM and CH 40% restriction group were not up-regulated (Fig.3.2 A).

While the levels in all other restriction groups remained unalteredafter 3 weeks of the study, the liver iNOS relative gene expression levels were notably up-regulated, though not significantly, for the KU 55% dietary energy restriction group (Fig. 3.1 B).The iNOS relative gene expression levels were up-regulated for CH 55% restriction group ($p<0.05$)and both restriction groups of KU after 7 weeks of the study (Fig. 3.2 B). Both dietary energy restriction groups for MM and CH 40% restriction group were not altered by low energy diets.

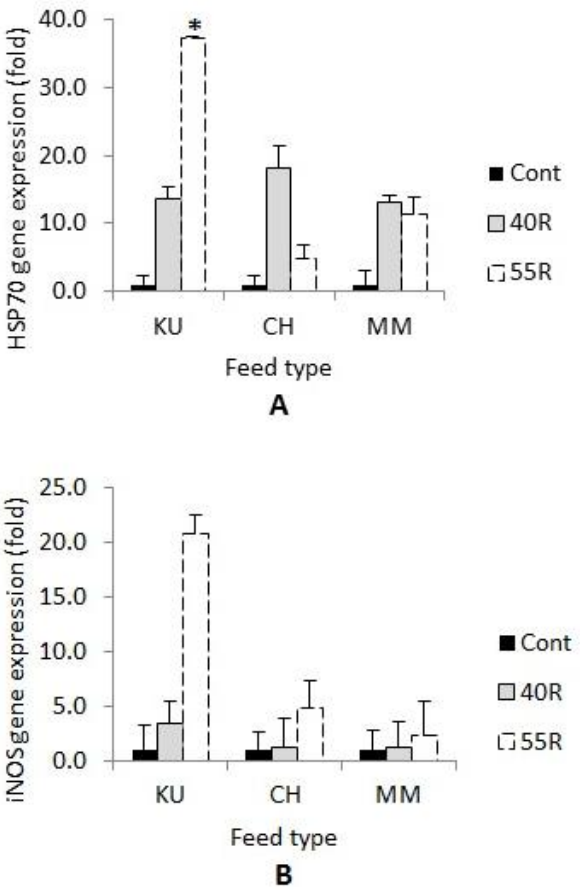

Figure 3.1:Liver hsp70 (A**) and iNOS (**B**) gene expression after 21 days of dietary energy restriction;** *significantly different ($p<0.05$) from the control; Values are presented as Mean±SE. *Cont: control, 40R: 40% dietary energy restriction, 55R: 55% dietary energy restriction, KU: Kuchi, CH: Ching'wekwe, MM: Morogoro medium*.

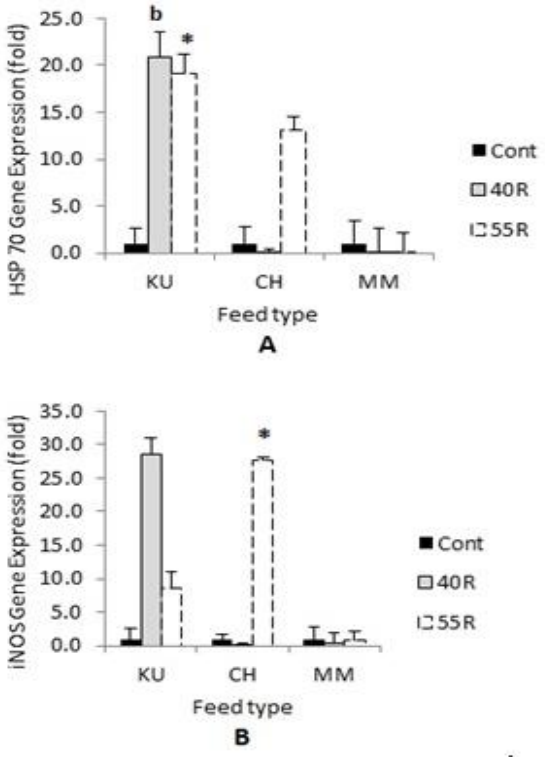

Figure 3.2:**Liver hsp70 (**A**) and iNOS (**B**) gene expression after 49 days of dietary energy restriction;** *significantly different ($p<0.05$) from the control; b: significantly higher than in CH. Values are presented as Mean±SE. *Cont: control, 40R: 40% dietary energy restriction, 55R: 55% dietary energy restriction, KU: Kuchi, CH: Ching'wekwe, MM: Morogoro medium*.

3.2.2Corticosterone

Results for serum corticosterone concentrations are presented in Fig.3.3. After a week of feed restriction, significant elevations ($p<0.05$) of serum corticosterone levels were noted for KU whilst MM recorded a drop. Ecotype-specific differences in the levels were notable, with KU 40% restricted ground showing a significantly higher level than CH 40%. Apparently, MM had the least amount of serum corticosterone ($p<0.05$) for the 55% restricted groups. After 3 weeks, the low energy diet induced markedly elevated corticosterone levels ($p<0.05$)only for KU 55% restricted group. Among controls MM had significantly higher ($p<0.05$) levels of serum corticosterone than KU and CH. Moreover, MM 40% restricted group similarly had a markedly higher level than CH 40%. The serum corticosterone levels were not altered by low dietary energy intake after 5 weeks of feed restriction, and ecotype-specific differences in the levels were absent.

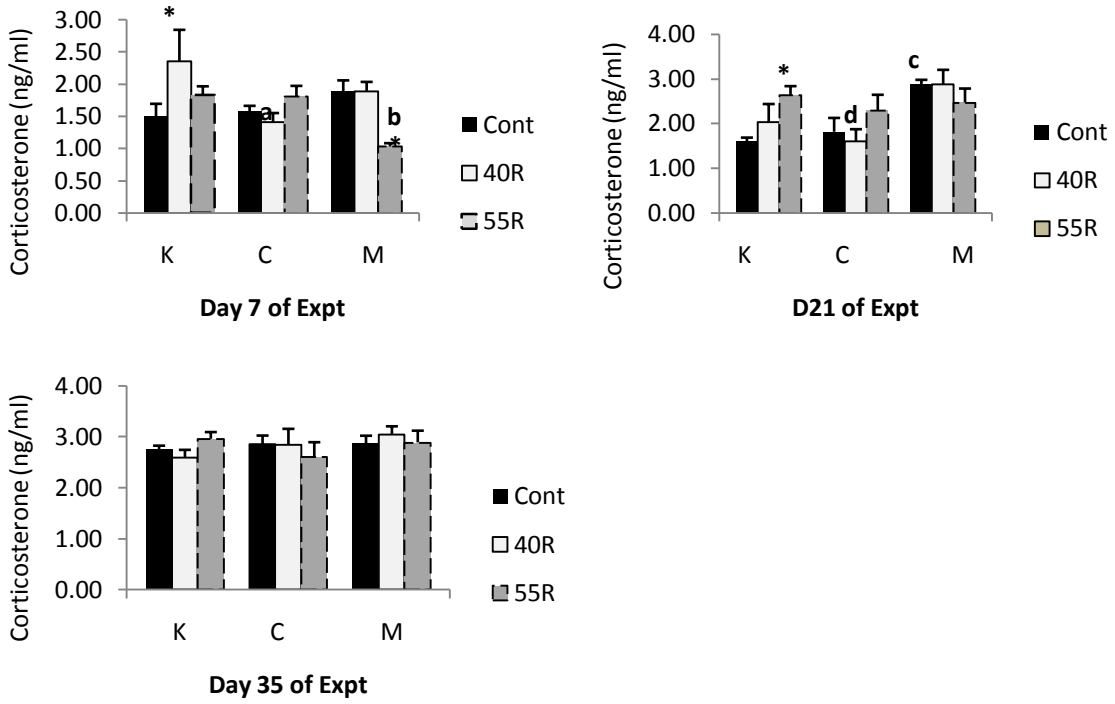

Figure 3.3: Serum corticosterone levels after 7, 21 and 35 days of dietary energy restriction; **: significantly different from the control; a: significantly lower (p<0.05) than K40R; b: significantly lower (p<0.05) than K55R and C55R; c: significantly higher (p<0.05) than cont in K and C; d: significantly lower (p<0.05) than M40R; K: Kuchi; C: Ching'wekwe; M: Morogoro medium; 40R: 40% dietary energy restriction; 55R: 55% dietary energy restriction; Cont: control.*

3.2.3Uric acid

Serum uric acid concentration results are presented in Fig. 3.4. After a week of dietary energy restriction, there were significant elevations ($p<0.05$) in serum uric acid levels but no inter-ecotype differences were observed in both 40% and 55% restricted groups in all ecotypes. Serum uric acid levels for MM (both groups) and KU (55% restriction group)weremarkedly elevated ($p<0.05$) after 3 weeks of dietary energy restriction. Moreover, the KU 55% restriction group had significantly higher amounts ($p<0.05$) than both CH and MM at the same restriction level. Similarly, after 7 weeks of dietary energy restriction marked levels ($p<0.05$) of serum uric acid were evident in both 40% and 55% restriction groups for all the ecotypes. For the controls, CH had significantly higher ($p<0.05$) levels than both KU and MM; and similarly for the 55% restriction group, CH had significantly higher ($p<0.05$) levels than KU.

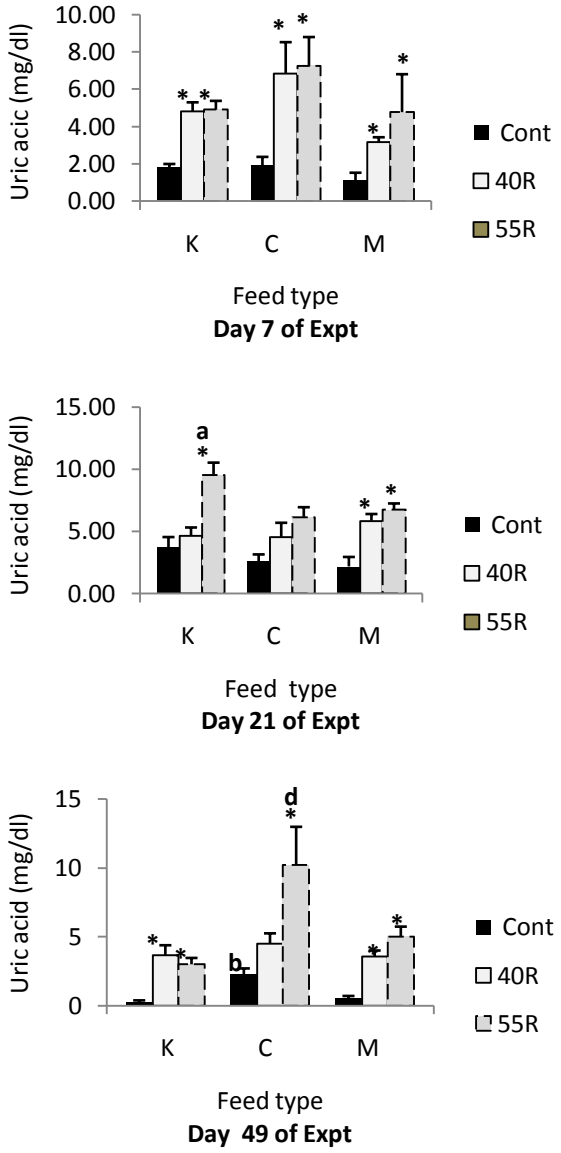

Figure 3.4: **Serum uric acid levels after 7, 21 and 49 days of dietary energy restriction;** **: significantly different from the control; a: significantly higher (p<0.05) than in C and M; b: significantly higher (p<0.05) than in K and M; d: significantly higher (p<0.05) than in K; K: Kuchi; C: Ching'wekwe; M: Morogoro medium; 40R: 40% dietary energy restriction; 55R: 55% dietary energy restriction; Cont: control*

3.2.4Glucose and triglycerides

Resultsfor serum glucose and triglycerides concentrations are shown in Table 3.3. After a week of feed restriction,serum triglycerides levels in both 40% and 55% restricted groups markedly reduced ($p<0.05$) in all the ecotypes, with no ecotype-specific differences. A similar trend was maintained by both restriction groups for all ecotypesafter 3 weeks, though at 40% restriction level CH had significantly higher ($p<0.05$) levels than MM. After 7 weeks, the serum levels of triglycerides in KU for both restriction levels were not altered but levels in CH and MM markedly declined ($p<0.05$) for the 55% restricted groups. The CH control group had significantly higher amounts ($p<0.05$) than both KU and MM controls.

Serum glucose levels were notably reduced for the MM 55% restriction group after a week of dietary energy restriction, and for the 40% restricted group, MM had significantly lower levels than KU. After 3 weeks, serum glucose levels were not altered by low energy diets in all groups except the CH 40% restricted group which had a surge. The KU 55% restricted group recordedmarkedly higher ($p<0.05$) levels of serum glucose, which were higher than that of MM at the same restriction level after 7 weeks of dietary energy restrictions.

Table 3.3: Effects of low dietary energy on glucose and triglycerides after 1, 3 & 7 weeks (Mean±SD)

Feed type	Wk1 Gluc.	Trigly.	Wk3 Gluc.	Trigly.	Wk 7 Gluc.	Trigly.
K Cnt	180.2±31.5	204.4±60.9	84.3±30.4	155.9±27.2	130.6±17.3	84.9±22.8***b***
K 40	189.2±14.1***b***	117.5±38.6$^{n\,i}$	67.5±23.6***b***	90.8±27.6$^{n\,i}$	135.5±15.2	90.9±46.4
K 55	175.0±26.6	98.2±21.0$^{n\,i}$	58.1±43.0	88.6±25.1$^{n\,i}$	161.6±17.9$^{n\,i}$***b***	80.0±19.3
C Cnt	150.6±33.1	150.6±33.1	77.5±25.0	208.6±74.4	147.7±23.5	180.7±60.4***bc***
C 40	164.7±17.6	164.7±17.6	115.8±18.8$^{n\,i}$***bc***	157.6±61.8***b***	129.1±41.5	133.7±39.2
C 55	168.2±22.8	168.2±22.8	84.0±20.2	90.9±56.4$^{n\,i}$	152.2±28.8	76.6±18.3$^{n\,i}$
M Cont	174.8±25.1	174.8±25.1	95.0±24.5	151.2±26.8	132.8±17.4	124.2±21.3***c***
M 40	160.9±23.5***b***	160.9±23.5***b***	70.2±17.2***c***	83.4±30.6$^{n\,i}$***b***	143.6±10.3	103.3±47.8
M 55	144.7±28.0$^{n\,i}$	144.7±28.0$^{n\,i}$	85.7±20.6	90.2±45.8$^{n\,i}$	123.0±26.0***b***	70.8±15.4$^{n\,i}$

$^{n\,i}$; means in a column for each ecotype are significantly different from the control ($p<0.05$); b and c: means bearing the same letter within a column between ecotypes are significantly different, and those with a pair are significantly different from those bearing either letter of the pair ($p<0.05$). *K: kuchi; C: ching'wekwe; M: Morogoro medium; 40: 40% restriction; 55: 55% restriction; Cont: control; Gluc: glucose (mg/dl); Trigly: triglycerides (mg/dl).*

3.2.5Hb and Hct

Results for Hb and Hct levels are shown in Table 3.4. The Hb and Hct levels were not significantly altered by low energy diets during the first four weeks of the study in all ecotypes. Nonetheless, after 5 and 7 weeks of the study, Hb and Hct levels for CH markedly declined ($p<0.05$) for both restricted groups and ecotype-specific differences were not evident.

Table 3.4: Effects of dietary energy restriction on Hb and Hct after 5 & 7 weeks (Mean ± SD)

Wk 5	**Treatments**								
	K Cont	**K 40**	**K 55**	**C Cont**	**C 40**	**C 55**	**M Cont**	**M 40**	**M 55**
Hb (g/dL)	9.2±0.9	9.4±0.9	9.4±0.7	9.6±1.0	8.0±2.4	8.6±0.8$^{n\,i}$	9.4±2.0	8.5±1.0	9.8±0.9
Hct (%)	24.8±2.9	25.8±2.2	25.9±1.6	24.8±2.2	22.5±4.4	21.3±4.0$^{n\,i}$	24.7±4.0	23.1±2.5	25.5±3.1
Wk 7									
Hb (g/dl)	11.3±1.6	10.7±2.5	11.3±2.2	11.9±1.5	9.9±0.9$^{n\,i}$	10.1±2.0$^{n\,i}$	11.6±1.1	11.4±1.5	11.1±1.2
Hct (%)	25.8±3.2	24.7±3.9	24.3±3.9	26.7±3.3	21.4±2.9$^{n\,i}$	22.3±3.4$^{n\,i}$	25.8±1.9	26.1±4.2	24.8±2.3

$^{n\,i}$; means in a row for each ecotype that are significantly different from the control ($p<0.05$). *K: kuchi; C: ching'wekwe; M: Morogoro medium; 40: 40% restriction; 55: 55% restriction; Cont: control;Hb: hemoglobin; Hct: hematocrit; RBC: red blood cells; wk: week*

3.3 Discussion

Dietary energy is important for basal metabolism, maintenance, growth and tissue accretion in chickens, and therefore, reduction in dietary energy intake results in reduced growth and tissue accretion (Veldkamp *et al*., 2005). In the current study, liver hsp70 and iNOS up-regulation at 3 weeks of dietary energy restriction may be linked to cytoprotection under escalated stressful conditions. In consistent with this, Al-Aqil and Zulkifli (2009) showed that 60% feed restricted female broiler chicks had higher hsp70 density than those of the *ad libitum*-fed group. Delezie*et al*. (2007) also reported increased hsp70 gene expression levels in broiler chickens after feed deprivation. Upon a variety of stresses, hsp70 expression is rapidly induced through MAPK/SAPK signaling cascades activating HSFs (Morimoto, 1993;Juhasz *et al*., 2014). Hsp70 restores the balance of cell proteome by normalizing the concentration of unfolded and denatured proteins (Juhasz *et al*., 2014). The current findings indicated significantly higher liver hsp70 expression levels for KU 55% group than CH and MM, suggesting that it was the most affected ecotype with low dietary energy at the time (after 3 weeks of study). It appears the chickens were

affected similarly at 40% energy restriction level but the impact of low dietary energy stress was minimal.

The up-regulated liver hsp70 and iNOS expressions for KU and CH 55% restriction groups even at 7 weeks of study suggests that the stress effect remained high for these groups. The MM ecotype appeared to be better tolerant and/or was able to quickly adapt to low dietary energy stress by 7 weeks of the study and this is reflected in the low liver hsp70 and iNOS expression levels, unlike KU and CH chicken ecotypes, which had considerable up-regulations. This finding is in agreement with work on the same chickens which showed that MM performed better than KU and CH at lowest energy levels used in the study (1319 Kcal/kg ME) with respect to growth rate, mean percent weight gain, feed efficient utilization, behavioral and mortality indicators (Chapter two). These results therefore, may imply that liver injury or inflammation due to low energy diets was evident in KU after 3 and 7 weeks but for CH ecotype inflammation was only evident by the 7^{th} week of the study. Consistent with some of the findings of the current study, Kang *et al*. (2011) reported increased liver iNOS gene expression after stress caused by feed restriction (75% of voluntary) and high stocking density in White Leghorn laying hens. Liver iNOS expression may function as an adaptive response to minimse inflammatory injury (Taylor *et al*., 1998). Generally iNOS is absent in normal liver but is markedly increased in response to inflammation and some oxidative stresses (Clemens, 1999).The molecular regulation of iNOS expression (and eventual NO synthesis) is complex and occurs at multiple levels in the gene expression pathway (Taylor *et al*., 1998). NO exerts protective effects in part, by neutralizing toxic oxygen radicals and by blocking the cytokine TNF-alpha-induced apoptosis and hepatotoxicity, partly by a thiol-dependent inhibition of caspase-3-like protease activity (Taylor *et al.*, 1998).

Significant elevations of serum corticosterone levels were noted for the KU ecotype restriction groups after 1 and 3 weeks and this is consistent with previous research whereby feed restriction caused a significant elevation in plasma corticosterone concentration in broiler chickens (Al-Aqil and Zulkifli, 2009;Prieto and Campo, 2011). In response to stress, CRF is released into hypophysial portal vessels that access the anterior pituitary gland, and binding of CRF to its receptor on pituitary corticotropes induces the release of ACTH into the systemic circulation whose principal target is the adrenal cortex, where it stimulates corticosterone synthesis and secretion (Smith and Vale, 2006).The biological effects of corticosterone are usually adaptive; however, inadequate or excessive activation of the HPA axis may contribute to the development of pathologies (Munck*et al.*, 1984). The secretion of corticosterone causes metabolic alterations by promoting gluconeogenesis leading to the liberation of substrates from bodytissues necessary for endogenous glucose production(Virden and Kidd, 2009).In the current study, the trends in corticosterone levels coupled with liver hsp70 and iNOS gene expression levels, suggest that KU was the most stressed local chicken ecotype by low dietary energy levels. Although the body weight differences were still not very pronounced at this age, it appears that the larger mean body weight for KU may have partly contributed to the differences due to escalated metabolic needs for basal metabolism and maintenance.

At 5 weeks of dietary energy restriction, serum corticosterone levels were not altered by low energy diets in all ecotypes. This may entail that although differences may exist on how these chickens respond to low dietary energy stress, the period of time in which adaptation takes place might be similar. Surprisingly for CH and MM ecotypes, the levels in serum corticosterone were not significantly different from the controls even earlier at 1 and 3 weeks of the study. While this is a good tolerance and adaptation indicator in these chickens, it may also be possible that elevations might have occured earlier such that by

the time of blood sampling done after a week, levels would have dropped already toprevent chronic elevations.Chronic stressors such as feed restriction cause corticosterone use to be up-regulated earlier than expected, but in cases of extended chronic stress, down-regulation may ensue, thereby avoiding the adverse effects of chronically elevated levels (Walker *et al*., 2005).Feed restriction initially causes a physiological stress response, although chickens quickly habituate and the response is minimized (Prieto and Campo, 2011).Other studies have also shown that repeated feed restriction or deprivation can lead to habituation of the corticosterone responses in poultry (Zulkifli *et al*., 2006). In the current study, while all the three ecotypes seemed to be well adapted to their environments, the better tolerance and adaptation exhibited by the MM ecotype might be due to natural genetic selection overtime.

Serum glucose levels for the restricted groups were generally maintained at similar levels with controls except for MM 55% restricted group at 1 week (lowered), CH 40% restricted group (elevated) at 3 weeks, and KU 55% restricted group (elevated) at the end of the study. Plasma levels of glucose are typically very stable in birds, even during fasting or starvation (de Jong *et al*., 2002).However,the continuous stimulation of the adrenal cortex leads to intermittent increase in the level of corticosterone, which is responsible for the formation of glucose molecules from reserves of carbohydrates, lipids and proteins (Ognik and Sembratowicz, 2012).In the current study, it seems that the chickens' physiological response progressed at different rates having been affected differently as shown by inconsistent changes in levels of serum glucose between ecotypes. On the other hand serum triglyceride levels were consistently significantly reduced in both restricted groups after 1 and 3 weeks for all the ecotypes and at both times there were no between-ecotype differences. After 7 weeks of the study, triglyceride levels did not significantly differ from the control except for CH and MM 55% restricted groups. Therefore it shows that CH and

MM 55% restricted groups had lowered triglyceride levels throughout the study period. This is consistent with studies by Zhan *et al*. (2007), who reported decreased triglycerides serum levels in feed restricted broilers on day 21 of feed restriction. The major fuels of muscle include glucose and fatty acids; and fatty acids in muscle are derived from circulating triglycerides and endogenously stored intramuscular triglycerides (Zhan *et al*., 2007). In the current study, therefore, as low energy levels persisted for the chickens, there was probably high demand of these metabolites to fuel muscular function. Since circulating triglycerides were targeted in this study, chickens' responses to lower energy levels seemed similar in all ecotypes with respect to stimulating triglyceride uptake from the blood though it seems CH and MM triggered a continuous response thus, better copping up than KU.

The results of the current study have also shown markedly higher ($p<0.05$) serum levels of uric acid in the restricted groups for the entire period of study in all chicken ecotypes. The elevations are consistent with Chen *et al*. (2012) who reported that energy restriction significantly increased serum uric acid in 30% energy restricted broilers. The ecotype-specific differences in the levels of uric acid were seen in the later stages of the study, with KU 55% and CH 55% restricted groups recording higher levels after 3 and 7 weeks, respectively. Despite these differences in levels, it seems, the consistence and rate of the physiological response to low energy levels with respect to release of uric acid among the ecotypes was similar. Avoidance of oxidative stress relies on antioxidants and antioxidative enzymes; and uric acid is an important antioxidant and primary end-product of nitrogen metabolism in birds (Hartman *et al*., 2006). It has the ability to inactivate strong oxidants like nitrite and hydroxyl generated radicals,via an electron transfer before the oxidant can react with the targeted biological molecule (Simic *et al*., 1989).

The low energy diets did not induce changes in the levels of Hb and Hct for the entire study period except for the CH ecotype, which showed significantly lower Hb and Hct after 5 and 7 weeks for both restricted groups. Findings by previous researchers (Boostani *et al*., 2010;Tamzil *et al*., 2014) reported reductions in Hb and Hct after feed restriction in broiler chickens. Reductions in these parameters for the CH ecotype may have led to a decrease in oxygen carrying capacity and the acid base balance could be compromised as evaluated Hct is advantageous in adaptation to stress through maintenance of high oxygen carrying capacity. However, Junqueira *et al*. (2003) reported that Hb and Hct values were not affected by feed restriction of broilers from 22 to 42 days of age. This is consistent with responses of KU and MM ecotypes in the current study. Therefore, ecotype-specific differences in Hb and Hct responses to low dietary energy may have been influenced by apparent differences in the genotypes of the chickens.

3.4 Conclusion

Low energy diets induced stress in all the chicken ecotypes studied and ecotype-specific responses and tolerance were manifested in the liver iNOS and HSP70 up-regulations. Adaptation patterns through changes in serumcorticosterone, Hb, Hct and uric acid in some cases also show inter-ecotype differences. The MM ecotype was better tolerant at lowest energy levels used in this study whilst KU appeared to be the least tolerant.

3.5 Acknowledgements

The authors acknowledge financial support from Intra-ACP Mobility Scholarship Program through the Education, Audiovisual and Culture Agency of the EU. Chicken flocks utilized in this study were generated through the support by the US Agency for International Development on Feed the Future Innovation Lab for Genomics to Improve Poultry.

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CHAPTER FOUR

Growth and Hematological Responses to Heat Stress and Low Dietary Energy in Tanzanian Local Chicken Ecotypes

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ABSTRACT

Two studies, each with three replicates, were conducted to determine and compare effects of heat stress and a combination of heat stress and low dietary energy on growth and hematological parameters in three Tanzanian local chicken ecotypes. In Studyone, for each replicate, 78 five weeks old hens belonging to Kuchi (KU), Ching'wekwe (CH) and Morogoro medium (MM) ecotypes were weighed and allocated into separate pens in two adjacenttemperature controlled rooms (39 chickens per room and 13 per ecotype per pen). The hens had*ad libitum* access to waterandwere fed a control diet consisting of 18% crude protein and 2864 kcal ME/kg.In one room ambient temperature was maintained at 26.5±0.5^{o}C (control)for 17 days whilst in the adjacent room it was raised and maintained at 32±1°C for 7 days and thereafter raised and maintained at 37±1°C from 08:00hrs to 16:00hrs per day for 10 days. A similar design was used in Study two except that chickens in the high temperature group were fed with 55% less dietary energythan the control diet.Exposure of chickens to 32±1^{o}C for 7 days and 37±1^{o}C for 10 days caused significant reductions ($p<0.05$) in mean percent weight gains for MM and CH, but not for KU. At the end of Study one MM had lower ($p<0.05$) mean percent weight gain than KU and CH. On the other hand, exposure of chickens fed low energy diet to 32±1^{o}C for 7 days (Study two) had no significant effect on percent weight gains of all the ecotypes. However, after exposure to 37±1^{o}C for 10 days, chickens of all ecotypes had significantly lower ($p<0.05$) percent weight gains than controls. Reduction in weight gain was more pronounced ($p<0.05$) in MM than in KU and CH. In both studies and in all ecotypes feed utilization efficiencies were significantly reduced. Heterophil/Lymphocyte ratios were markedly ($p<0.05$) increased and did not show inter-ecotype differences when chickens were exposed to 32±1^{o}C for 7 days and 37±1^{o}C for 10 days and fed with lowenergy diet. Meanwhile,changes in mean Hb and Hct at higher temperatures in both studies showed

ecotype differences. The results of these studiessuggest that as the magnitude of heat stress increased, the responsesbecameecotype dependent and that growth was synergistically suppressed by a combination of heat stress and low dietary energy in an ecotype-specific manner at much higher temperatures.WhileMMecotypedemonstrated better toleranceto moderately high temperature, KU and CH were more tolerant to higher temperature with respect topercent weight gain.

Key words:*ecotype, growth, poultry, temperature, tolerance, weight gain*

4.0 INTRODUCTION

In many developing countries, a majority of rural households and small scale farmers are actively involved in the production of local chickens, whichare important sources of protein and income(Mwalusanya *et al*., 2001; Ayo*et al*., 2011). In Tanzania, local chicken ecotypes bred from different regions of the country are recognized (Msoffe *et al*.,2002;2005) and these include: Kuchi (KU), Singamagazi, Ching'wekwe (CH), Morogoro medium (MM), Mbeya,Pemba, Tanga, Unguja, and N'zenzegere(Msoffe *et al*., 2005).The naming of some of these ecotypes relate to the phenotypic characteristics an to some extent, areas of origin in Tanzania (Msoffe *et al*., 2001).These chickens are mainly reared as free scavenging and their production generally remains low due to a myriad of seasonal challenges associated with changes inambient temperature and diets that donot meet their nutritional demands insome regions of the country (Mwalusanya *et al*., 2001). The present study focused on CH, KU and MM local chicken ecotypes, which have been fairly studied and their productive and disease resistance potentialsare known (Msoffe *et al*., 2002; 2005; Lwelamira, 2012). KU is a heavier ecotype originally bred from largely wet and warm north-west regions of the country,whilst both CH (characteristically with

shorter shanks) and MM are originally from relatively dry and hot regions ofcentral Tanzania.

The optimum ambient temperature range for poultry is 12 to 26^{o}C, and in cases where the environmental temperatures exceedthe thermoneutral zone, core body temperature becomes elevated and as a result a number of responses are initiated, leading to the neutralisationof heat stress-induced metabolic changes (Ayo *et al.*, 2011; Melesse *et al*., 2013).One of the physiological responses of exposure to stress is the releaseofglucocorticoids,causingdissolutionoflymphocytes in lymphoid tissues and leading to lymphopenia and eventual increase inheterophilrelease bythe bonemarrow (Zulkifli and Siegel, 1995; Borges *et al*., 2004). In addition, several blood parameters, including haemoglobin (Hb), CO_2 levels, saturated O_2 and pHare varied and these changes seem to be good candidates for predicting response to heat stress andfor use as biomarkers of heat tolerance (Lamont*et al*., 2015). Unlike in local chickens, the adverse effects of heat stress on broilers and commercial layers have been extensively studied (Lin *et al*., 2000; Deeb *et al*., 2002; Zhang *et al*., 2012) and these include lowering of cumulative feed consumption, feed utilization efficiencies, body weight,and production performance. In addition many researchers have reported breed and strain differences in tolerance to heat stress in chickens (Soleimani and Zulkifli, 2010;Felver-Gant *et al*., 2012; Melesse *et al*., 2013; Tamzil *et al*., 2014).

Differences in production attributes,adult body weight, anatomical features and resistance to disease among the local chicken ecotypes have been previously determined (Msoffe *et al*.,2002,2005; Lwelamira 2012; Guni *et al*., 2013).While it is generally considered that indigenous or local chickens in the tropical countries are able to withstand high ambient temperaturesbetter than exotic breeds (Soleimani and Zulkifli, 2010),itremainsimportant to assess if different common ecotypes can also show measurable differences in their heat

stress toleranceand further determine whether heat stress effects are compoundedbylow quality feed. The apparent differences taken together may be beneficial inmaking informed recommendations as to the most appropriate local chicken ecotype to be raised in a particular region in view of the changing climatic conditionsand the current drive by many communities in the country to raise these ecotypes for commercial purpose.

Local chickens will certainly be rearedin more temperature extreme conditions in the future due to both expansion into naturally hotter environments and global warming(Lamont *et al*., 2015).Therefore, selection of chickens for resilience to heat stress while maximizing their production potential could be a valid strategy to reduce the negative economic impact of climate change (Lan*et al*., 2016). The starting point and focus of such a selection should be to identify from among the local chicken ecotypes, those that have better production and heat tolerance potentials. Thus the current study was designed to investigate ecotype specific differences in growth performance and hematological responses to heat stress and a combination of heat stress and low dietary energy in selected Tanzanian local chicken ecotypes. This was done with an understanding that in the natural localities where these chickens are reared the dry season is characterized by high ambient temperatures, which are generally accompanied by a lack of adequate feed resources to meet their energy or nutritional requirements. Therefore, an assessment of growth and hematological stress indices under such conditions may provide a reflection of the chickens' tolerance levels.

4.1 Materials and Methods

4.1.1 Experimental chickens

Day-old MM, CH and KU local chicken ecotypes were obtained from the parent flock kept by the Feed the Future GIP Project at Sokoine University of Agriculture, Morogoro,

Tanzania. The chicks were brooded and reared under similar environmental, managerial and 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 were5 weeks old. All chickens were vaccinated against Newcastle disease, Infectious Bursal Disease (Gumboro), and Fowl pox, which are the common poultry diseases prevailing in the area.

All procedures used in this study were in compliance with the Sokoine University of Agriculture's guidelines for care and use of animals in research.

4.1.2 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 (i.e. 1319Kcal/kg ME) and served as energy restriction diet. Both diets were formulated using locally available feedstuffs and ground wood charcoal (Rezaei *et al.*, 2006) was used to dilute the experimental feed. The chemical (proximate) analyses of different feed ingredients were carried out using standard methods (FAO, 1994). Feed samples were analyzed for crude fiber, crude protein (Kjeldahl protein), moisture, ash, nitrogen-free extracts (digestible carbohydrates) and crude lipid; and then ME levels were estimated (Janssen, 1989;NRC, 1994). The composition of specific ingredients in the two feed formulations is depicted in Table4.1.

Table 4.1: **Composition and nutrient levels of experimental diets**

	Control diet 1319 Kcal/kg ME	**55% Energy Restriction 2864 Kcal/kg ME**
Ingredients	**(%)**	**(%)**
Maize meal	37.8	10
Maize bran	26	2
S.flr. meal	20.5	21
Fish meal	11	22.3
G. Charcoal	0	40
Limestone	2	2
Premix[n]	0.3	0.3
Methionine	0.3	0.3
Lysine	0.3	0.3
DCP	1.3	1.3
Salt	0.5	0.5

4.1.3 Study design

This work consisted of two studies which are detailed below:

Study one.A total of 78(26 per ecotype) five weeks old female chicks belonging to Kuchi (KU), Ching'wekwe (CH) and Morogoro medium (MM) ecotypes were weighed and randomly allocated into separate pens in two adjacenttemperature controlled rooms. Each room had three pens, with each having an average area of 2.5 m² floor space per 13 birds and hens were reared on littered (rice husks) floor. A three (3 ecotypes) x 1 (heat stress) 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 10L:14D cycle. To acclimatize to their new environment, all chickens had*ad libitum* access to waterandfeed consisting of 18% crude protein and 2864 kcal ME/kg, and were maintained at normal ambient temperature of 26.5±0.5^{o}C for5days.At the start of the study, 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 about 32^{o}C within 4 hours and thereafter was maintained at 32±1°C for 7 days. After 7 days temperature was raised again and maintained for 10 days at 37±1°C for 8hrs per day starting at 08:00hrs to 16:00hrs. 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%.

Studytwo. A similar design and chicken number (234) were used in Study 2 except that chickens in the high temperature group were fed with a diet formulated to contain 55% less energythan the control but having 18% crude protein.

4.1.4 Blood sampling and analysis

Whole blood was collected via the wing vein using a syringeand transferred immediately into ethylene diamine tetracetic acid (EDTA) – containing evacuated tubes.When birds were maintained at32±1°Cblood was collected at intervals of 6hr, 24hr,and 7 daysand after raising the temperature to 37±1°C, blood was taken at intervals of 4hr, 24hr, 7 days and 10 days. Blood sampling was conducted at similar time intervals between 10:00 and 12:00hrs in both studies. Blood collected was analysed for differential white blood cell count and other indices including Hb and Hct using the MS4S automated hematological analyser (Melet Schloesing Laboratories, Germany).

4.1.5 Growth performance data collection

For both studies, feed consumption was recorded daily,whilstchicken body weights were recorded on a weekly basis and at the end of the studies. Percent weight gains and FCRs (conversion index = daily feed consumption/daily weight gain) were subsequently calculated from the recorded data.

4.1.6 Statistical analysis

The Independent Sample t-test was used to compare means between treatment and control groups and One-way ANOVA (SPSS 20) 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 comparisonswere used to separate means, with significance statements based on $P < 0.05$. Results are presented as Means ± SE.

4.2 Results

Ecotype-specific differences in growth performance and hematological responses to heat stress (Study 1) and a combination of heat stress and low dietary energy (Study 2) wereassessed in CH, KU and MM local chicken ecotypes. The results of both studies are presented below:

Study one:

4.2.1 Growth performance

The growth performance parameters for the chickens are presented in Fig. 1 and Table 4.2. A marked reduction ($p<0.05$) in percent weight gain for MM and CH but not for KU was observed when the room temperature was raised to 32±1^{o}C for a 7-day period (Fig.4.1 A). Similarly, when the temperature was raised to 37±1^{o}C, there were significant reductions ($p<0.05$) in percent weight gains for MM and CH, but not for KU. In addition, between ecotypes, MM had a significantly lower ($p<0.05$) percent weight gain than KU and CH.

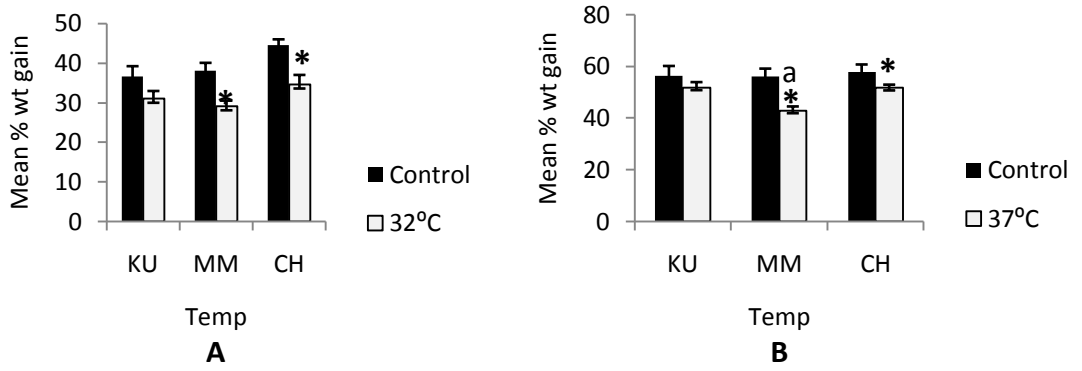

Figure 4.1: (A**) Mean percent weight gains of 5 week-old hens at control conditions (26.5°C) and at 32±1°C for a 7-day period. (**B**) Mean percent weight gains of 6 week-old hens at control conditions (26.5±0.5°C) and at 37±1°C (8 hours per day) for a 10-day period.** *Significantly lower ($p<0.05$) than the control, a: significantly lower ($p<0.05$) than KU and CH; *KU: Kuchi, CH: Ching'wekwe, MM: Morogoro medium*.

FCRs and feed intake were markedly ($p<0.05$) increased in all ecotypes after exposure of the chickens to both temperatures of 32±1 and 37±1°C. KU had the lowest ($p<0.05$) feed conversion ratio (FCR) among the control groups (Table 4.2). Between-ecotypes, after exposure to 37±1°C KU had the highest ($p<0.05$) mean feed intake followed by MM and the least was in CH. This is also depicted in higher ($p<0.05$) body weights for KU and MM than CH.

Table 4.2: Growth parameters of the chickens at control temperature (26.5^{0}C) and after exposure to 32±1^{0}C (constantfor 7 days) and 37±1^{0}C (8hrs a day for 10 days). Values are presented as Mean ± SE.

		KU	MM	CH		KU	MM	CH
Bw(g)	**Cont**	195.2±9.9^{e}	212.2±11.3^{d}	152.6±4.7^{cd}	**Cont**	305.4±17.8^{m}	331.1±19.8^{0}	240.9±6.6^{0m}
	32^{0}C	186.2±6.1^{g}	184.2±5.8^{k}	146.9±5.7^{gk}	**37^{0}C**	282.7±9.1^{r}	263.2±10.1*^{ñ}	222.9±8.9^{rñ}
FI^1	**Cont**	153.8±2.8^{n}	243.2±4.1^{n}	164.5±2.8	**Cont**	297.9±2.2^{i}	405.5±2.6^{ia}	313.4±1.6^{a}
	32^{0}C	280.1±5.0*	293.9±3.4	233.5±3.5*	**37^{0}C**	561.3±2.4*^{b}	485.5±1.8*^{b}	415.6±1.8*^{b}
Wg(g)	**Cont**	52.3±3.7	58.5±4.2	47.0±1.6	**Cont**	110.2±9.7	118.9±7.9^{l}	88.3±5.3^{l}
	32^{0}C	44.1±2.6	41.5±2.2*	37.8±2.7*	**37^{0}C**	96.5±4.3^{ut}	79.0±4.0^{t}*	76.0±3.2^{u}*
FCR	**Cont**	2.9±0.5^{j}	4.2±0.7^{j}	3.5±0.4	**Cont**	2.7±0.4	3.4±0.4	3.5±0.3
	32^{0}C	6.3±1.1*	7.1±0.8*	6.2±0.7*	**37^{0}C**	5.8±0.5*	6.1±0.4*	5.5±0.5*

*Significantly lower ($p<0.05$) than control; similar superscript letters within a row for each temperature treatment are significantly different ($p<0.05$). *KU: Kuchi, CH: Ching'wekwe, MM: Morogoro medium, Cont: control, Bw: body weight, FI: feed intake, Wg: weight gain, FCR: feed conversion ratio,^{1}:g/hen/period ofexposure, that is, either 7 days at 32^{o}C or 10 days at 37^{o}C.*

4.2.2 Hematological indices

The changes in levels of Hb and Hct during the study period are presented in Fig.4.2 and 4.3. Exposure of the birds to 32±1^{o}C for24hrs resulted in significant decline ($p<0.05$) in mean Hct and Hb levels for KU and MM(Fig.4.2). The Hb levels for KU returned to control levels after one week exposure while those for MM remained significantly lower ($p<0.05$) during the same period. There were no notablechanges in mean Hb values for CH at this temperature. However, the mean Hct levels for CHand KU but not MM were markedly increased ($p<0.05$) after one week exposure to 32±1^{o}C.

When the temperature was raised to 37±1^{o}C no significant changes in mean Hb and Hct values for KU and MM were observed for the entire 10-day period of exposure but CH showed a significant increase in Hb and Hct values within 4hrs of temperature rise

(Fig.4.3). In addition, the mean Hct for CH was significantly lowered ($p<0.05$) after 24hrs of exposure.

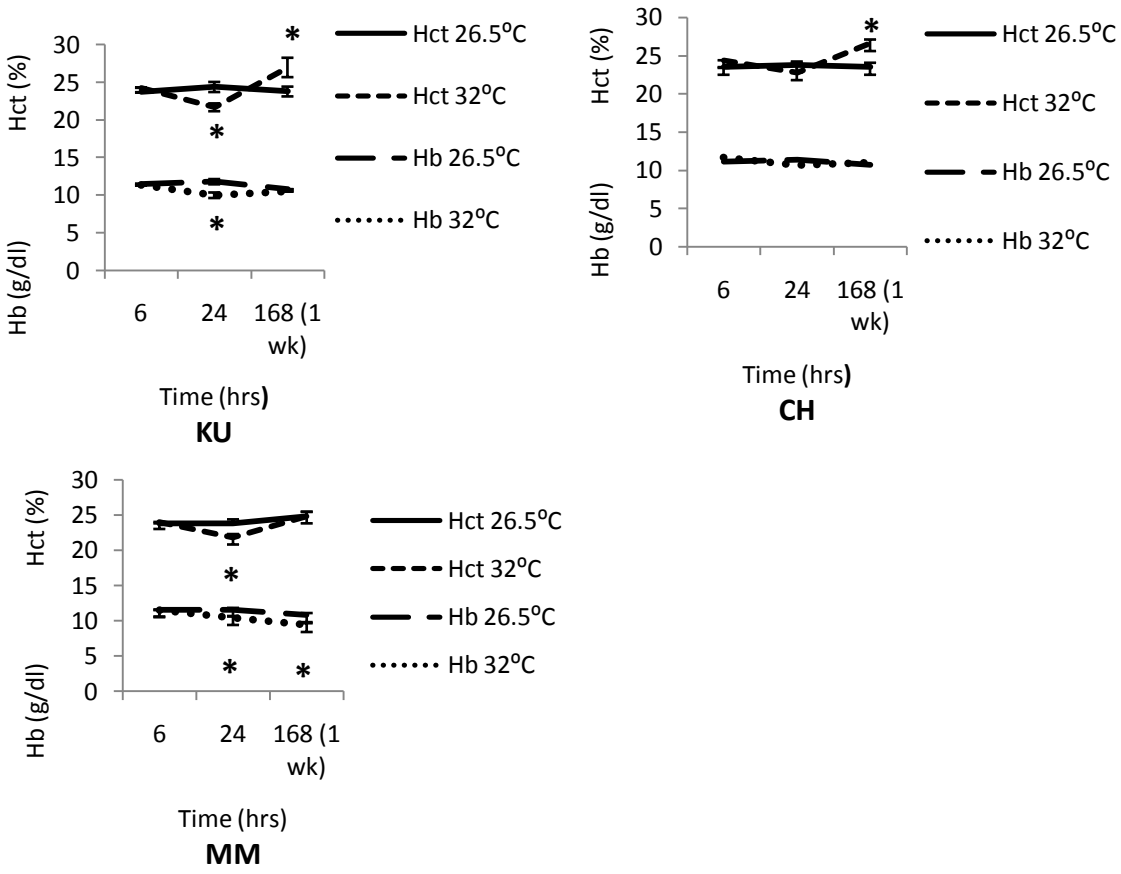

Figure 4.2: **Hct and Hb of 5 week-old KU, CH and MM hens at control conditions (26.5±0.5°C) and 32±1°C after 6hrs, 24hrs and 1 week.** *significantly different ($p<0.05$) from the control.*KU: Kuchi; CH: Ching'wekwe, MM: Morogoro medium. Hct: hematocrit; Hb: hemoglobin.*

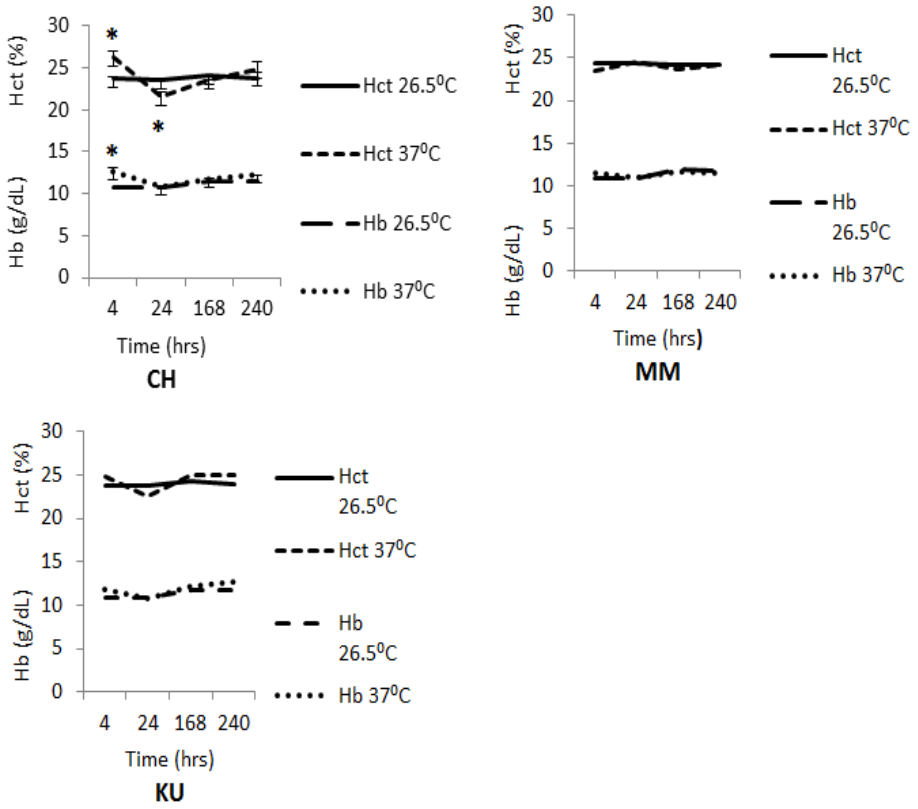

Figure 4.3: **Hct and Hb of 5 week-old KU, CH and MM hens at control conditions (26.5±0.5°C) and 37±1°C after 4hrs, 24hrs 7 days and 10 days.** *significantly different ($p<0.05$) from control; *KU: Kuchi; CH: Ching'wekwe, MM: Morogoro medium*. *Hct: hematocrit; Hb: hemoglobin.*

Study two:

4.2.3 Growth performance

The growth performance parameters for the chickens fed a low energy diet are presented in Fig. 4.4 and Table 4.3. After exposure to 32±1°C for 1 week (Fig.4.4 A), no significant changes in percent weight gains were observedfor all ecotypes when compared to control birds maintained at 26.5±0.5°C during the same period.However, when these birdswere subjected to37±1°C (8hrs a day for10 days), the percent weight gains were significantly

reduced ($p<0.05$) in all the ecotypes (Fig.4.4 B) and this reduction was greaterin MM($p<0.05$) than in KU and CH.

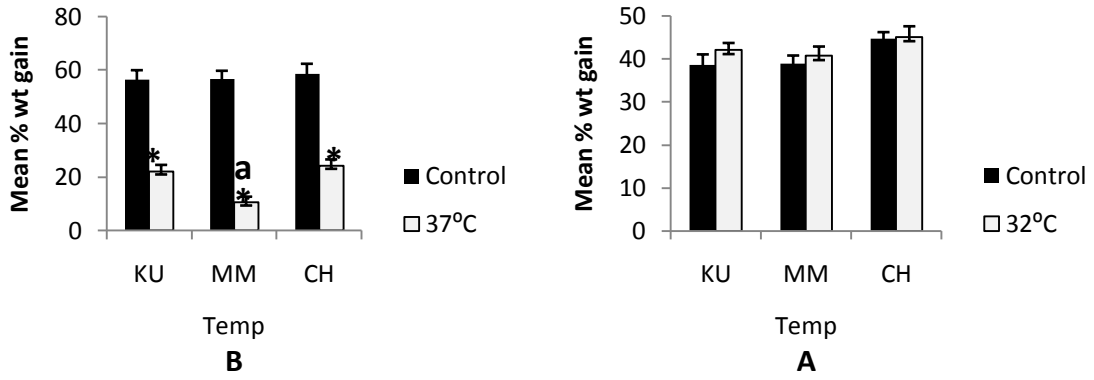

Figure 4.4: **Mean percent weight gains of: (**A**) 5 week-old hens at control conditions (26.5±0.5°C) and at 32±1°C with 55% dietary energy restriction for a 7-day period; (**B**) 6 week-old hens at control conditions (26.5±1°C) and at 37±1°C (8 hours per day) with 55% dietary energy restriction for a 10-day period**.*Significantly lower ($p<0.05$) than the control, a: significantly lower ($p<0.05$) than KU and CH. *KU: Kuchi, CH: Ching'wekwe, MM: Morogoro medium*.

The KU and CHchickens reared at 32±1^0 C for 7 days depicted amarked increases($p<0.05$) in feed intake and body weights when compared with the controls (Table 4.3). Between ecotypes, KU had significantly higher ($p<0.05$) mean feed intake and body weight than both MM and CH. Mean FCR values were significantly higher ($p<0.05$) than controls for KU and CH but not MM.

After exposure to 37±1°C (8hrs for 10 days) the amount of feed consumed was significantly higher ($p<0.05$) than the controlsin all ecotypes. The mean body weights were not significantly different from respective controls for KU and CH but were markedly lower ($p<0.05$) for MM. Mean FCR values were higher ($p<0.05$) than controls in

all ecotypes, and between ecotypes, MM depicted a significantly higher ($p<0.05$) mean value than both CH and KU.

Table 4.3: Growth parameters of chickens at control temperature (26.5±0.5^{0}C) and after exposure to 32±1^{0}C (constantly for 7 days) and 37±1^{0}C (8hrs a day for 10 days) and fed low dietary energy. Values are presented as Mean ± SE.

		KU	MM	CH		KU	MM	CH
Bw(g)	**Cont**	195.2 ±10.0^{m}	212.2±13.3^{i}	152.6±4.7^{im}	**Cont**	305.4±17.8^{t}	331.1±19.8^{0}	240.9±6.6^{0t}
	32^{0}C	273.8±7.3^{u}*	233.7±6.4^{u}	193.3±7.8^{u}*	**37^{0}C**	325.0±9.4^{nr}	260.4±9.2^{r}*	240.5±9.8^{n}
FI^1	**Cont**	153.8±2.8^{b}	243.2±4.1^{b}	164.5±2.8	**Cont**	297.9±2.2^{l}	405.5±2.6^{lj}	313.4±1.6^{j}
	32^{0}C	370.0±2.9^{a}*	319.0±3.6	279.6±3.6^{a}*	**37^{0}C**	597.9±4.5*	537.7±3.4*	635.6±4.9*
Wg(g)	**Cont**	52.3±3.7	58.5±4.2	47.0±1.6	**Cont**	110.2±9.7	118.9±7.9$^{\text{ɯ}}$	88.3±5.3$^{\text{ɯ}}$
	32^{0}C	80.8±4.3^{ck}*	66.7±3.3^{e}	56.9±4.7^{k}	**37^{0}C**	51.2±6.4^{v}*	26.7±5.5$^{v\chi}$*	47.2±6.9$^{\chi}$*
FCR	**Cont**	2.9±0.5^{g}	4.2±0.7^{g}	3.5±0.4	**Cont**	2.7±0.4	3.4±0.4	3.5±0.3
	32^{0}C	4.6±0.5*	4.8±0.6	4.9±0.6*	**37^{0}C**	11.2±2.0$^{\text{ñ}}$*	20.1±2.3$^{d\text{ñ}}$*	13.5±1.9^{d}*

*Significantly lower ($p<0.05$) than control; similar superscript letters within a row for each temperature treatment are significantly different ($p<0.05$). *KU: Kuchi, CH: Ching'wekwe, MM: Morogoro medium, Cont: control, Bw: body weight, F Int: feed intake, W gain: weight gain, FCR: feed conversion ratio, ¹: g/period ofexposure, that is, either 7 days at 32^{o}C or 10 days at 37^{o}C.*

4.2.4 Hematological indices

H/L ratios for the chickens fed low energy diet during the study period are shown in Table 4.4.

Table 4.4: H/L ratios for the chickens at control (26.5±0.5^{0}C) and heat stress (32±1 and 37±1^{0}C) with low dietary energy (55%) conditions

	[5wk old]	6hrs	24hrs	1 week	[6wk old]	4hrs	1 week
KU	**Control**	0.17±0.02	0.18±0.02	0.16±0.03	**Control**	0.20±0.03	0.20±0.03
	32±1^{0}C	1.63±0.99*	1.49±0.86*	0.14±0.01	**37±1^{0}C**	0.15±0.03	0.53±0.02*
CH	**Control**	0.15±0.01	0.17±0.02	0.15±0.01	**Control**	0.10±0.04	0.10±0.02
	32±1^{0}C	1.24±0.58*	1.50±0.69*	0.10±0.01	**37±1^{0}C**	0.16±0.03	0.60±0.03*
MM	**Control**	0.18±0.01	0.19±0.01	0.18±0.02	**Control**	0.12±0.02	0.11±0.02
	32±1^{0}C	0.77±0.27*	1.62±0.72*	0.16±0.03	**37±1^{0}C**	0.11±0.01	0.57±0.03*

*Significantly different ($p<0.05$) from the control; *KU: Kuchi, CH: Ching'wekwe, MM: Morogoro medium. H/L: heterophyl/lymphocyte ratio.*

Raising the rearing temperature from 26±0.5^{o}C to 32±1^{o}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^{o}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.4.5 and 4.6. Exposure of chickens to 32±1^{o}C for 7 days did not alter Hb levels except after 24hrs when CH had markedly lower values ($p<0.05$) and after 1 week when KU had significantly higher ($p<0.05$) values (Fig.4.5). While the Hct levels for MM were not altered during the entire 7-day period at 32±1^{o}C, the levels for KU weremarkedly elevated ($p<0.05$) after 6hrs of exposure and CH had a decline ($p<0.05$) after 24hrs.Raising the temperature to 37±1^{o}C did not significantly change Hb levels in KU and MM for the entire period of

exposure but a marked reduction ($p<0.05$) was observed after 24hrs in CH (Fig.4.6). Meanwhile,there was a reduction ($p<0.05$) in Hct levels at this temperature in all ecotypes.

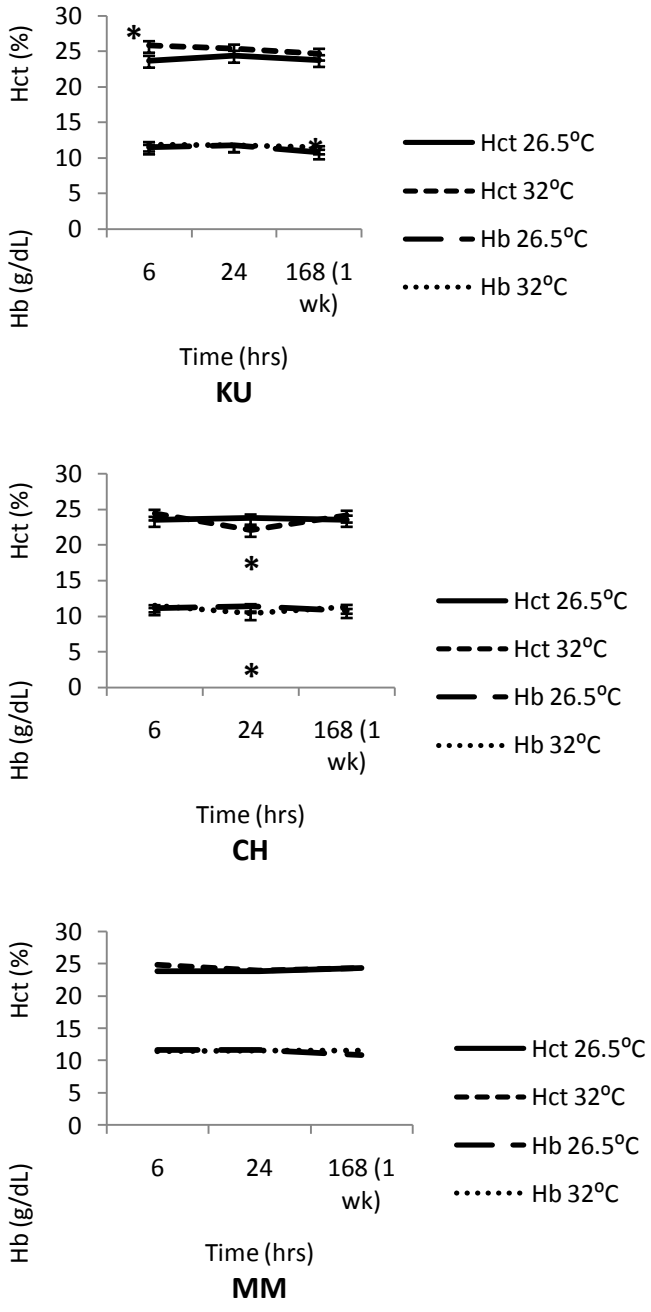

Figure 4.5: **Hct and Hb of 5 week-old KU, CH and MM hens at Control Conditions (26.5±0.05°C) and at 32±1°C with 55% dietary energy restriction for a 7-day period.** *Significantly different ($p<0.05$) from the control; *KU: Kuchi, CH: Ching'wekwe, MM: Morogoro medium*.

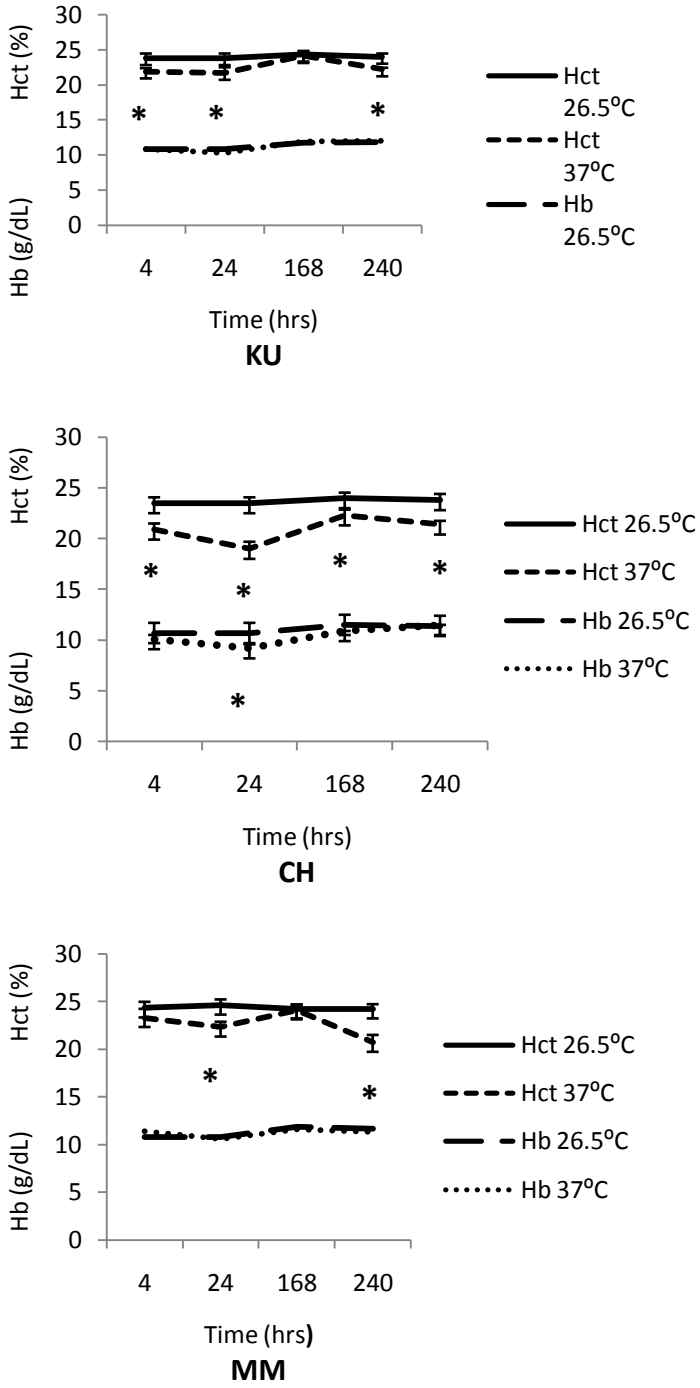

Figure 4.6: **Hct and Hb of 5 week-old KU, CH and MM hens at Control Conditions (26.5±0.5°C) and at 37±1°C with 55% dietary energy restriction for a 10-day period.** *Significantly lower ($p<0.05$) than the control; *KU: Kuchi, CH: Ching'wekwe, MM: Morogoro medium*.

4.3 Discussion

One of the viable strategies to alleviate the adverse effects of high ambient temperatures would be to selectfor chickens from among the local chicken ecotypes with better performance traits and those that are more resilient to heat stress. The current study aimed at investigating if viable performance characteristics and resiliencies to heat stress do exist in some selected ecotypes. In addition,an investigation was further done to assess if heat stress when compounded with low energy intake commonly seen in free ranging birds living in the tropics could provide measurable response differences within ecotypes under study. The assessed parameters between and within ecotypes were growth performance, Hb, Hct and H/L. The choice of the ecotypes used in the study represents the common local chicken ecotypes raised under free range in the mid and northwest Tanzania and their genetic and performance information is fairly available (Msoffe *et al*., 2002). Only female chicks were used to minimize sex-induced response differences.

For birds maintained under control dietary energy (2864 kcal ME/kg), an ecotype-specific response to heat stress was observed in all growth parameters measured when the room temperature was raised and maintained at 32±1^{o}C for 7 days (Study one). Heat stress induced by this temperature did not significantly reduce percent weight gain for KU but did for MM and CH. However, when energy content in feed was reduced by 55%, no notable differences in percent weight gains were observed in all ecotypes after exposure to 32±1^{o}C for 1 week (Study two). This observation may be an indication that stress induced by this temperature was ameliorated by low dietary energy level and therefore did not affect mean percent weight gain of the chickens. Low dietary energy levels induced hyperphagia through hypothalamic signalingand led to increased feed intake that subsequently promoted weight gain, andheat dissipation through thermogenesis was avoided.Under these conditions, chickens were all observed to consume more feed as a

metabolic adaptation to meet their energy requirements (NRC, 1994;Nakkazi *et al*., 2015),thusensuring that the calories consumed are similar andimproving their percent weight gains to control levels (dePersio *et al*., 2015). Mean FCR values were significantly increased ($p<0.05$) in all ecotypes maintained at 32±1^{o}C and fed control dietary energy, signifying reduced feed utilization efficiency. Apparently, better feed utilization efficiency was observed in MM after exposure to 32±1^{o}C and fedlow dietary energy, signifying better performance under these conditions.

An ecotype specific difference was observed in mean percentage weight gain when birds were exposed to 37±1^{o}C and fed normal control energy diets. This was evidenced by marked reductions ($p<0.05$) in mean percent weight gains in CH and MM but not in KU, with a more pronounced reduction in MM. This observation may imply least growth performance for MM at this temperature. Moreover it is worth noting that percent weight gain reductions at this temperature are lower, and this may be attributed to earlier conditioning of the chickens at 32±1^{o}C thereby lessening the effects when later exposed to a higher temperature (Vinoth *et al*., 2016). On the other hand, significant ($p<0.05$) reductions in percent weight gains were observed in all ecotypes fed low energy diet when they were exposed to 37±1^{o}C for 10 days. This response differed between ecotypes as it was more pronounced in MM (81%)than in KU (61%)and CH (59%). It appears the stress level was too severe and chronic to be ameliorated by low energy diet at this stage as shown by very high percent weight gain reductions by the chickens of all ecotypes. This implies that the apparent increase in feed intake was not adequate to meet the daily energy requirements for the birds during this period. Feed utilization efficiencies were significantly reduced for chickens fed normal control diet, with no inter-ecotype differences observed, but for those fed low energy dietMM showed the greatest reduction, as shown by increased FCR values.

In commercialbroilers and layers heat stress causes decreased body weight gainand feed utilization efficiency(Quinteiro-Filho *et al*., 2010; Felver-Gant *et al*., 2012; Mello*et al*., 2015) in consistent with findings of the current study in local chicken ecotype stocks.Quinteiro-Filho *et al*. (2010) attributed the decrease in food consumption and consequently a decrease in an animal's body weight gain under heat stress to corticosterone that could be acting in the hypothalamic feeding control nuclei that regulate food intake and satisfaction. However, in the current study, the amount of feed consumed was unexpectedly significantly increased ($p<0.05$) in chickens fed normal control diet at the end of exposure to 32±1^{o}C and 37±1^{o}C, with KU having the highest increase. The higher amount of feed consumed for KU even at high temperature seems to have contributed to its better performance in terms of percent weight gain. Since the exposure to 37±1^{o}C was only between 08:00 and 16:00hrs, it maybe that the chickens maximized feeding at times when the temperature was reduced to 32±1^{o}C between 16:00hrs and 08:00hrs. These observations are contrary to previous findings in commercial chickens by other researchers (Quinteiro-Filho *et al*., 2010; Mello*et al*., 2015) but in agreement with Melesse *et al*. (2013) who reported a higher feed consumption for the chronically heat stressed layer commercial hens than those at thermoneutral conditions. The differences with current findings may also be attributed to chicken strains and age, as the chickens used in the current study were in their early growth phase that is characterized by faster growth and may have different metabolic requirements. Similarly, feed consumption significantly increased ($p<0.05$) in chickens fed low energy diet when they were exposed to 32±1^{o}C and 37±1^{o}C for 1 week and 10 days, respectively. This was likely because the chickens were able to increase low energy feed consumption so that they could ensure adequate energy and nutrient intake(dePersio *et al*., 2015).

The H/L ratios for the chickens fed low energy diet were increased when temperature was raised to 32±1^{o}C for 1 week and thereafter to 37±1^{o}C for 10 days and did not show inter-ecotype differences. The returnof H/L ratios to control levels after 1 week exposure to 32±1^{o}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 and 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^{o}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 increase in H/L ratio could be due to glucocorticoidsreleasecausingdissolutionoflymphocytes in lymphoid tissues and leading to lymphopenia, with an accompanying increase inheterophilrelease bythe bonemarrow (Zulkifli and Siegel, 1995; Borges *et al*., 2004).

The changes in levels of mean Hb and Hct in chickens fed control diet and exposed to 32±1^{o}C show ecotype related differences. The significant reductions ($p<0.05$) in mean Hb and Hct for KU and MM but not for CH after 24hr-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 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 studies, significant changes in Hb and Hct for the chickens fed low energy diet after exposure to 32±1^{o}C were only observed in KU and CH. 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^{o}C showed between-ecotype differences in Hb and Hct changes both for those fed control diet andlow dietary energy. Marked changes in mean Hb for CH but not KU and MM after 4hrs and 24hrs for chickens fed control diet and low energy diet, respectively shows that at this temperature, unlike at32±1^{o}C, there were more physiological adjustments and changes in CH than in KU and MM. These changes may entail that MM and KU had tolerated these stress conditions better than CH. Meanwhile, there was a similar pattern of Hct reduction in all the chicken ecotypes fed low energy diet, which was not the case for chickens fed normal control diet. This may imply that low dietary energy compounded the stress levels at high temperature that might have triggered significant changes in physiological components.Energy intake may have been inadequate for energy costs of blood cells synthesis and the general implication of reduced Hct is a decrease in circulating concentrations of oxygen.The findings in the current study are consistent with Lamont *et al*. (2015) who reported decreased Hb in Fayoumichickens under heat stress. Decreases of Hct and Hb could be also potentially important parameters, contributing to chicken'sheat stress resistance(Lamont *et al*., 2015). Meanwhile Oladele *et al*. (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. 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 is only consistent with findings relating to KU and MM but not CH at 37±1^{o}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.

4.4 Conclusion

As the magnitude of heat stress increased adaptation and tolerance becameecotype-dependent.WhileMMecotypedemonstrated bettertoleranceto moderately high temperature, KU and CH were more tolerant to higher temperature with respect to percent weight gain. Some effects of stress induced by moderately high temperatures can be ameliorated by low dietary energy. Growth performance was synergistically suppressed by a combination of chronic heat stress and low dietary energy in an ecotype-specific manner at much higher temperatures.The variability in dynamism and ecotype-specific nature in some hematological parameters' responses suggest that they may be reliable indicators of heat tolerance in local chickens.

4.5 Acknowledgements

The authors acknowledge financial support from Intra-ACP Mobility Scholarship Program through the Education, Audiovisual and Culture Agency of the EU. Chicken flocks utilized in this study were generated through the support by the US Agency for International Development on Feed the Future Innovation Lab for Genomics to Improve Poultry.

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CHAPTER FIVE

Effects of Heat Stress and Low Dietary Energy on Behaviour, Blood Indices, Liver hsp70 and iNOS Gene Expressionsin Selected Tanzanian Local Chicken Ecotypes

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ABSTRACT

Two studies, each with three replicates, were conductedto compare the effects of heat stress and a combination of heat stress and low dietary energy on behaviour, blood indices, liver hsp70 and iNOS gene expressionsin Tanzanian local chickens.In Study one, 78 five weeks old Kuchi (KU), Ching'wekwe (CH) and Morogoro medium (MM) ecotypes were allocated to separate pens in two temperature controlled rooms (39 chickens per room and 13 per ecotype per pen). In one room temperature was maintained at 26.5±0.5^{o}C (control) for 17 days whilst in the adjacent room it was raised and maintained at 32±1°C for 7 days and thereafter raised and maintained at 37±1°C from 08:00-16:00hrsfor 10 days. A similar design was used in Study two except that chickens in the high temperature group were fed 55% less dietary energy than the control.Heat stress induced an increase ($p<0.05$) in serum corticosterone after exposure to 32^{o}C for 7 days and 37^{o}C for 24hrs in KU and to 37^{o}C for 24hrs in CH.On the other hand, in Study two, serum corticosterone markedly increased ($p<0.05$) after exposure of chickens to 32^{o}C for 7 days, 37^{o}C for 24hrs in CH and KU, and to 37^{o}C for 10 days in all ecotypes. While serum uric acid declined ($p<0.05$) in all chickensafter exposure to37^{o}C for 24hrs in Study one, levels remained unchanged in Study two. Apparently, in both studies,there was a marked reduction ($p<0.05$) inserum total protein levels after exposure to 37^{o}C for 24hrs (all ecotypes) and for 10 days (CH and MM). Meanwhile there were marked reductions in serum triglyceride after 7 days at 32^{o}C (KU and MM) and 10 days at 37^{o}C in all ecotypes in Study two. Both hsp70 and iNOS gene expression levels remained unchanged at the end of Study one but hsp70 expression for KU was markedly higher ($p<0.05$) than for CH and MM. At the end of Study two, liver hsp70 gene expression levels were significantly ($p<0.05$) up-regulated in all ecotypes, with levels in KU higher ($p<0.05$) than in MM. Similarly, liver iNOS relative gene expression levels were significantly increased ($p<0.05$) in all ecotypes but without

between-ecotype differences.KU showed a greater ($p<0.05$) frequency of panting than CH and MM, whilst wing droop frequencies were markedly lower in CH than in KU and MM. Heat stress and a combination of heat stress with low dietary energy further induced significantreductions in feeding, preeningand locomotion in all ecotypes, and preening was lower ($p<0.05$) in KU than in CH. These results suggest that there are ecotype-based differences in the local chickens' adaptive responses to heat stress and a combination of heat stress with low dietary energy.MM and CH demonstrated better tolerance than KU when only heat stress was applied but a synergistic effect of heat stress and low dietary energy suggested that MM is more tolerant.

Key words:*behaviour, corticosterone, ecotype, panting, stress, tolerance*

5.0 INTRODUCTION

Livestock production is increasing throughout Africadue to demand for meat and other animal products,driven by growth of human population, living standards and urbanisation (IUCN, 2010). 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 likeTanzania (Queenan*et al*., 2016). The chickens are generally left to scavenge for food on their own, with the farmer only providing shelter and thus, they are seasonally faced with stressors, mainly in the form of elevated temperatures, low quality nutrition and disease (Mwalusanya *et al*., 2001;Ayo *et al*., 2011). These stressors contribute to the low production capacity in this sector. Although local chickens are supremely adapted to the harsh environments in areas where they are bred and can produce under conditions where exotic breeds may not survive, ecotype-differences in production performance and disease resistance have been shown(Msoffe *et al*., 2002;Lwelamira, 2012). 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 some local chicken ecotypes which show better performance traits when exposed to various common stressors are highly needed.These will fill the missing gaps useful in selection.

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 the impairment of the bird's well-being (Cheng and Jefferson, 2008) and marked reduction in production capabilities (IUCN, 2010).Similarly, biochemical parameters in the blood may reflect the physiological state of the birds (Lin *et al*., 2000;Hrabcakova *et al*., 2014). Heat stress is well known to increase plasma glucose levels (Lin *et al*., 2000;Garriga *et al*., 2006), body core temperature (Soleimani *et al*.,

2011), and to alter the electrolyte balance and blood pH (Van Goor, 2016)in commercial broilers and layers.The hypothalamus-pituitary-adrenocortical (HPA) axis is usually activated, leading to a rapid increase in circulatory corticosterone levels (Quinteiro-Filho *et al*., 2010;Rimoldi *et al*., 2015;Akbarian *et al*., 2016).A major change in blood components seems to be associated with cellular damage (Bogin *et al*., 1996) and heat - induced increased respiration, which results in respiratory alkalosis (Van Goor *et al*., 2016).

Under heat stress, dysregulation of concentrations of hormones such as corticosterone has been associated with induction of varied behavioural changes (Cheng and Jefferson, 2008) aimed at aiding thermoregulation and increase in the flux of heat from the tissues to the environment. In turn this promotes the dissipationof heat from the body leading to decreased body temperature (Syafwan *et al*., 2011;Mack *et al*., 2013). Such behaviours include panting, sand bathing and standing with wings drooped and lifted slightly from the body to maximize heat loss (Cheng and Jefferson, 2008). In general, birds react similarly to heat stress but express individual variation in the intensity and duration of their responses (Mack *et al*., 2013). Whereas, most of the physiological studies related to heat stress in birds have mainly been done in commercial broiler and layer chickens (Mack *et al*., 2013;Li *et al*., 2015), there is paucity of such studies in local chickens.

Oxidative stress can be detrimental to gene expression,leading to posttranscriptional changes to signaling genes and disruption of the health of an animal at the genetic level (Allen and Tresini, 2000; Fleming *et al*., 2016).The vulnerability of poultry to heat stress varies according to genetic potential, life stage and nutritional status (IUCN, 2010).The liver is more susceptible to oxidative stress and injury than other body organs and plays an important role in energy metabolism (Xie *et al*., 2014; Lan *et al*., 2016). Heat stress retards

synthesis of most proteins but a group of highly conserved proteins known as heat shock proteins (Hsps) are rapidly synthesized (Al-Aqil and Zulkifli, 2009). Hsps are molecular chaperones essential for maintaining cellular functions by preventing misfolding and aggregation of nascent polypeptides and by facilitating protein folding (Zeng *et al*., 2014). Among Hsp families, Hsp70 is the most extensively studied because of its prominent response to diverse stressors (Zhao *et al*., 2013). Increased synthesis of these inducible proteins is involved in the protection of stressed cells (Gabriel *et al*., 2002; Zhao *et al*., 2013).Acute heat stress elicits rapid Hsp synthesis and causes dramatic changes in gene expression (Pardue *et al*., 1992; Xie *et al*., 2014). In contrast, long-term heat exposure may induce adaptations (Xie *et al*., 2014). On the other hand, inflammation is an important indicator of animal tissue damage due to heat stress.Generally iNOS is absent in normal liver but is markedly increased in response to inflammation and a variety of oxidative stresses, and is responsible for the synthesis and catalysis of NO, which has anti-inflammatory activities (Clemens, 1999; Zhao *et al*., 2013).

It has been observed in previous studies that Liver hsp70 concentrations are increased whereas liver weights are reduced in hens exposed to heat stress (Felver-Gant *et al*., 2012). In broiler chickens for example, HSP70 is highly induced after acute heat exposure (Yu and Bao, 2008; Lowman *et al*., 2014; Xie *et al*., 2014). 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 Tanzanian 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 and Jefferson, 2008).Information on the relationships among behaviour, biochemical and hormonal homeostasis in local chickens when responding to stressful stimulations caused by heat stress and low dietary energy is needed. In the present study, three chicken ecotypes, Ching'wekwe (CH), Kuchi (KU), and Morogoro medium (MM),which represent unique local ecotypes raised under free range in the mid and northwest Tanzania were recruited. These ecotypes are generally considered to have good productionand disease resistantpotentials (Msoffe *et al*., 2002). The assumption is that the differences in resistance to disease between ecotypes shown by other studies on the same ecotypes may also be reflected in their responses to heat stress, low dietary energy or even a combination of these stressors.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 behaviour, blood indices, liver hsp70 and iNOS gene expressionsin Tanzanian local chickens.Identification of 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 or even under a combination of high ambient temperatures and low dietary energy intake.

5.1 Materials and Methods

5.1.1 Study chickens

Day-old MM, CH and KU local chicken 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 under similar environmental, managerial and 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 minimize sex-induced response differences.

5.1.2 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 (i.e. 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 (Chapters two and three) 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 and ground wood charcoal (Rezaei *et al*., 2006) was used to dilute the experimental feed. The chemical (proximate) analyses of different feed ingredients were carried out using standard methods (FAO, 1994). Feed samples were analyzed for crude fiber, crude protein (Kjeldahl protein), moisture, ash, nitrogen-free extracts (digestible carbohydrates) and crude lipid; and then metabolisable energy levels were estimated (Janssen, 1989;NRC, 1994). The composition of specific ingredients in the feed is depicted in Table 5.1.

Table 5.1: **Composition and nutrient levels of experimental diets**

	Control diet 2864 Kcal/kg ME	**55% Energy Restriction 1319 Kcal/kg ME**
Ingredients	(%)	(%)
Maize meal	37.8	10
Maize bran	26	2
S.flr. 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
DCP	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: 10mg, vitamin K3: 200mg, vitamin B12: 2.5mg, niacin: 6mg, pantothenic acid: 5mg, selenium: 0.2mg, Fe: 80mg, Cu: 80mg, Zn: 100mg, and Mn: 120mg, S. flr.: Sun flower.

5.1.3 Study Design

Two studies were conducted designated Study one and Study two.

Study one:A total of 78(26 per ecotype) five weeks old female chicks belonging to Kuchi (KU), Ching'wekwe (CH) and Morogoro medium (MM) ecotypes were weighed and randomly allocated into separate pens in two adjacenttemperature controlled rooms. Each room had three pens, with each having an average area of 2.5 m² floor space per 13 birds and hens were reared on littered (rice husks) floor. A 3 (3 ecotypes) x 1 (heat stress) 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 10L:14D cycle. To acclimatize to their new environment, all chickens had*ad*

libitum access to waterandfeed consisting of 18% crude protein and 2864 kcal ME/kg, and were maintained at normal ambient temperature of 26.5±0.5^{o}Cfor 5days.At the start of the study, 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 hours and was maintained at this temperaure for 7 days. After 7 days temperature was raised again and maintained for 10 days at 37±1°C for 8hrs per day for starting at 08:00hrs to 16:00hrs; 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%.

Study two: Effects of a combination of heat stress and low dietary energy on physiological, biochemical and behavioural responses.

A similar design and chicken number (234) were used in Study 2 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. The basis of using 55% less dietary energy than the control as an additional stressor is from earlier studies (Chapters two and three) that evaluated the responses of the same local chicken ecotypes to low dietary energy and showed that this level of energy restriction was low enough to induce stress.

5.1.4 Blood sampling and analysis

Whole blood was collected via the wing vein at similar times of the day (between 10:00 and 12:00hrs) using syringes and immediately transferred into ethylene diamine tetracetic acid (EDTA) containing vacutainers and/or plain vacutainer tubes (for serum preparation).

During the period birds were reared at 32±1°C, blood was collectedat intervals of 6hr, 24hr, and 7 days and when the temperature was raised to 37±1°C blood was taken at intervals of 4hr, 24hr, 7 days and 10 days. 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).

5.1.5 Liver tissue collection, RNA extraction and quantitative real-time PCR

At the end of the study (17days), 5 chickens from each pen were randomly selected, weighedand then humanly sacrificed and decapitated. Liver samples were quickly collected, weighed on a kitchen scale and placed on ice beforestorage at -80^{o}C. Liver weights were recorded in g/kg of chicken weight. Total RNA was extracted from liver samples (50mg) using the Quick-RNA™ MiniPrep Plus kit (Zymo Research) following manufacturer's instructions of preparation and purification. The integrity of the isolated RNA was examined using 1.2 % agarose gel containing 0.1 % ethidium bromide. First-strand complementary DNA was synthesized from about 5µg of total RNA according to manufacturer's instructions in a 20µL reaction volume by using RevertAid First-Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's instructions. Predesigned primers for hsp70, iNOS(Zhao *et al*., 2013) and GAPDH (Xie *et al*., 2014) were used, and are depicted inTable 5.2. The quantitative real-time PCR (qPCR) was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) on an ABI 7500 (Applied Biosystems USA). Reactions were performed in a 25-µL reaction mixture. The cycling protocol included an initial denaturation step at 95°C for 10 min,

followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 60sec. A dissociation curve was run for each plate to confirm the production of a single product. 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.

Table 5.2: Target gene Primers used

Gene	Primer set	Product (bp)	Tm (^{o}C)
hsp70	F 5′-CGGGCAAGTTTGACCTAA-3′	250	58
	R5′-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

5.1.6Behaviour observations

Behaviour observations were done using a combination of the direct observation method (Lolli *et al*., 2013) and using a video camera recorder (Samsung SM-G361H). Behaviour was classified into 7 categories, namely: feeding, drinking, resting (sitting and standing), preening, locomotory activities (moving around), panting and wing droop. The number of birds in each pen engaged in particular behavior was counted (and expressed as percentages) at 5 minute-intervals and repeated 6 times. Mean values for each pen incuding all the replicates were then computed. The observations and video recordings were made between 11:00 and 14:00 hours after24hr and 7 days for birds reared at 32±1°C, and after 24hr, 7 days and 10 days for birds reared at 37±1°C.A single observer

was stationed wihin the chicken house and precaution was taken not to disturb the natural behaviour of the chickens.

5.1.7Statistical analysis

The Independent Sample t-test was used to compare means between treatment and control groups and One-way ANOVA (SPSS 20) 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 comparisonswere used to separate means, with significance statements based on $p< 0.05$. Correlation analysis was performed by linear regressiontest using SPSS 20 softwareand the correlation coefficients were considered significant at $p< 0.05$. Results are presented as Means ± SE.

5.2 Results

Comparisons were made onCH, KU and MM local chicken ecotypes' responsesto heat stress and a combination of heat stress and low dietary energy with respect toblood indices, behavioural responses, liver hsp70 and iNOS gene expressions after conducting 2 studies. The results of both studies are presented below:

Study one

(a) 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 $32\pm1^{o}C$ for 7 days and thereafter to $37\pm1^{o}C$ (8hrs per day) for 10 days, and the results are depicted in Fig. 5.1and Fig. 5.2. At the end of the study (17 days), whereas the expression for CH and MM clearly remained unchanged, the relative gene expression of hsp70 for KU was up-regulated, though not significantly. The levels of expression of hsp70 for the KU ecotype were markedly higher ($p<0.05$) than in CH and

MM (Fig. 5.1). There was no change in the relative gene expression levels of iNOS in all ecotypes and no between-ecotype differences were observed (Fig. 5.2).

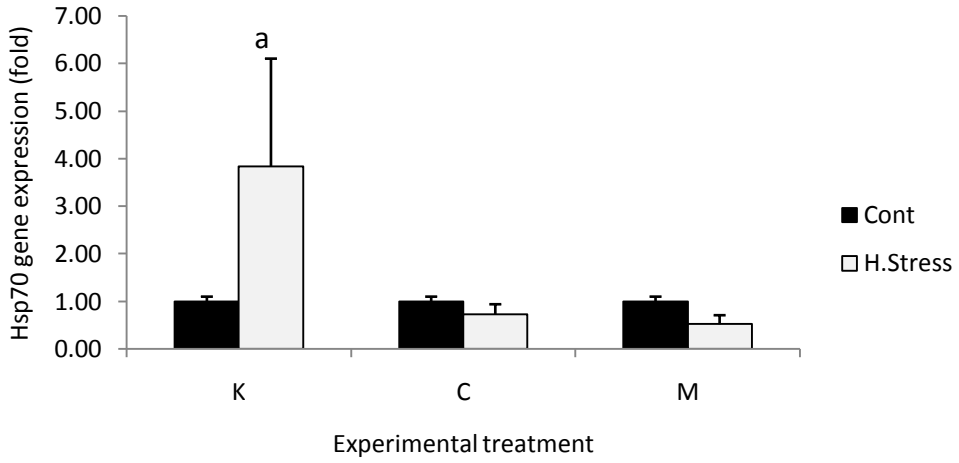

Figure 5.1: **Liver HSP70 gene expression at control conditions (26.5±0.5^{o}C) and after exposure to 32±1^{o}C for 7 days and thereafter to 37±1^{o}C (8hrs per day) for 10 days; ^{a}significantly higher than C and M; K: Kuchi, C: Ching'wekwe, M: Morogoro medium; Cont: control, H: heat.**

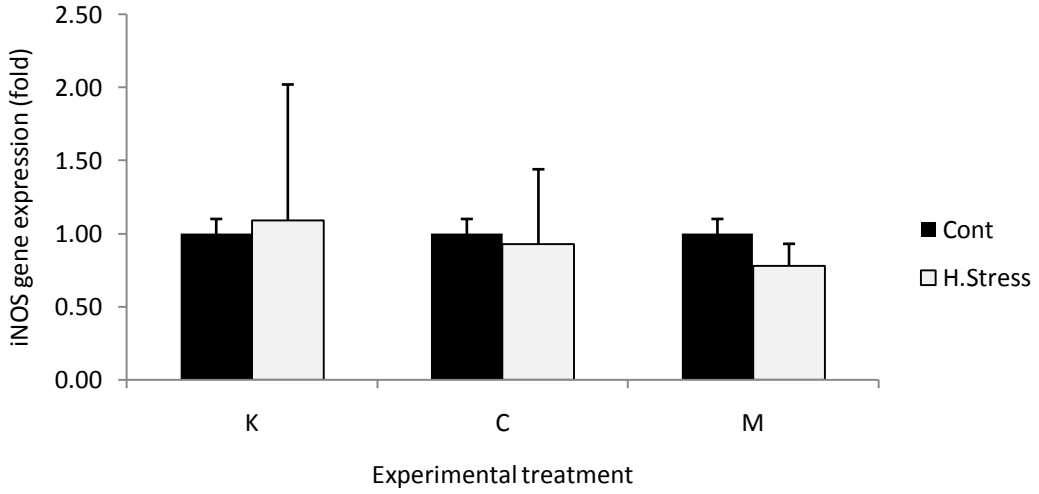

Figure 5.2: **Liver iNOS gene expression at control conditions (26.5±0.5^{o}C), exposure to 32±1^{o}C for 7 days and to 37±1^{o}C (8hrs per day) for 10 days; K: Kuchi, C: Ching'wekwe, M: Morogoro medium; Cont: control, H: heat.**

(b) Corticosterone

The results for serum corticosterone concentration of the hens at control (26.5±0.5^{o}C) conditions for 17 days and after exposure to 32±1^{o}C for 7 days and 37±1^{o}C for 24hrs and 10 days are shown in Fig.5.3. Exposure of the chickens to 32±1^{o}C for 7 days caused a significant rise ($p<0.05$) in serum corticosterone for KU but not CH and MM. Within 24hrs of raising the temperature to 37±1^{o}C, KU and CH but not MM showed a marked increase ($p<0.05$) in corticosterone levels. After a 10 day-exposure to 37±1^{o}C, no significant increases in serum corticosterone level were observed in all the chicken ecotypes.

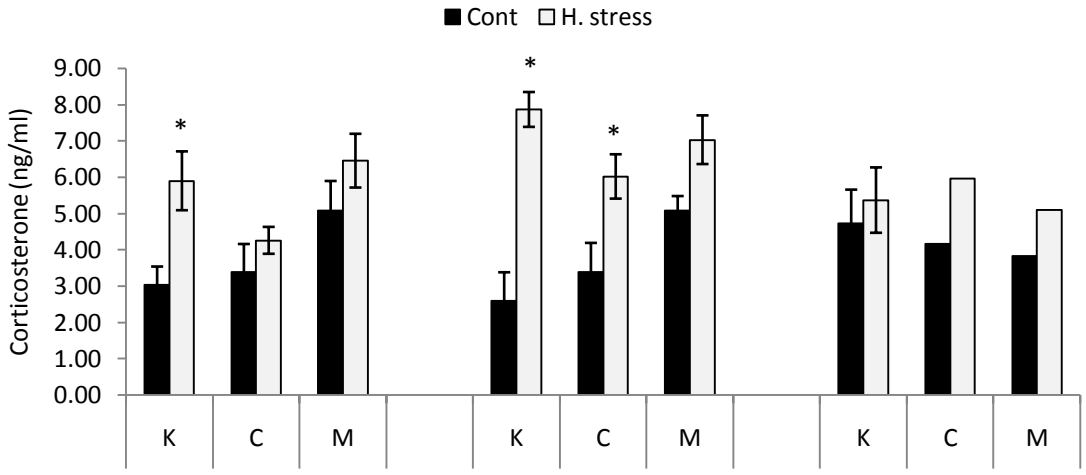

Figure 5.3: **Serum corticosterone concentration of the hens at control (26.5±1^{o}C) conditions and after exposure to 32±1^{o}C for 7 days and 37±1^{o}C for 24hrs and 10 days;** *significantly higher than the control; K: Kuchi, C: Ching'wekwe, M: Morogoro medium; D: day, H: heat, Cont: control.

(c) Uric acid

The results for serum uric acid concentration of the hens at control (26.5±0.5^{o}C) conditions and after exposure to 32±1^{o}C for 7 days and 37±1^{o}C for 24hrs and 10 days are shown in Fig.5.4. Exposure of the chickens to 32±1^{o}C for 7 days caused a significant reduction ($p<0.05$) in serum uric acid levels for CH but not for KU and MM. Serum uric levels were further reduced equivocally ($p<0.05$) in all ecotypes when exposed to 37±1^{o}Cfor 24hrs. Exposure of the chickens to 37±1^{o}C for 10 days led to an increase ($p<0.05$) in serum uric acid levels for KU only but caused a marked decline for CH and had no effect for MM.

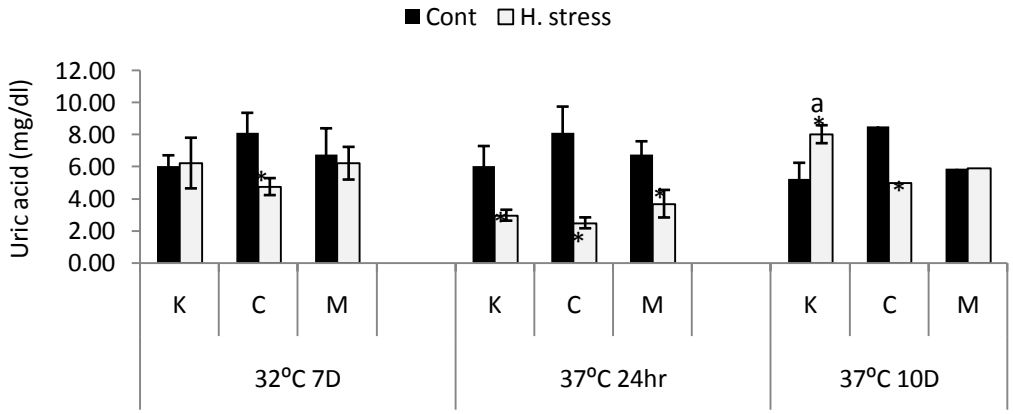

Figure 5.4: **Serum uric acid concentration of the hens at control (26.5±1^{o}C) conditions and after exposure to 32±1^{o}C for 7 days and 37±1^{o}C for 24hrs and 10 days;** *significantly different from the control; [a]significantly higher than C; K: Kuchi, C: Ching'wekwe, M: Morogoro medium; D: day, H: heat, Cont: control.

(d) Total protein, glucose and triglycerides

Serum total protein, glucose and triglyceride concentrations are depicted in Table 5.3. Whileserum levels for glucose and triglyceride were not changed in all ecotypes,exposure of chickens to 32±1^{o}C for 7 days caused a marked decrease ($p<0.05$) in total protein levels

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

Table 5.3: Serum total protein (T.P), glucose (Glu) and triglyceride (T.G) concentrations of the hens at control (26.5±0.5^{o}C) conditions and after exposure to 32±1^{o}C for 7 days and 37±1^{o}C for 24hrs and 10 days. Values are presented as Mean ± SE.

		7days		**24hrs**		**10days**
	Cont	**32^{o}C**	**Cont**	**37^{o}C**	**Cont**	**37^{o}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.

(e) Behaviour

Behavioural frequencies at control (26.5±0.5^{o}C) conditions and after 7 days exposure to 37±1^{o}C are shown in Fig. 5.5. Whilst panting and wing droop were significantly increased ($p<0.05$), a marked reduction ($p<0.05$) in feeding behaviour was observed for all chicken ecotypes after exposure to 37±1^{o}C for 7 days. Resting and locomotion showed notable reductions ($p<0.05$) for MM and KU, respectively, but both behaviour types were unchanged for CH. No significant reductions were observed for drinking and preening behaviours in all the chicken ecotypes. There were no notable between-ecotype differences in all behaviour-types except preening, which was significantly lower ($p<0.05$) in KU than in CH.

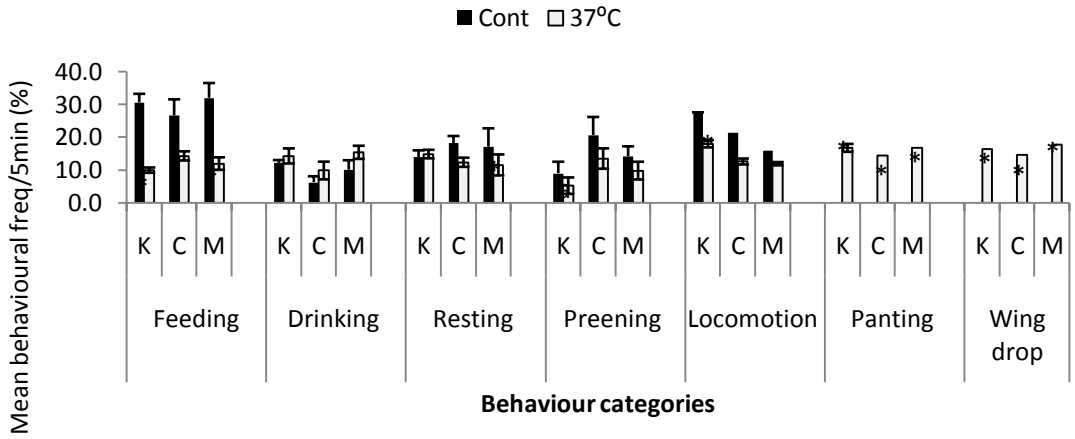

Figure 5.5: Behavioural frequency at control (26.5±1^{o}C) conditions and after 7 days exposure to 37±1^{o}C; *significantly different from the control; [a]significantly lower than C; *K: Kuchi, C: Ching'wekwe, M: Morogoro medium; Cont: control.*

(f) Correlation analysis of serum corticosterone and behavioural responses

Correlations between serum corticosterone and behavioural responses after heat stresss exposure are presented in Table 5.4. Corticosterone was strongly ($p<0.05$) negatively correlated with feeding and restingbehaviours in CH, while there was weak or no association in MM.Though not significant, corticosterone was negatively correlated with resting behaviour but had no association with feeding behavaviour in KU.The correlation with preening was negativeinKU and MM while in CH there was no association.Serum corticosterone was positively correlated with locomotion in KU and MM ($p<0.05$) but in CH, there was a weak ngative association.While panting and wing droop were positively correlated with corticosterone levels in CH and KU, the correlation was weak and negativein MM.

Table 5.4: Correlations between corticosterone and behavioural responses

Corticosterone KU CH MM			
Feeding	-0.004	-0.987* (p = 0.05)	0.275
Drinking	0.444	-0.093	0.460
Resting	-0.740	-0.984* (P = 0.05)	-0.06
Preening	-0.869	0.021	-0.817
Locomotion	0.864	-0.157	0.999* (P = 0.008)
Panting	0.267	0.931	-0.349
Wingdroop	0.214	0.995* (P = 0.032)	-0.260

*Significantly different; *K: Kuchi, C: Ching'wekwe, M: Morogoro medium*

Study two

(a) 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 (8hrs per day) for 10 days, and the results are depicted in Fig. 5.6 and Fig. 5.7. 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 KU ($p<0.05$) than in MM (Fig. 5.6). Similarly, iNOS relative gene expression levels were greatly increased ($p<0.05$) in all ecotypes but between-ecotype differences were absent (Fig. 5.7).

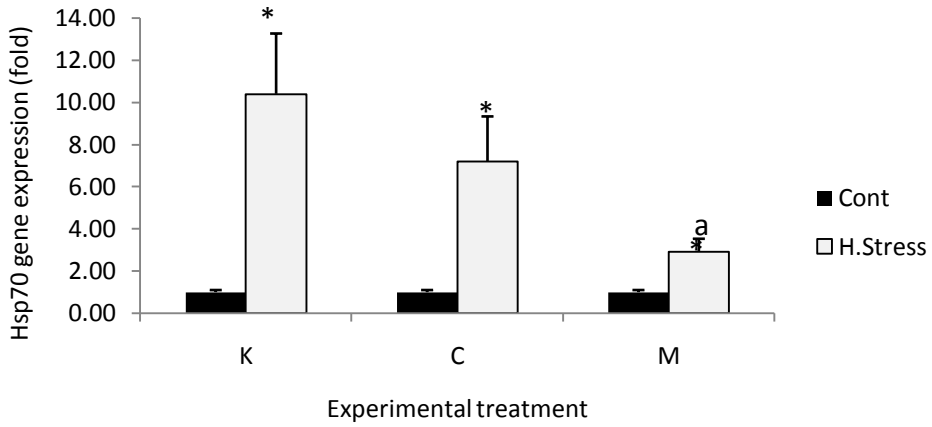

Figure 5.6: **Liver hsp70 gene expression at control conditions (26.5±0.5°C), exposure of chickens fed 55% of control energy to 32±1°C for 7 days and to 37±1°C (8hrs per day) for 10 days;** *significantly higher than the control; [a]significantly lower than K; K: Kuchi, C: Ching'wekwe, M: Morogoro medium; Cont: control, H: heat.

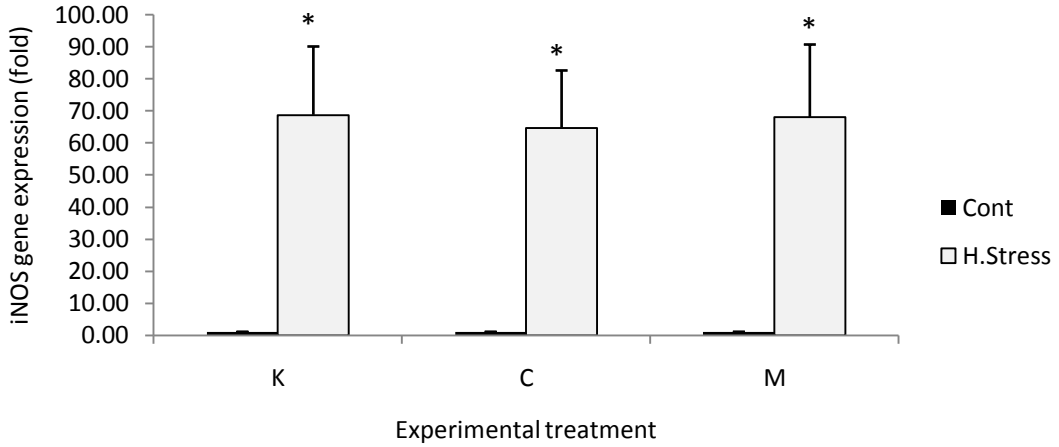

Figure 5.7: Liver iNOS gene expression at control conditions (26.5±0.5^{o}C), exposure of chickens fed 55% less energy than the control to 32±1^{o}C for 7 days and to 37±1^{o}C (8hrs per day) for 10 days; *significantly higher than the control; K: Kuchi, C: Ching'wekwe, M: Morogoro medium; Cont: control, H: heat.

(b) Corticosterone

Changes in serum corticosterone concentration of hens in Study 2 are shown in Fig.5.8. Exposure of chickens fed low energy diet to 32±1^{o}C for 7 days induced a marked rise ($p<0.05$) in serum corticosterone for KU and CH but not MM. Between-ecotypes, the serum levels of corticosterone for KU were significantly higher ($p<0.05$) than CH and MM. Within 24hrs of raising the temperature to 37±1^{o}C, KU and CH but not MM showed a marked increase ($p<0.05$) in corticosterone levels. A 10 day-exposure of the chickens to 37±1^{o}C induceda marked increase in serum corticosterone levels in all the chicken ecotypes. At this stage between-ecotype differences in serum corticosterone levels were absent.

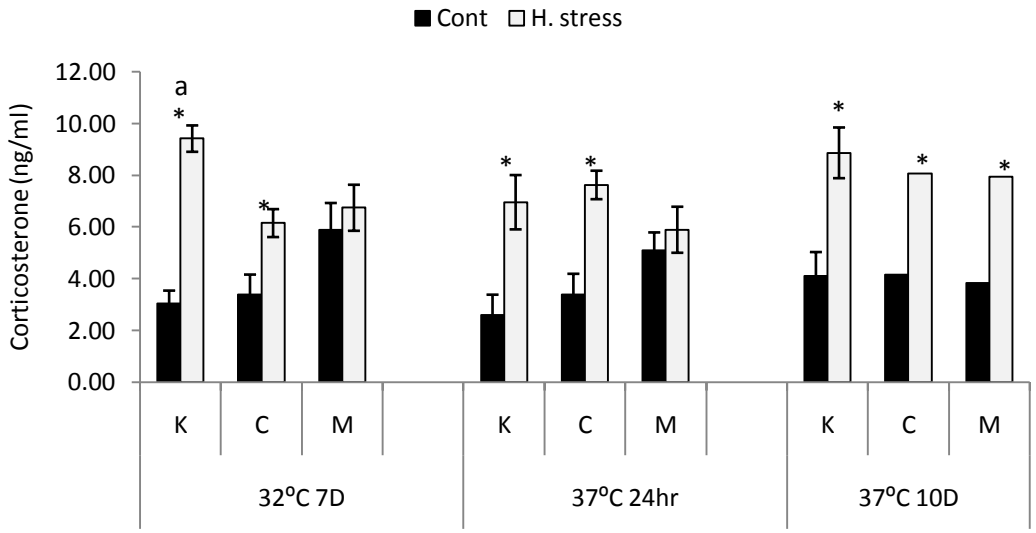

Figure 5.8: Serum corticosterone concentration of hens at control (26.5±1°C and control diet) conditions and of hens fed 55% less dietary energy than the control, after exposure to 32±1°C for 7 days and 37±1°C for 24hrs and 10 days. *significantly higher than the control; [a]significantly higher than C and M; K: Kuchi, C: Ching'wekwe, M: Morogoro medium; D: day, H: heat, Cont: control.

(c) Uric acid

The results for serum uric acid concentration of the hens in Study 2 are shown in Fig.5.9. Exposure of chickens fed lowenergy dietto 32±1°C for 7 days caused a significant increase ($p<0.05$) in uric acid levels for KU but not for CH and MM. After 24hrs of raising the temperature to 37±1°C, uric acid levels were not markedly changed in all the chicken ecotypes. Exposing of the chickens to 37±1°C for 10 days led to an increase ($p<0.05$) in serum uric acid levels for KU and MM but not for CH.

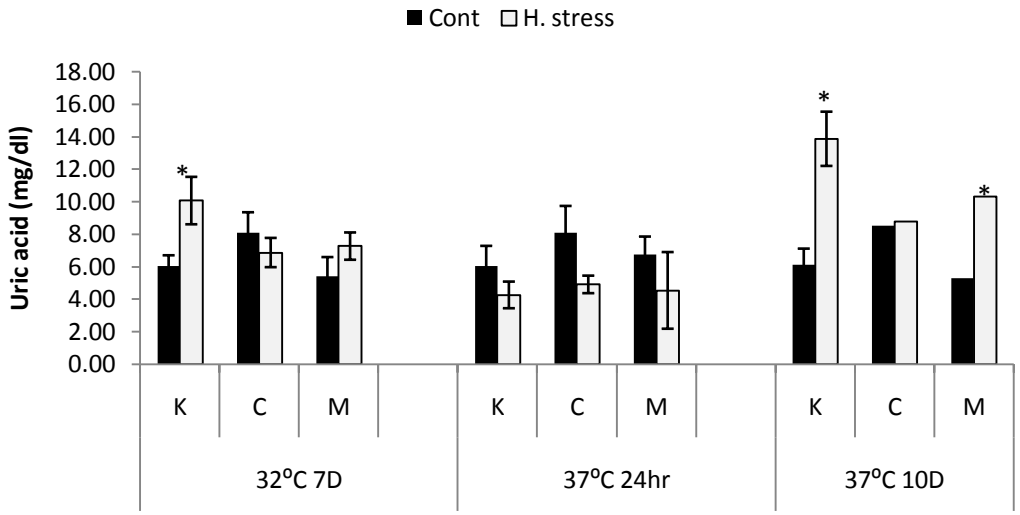

Figure 5.9: Serum uric acid concentration of hens at control (26.5±0.5°C and control diet) conditions and of hens fed 55% less dietary energy than control, after exposure to 32±1°C for 7 days and 37±1°C for 24hrs and 10 days. *significantly higher than the control; K: Kuchi, C: Ching'wekwe, M: Morogoro medium; D: day, H: heat, Cont: control.

(d) Total protein, glucose and triglycerides

Serum total protein, glucose and triglyceride concentrations for Study 2 are depicted in Table 5.5.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 CH but not for KU and MM, whilst glucose levels were not changed in all ecotypes. Between ecotypes, the serum glucose levels for KU were notably higher ($p<0.05$) than for CH and MM after exposure. The triglyceride concentration levels were markedly decreased ($p<0.05$) for KU and MM but were not affected for CH.While glucose levels were not significantly altered for CH and MM, there were marked reductions ($p<0.05$) in serum glucose levels for KU and in total protein levels for all the ecotypes after 24hr-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 MM having the highest drop. Total protein levels were significantly reduced ($p<0.05$) for CH and MM but not for KU. While a significant rise in

serum glucose levels for KU was noted, no notable differences were observed at this stage for CH and MM.

Table 5.5: Serum total protein (T.P), glucose (Glu) and triglyceride (TG) concentrations of hens at control (26.5±1^{o}C and control diet) conditions and of hens fed 55% less dietary energy than control, after exposure to 32±1^{o}C for 7 days and 37±1^{o}C for 24hrs and 10 days. Values are presented as Mean±SE.

		7days		24hrs		10days
	Cont	**32^{o}C**	**Cont**	**37^{o}C**	**Cont**	**37^{o}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.

(e) Behaviour

Results for behavioural frequencies for hens fed low dietary energy and exposed to 37±1^{o}C for 7 days are summarised in Fig.5.10.Whilst panting and wing droop behaviours were significantly increased ($p<0.05$), there was a marked reduction ($p<0.05$) in preening and locomotion behavioural frequencies for all chicken ecotypes. Between ecotypes, KU

showed a significantly higher ($p<0.05$) frequency of panting than CH and MM, whilst wing droop frequencies were markedly lower in CH than in KU and MM. Both CH and MM chicken ecotypes showed notable reductions and increases ($p<0.05$) in resting and drinking behaviours, respectively, but both behaviour types were unchanged by stress for KU.Feeding behaviour markedly decreased for KU but not for CH and MM.

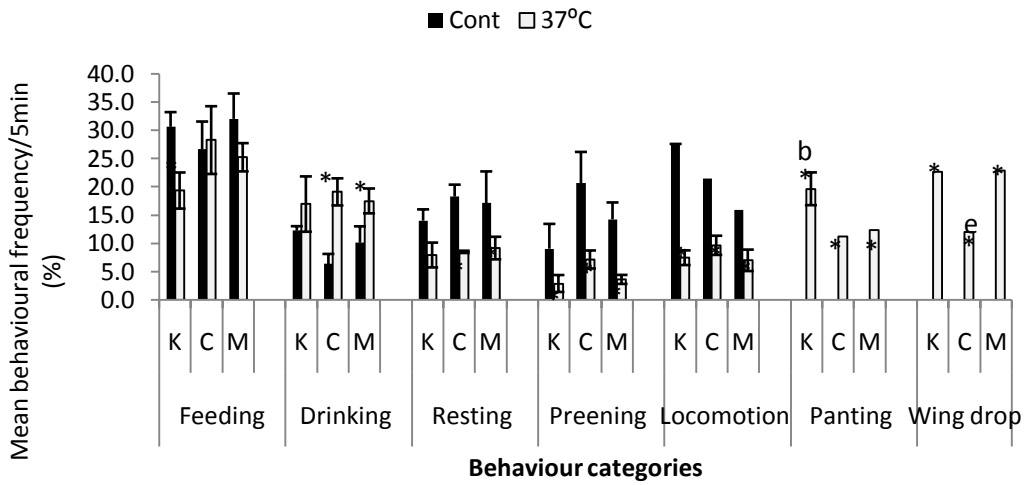

Figure 5.10: Behavioural frequencies of hens at control (26.5±1°C and control diet) conditions and of hens fed 55% less dietary energy than control and exposed to 37±1°C for 7 days. *significantly different from the control; [b]significantly higher than C and M; [e]significantly lower than K and M; K: Kuchi, C: Ching'wekwe, M: Morogoro medium; Cont: control.

5.3 Discussion

Heat stress is one of the most important environmental stressors challenging chicken production in hot climatic regions. Selection for ecotypes with increased heat resistance and tolerance is an opportunity for commercialisation and increased production of local chickens.The current studies aimed at determining the ecotype-based differences in physiological, biochemical and behavioral responses to heat stress and low dietary energy in Tanzanian local chickens.Low dietary energy was included as a stressor in order to

mimic the natural conditions whereby these chickens are faced with a seasonal combination of stressors in areas where they are bred. Identification of traits that make them cope and have ecotype-specific differences in response to heat stress and low dietary energy can provide a basis for selection of local chickens that perform better under such stressing conditions.

In the current studies, the observation thatbothheat stress and a combination of heat stress with low dietary energy markedly increased serum corticosterone concentration levels for the CH and KU ecotype after exposure to 32±1^{o}C for 7 days and 24hrs after temperature was raised to 37±1^{o}Cmaybe an indication that CH and KU had a stronger response and were the most affected by both stressors at this stage.Increases in corticosterone levels in the blood are linked to the HPA axis that controls animal adaptability in response to various stressors (Zulkifli and Siegel, 1995). On the other hand, both heat stress and its combination with low dietary energy did not significantly affect corticosterone levels for MM after exposure to 32±1^{o}C for 7 days and also 24hrs after the temperature was raised to 37±1^{o}C. It is probably an indication that by this time recovery and adaptation to stressors had already ensued and therefore corticosterone levels had since been down-regulated to baseline levels. Based on these observations, it is likely that MM showed greater adaptability to heat stress and its combination with low dietary energy than KU and CH but similar to CH when only heat stress was applied. The ecotype differences could be the result of different genetically mediated stress responses of the adrenal system (Cheng and Jefferson, 2008). These findings are consistent with earlier studies (Chapters two and three) that demonstrated that MM was the most tolerant ecotype to stress induced by low dietary energy under cyclic ambient temperatures of between 21.6 and 34.3^{o}C.In response to stress, CRF is released into hypophysial portal vessels, binds to the CRF type 1 receptor on pituitary corticotropes,thereby activating cyclic adenosine monophosphate (cAMP)

pathway events that induce the release of ACTH into the systemic circulation (Smith and Vale, 2006). In the presence of CRF, vasopressin elicits synergistic effects on ACTH release that are mediated through the vasopressin V1b receptor, and circulating ACTH binds to the melanocortin type 2 receptor in the adrenal cortex where it stimulates corticosterone synthesis and secretion into the systemic circulation (Smith and Vale, 2006).

Heat stress did not significantly alter serum corticosterone and the relative gene expression levels of both liver hsp70 and iNOS in all ecotypes after exposure of chickens fed control energy to 37±1^{o}C for 10 days. This is an indication that chickens from all ecotypes are able to adapt and recover from heat stress when exposed to high temperature for a longer period time. It is very likely that adaptation 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 KU was markedly higher ($p<0.05$) than levels in CH and MM highlights the suggestion that adaptation to heat stress was taking place at different rates and that tolerance levels may not be the same in these chickens. Short-term sub-lethal heat stress provokes 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 *et al.*, 1992; Xie *et al.*, 2014). The heat stress applied in the current studies was for a longer period of time, which appeared to have allowed some adaptation activity to take place by the end of 17 days of study. CH and MM appear to have coped and tolerated heat stress more efficiently than KU as shown by the clearly unchanged hsp70 levels. In broilers and chicken layers, previous studies have shown that acute heat challenge increased hsp70 expression levels (Yu and Bao, 2008; Felver-Gant *et al.*, 2012; Lowman *et al.*, 2014) in the liver but not the chronic heat stress treatment (Xie *et al.*, 2014).

A suggested mechanism for increased hsp70 expression within a cell involves an interaction of HSFs and HSPs through MAPK/SAPK signaling cascades activating HSFs (Morimoto, 1993; Juhasz *et al*., 2014). HSF3, a unique avian HSF, has been shown to function as a heat responsive transcription factor, though roles of distinct HSFs have been proposed to overlap depending on stimulatory signals (Pirkkala *et al*., 2001). The HSFs in the cytosol are bound by HSPs and are maintained in an inactive state (Kregel, 2002). Heat stress and/or energy depletion would activate HSFs, causing them to separate from HSPs, and the HSFs are then phosphorylated by protein kinases to form trimers in the cytosol and these HSF-trimer complexes enter the nucleus and bind to heat shock elements in the promoter region of the hsp70 gene; the hsp70 mRNA is then transcribed and leaves the nucleus for the cytosol, where new hsp70 is synthesized (Kregel, 2002).

In the current studies, the finding that there was no change in the relative gene expression levels of iNOS in all ecotypes may also remotely entail that heat stress of this level apparently did not induce inflammation, which is an important indicator of animal tissue damage under stress conditions. However, the significant increase in a similar pattern of iNOS relative gene expression 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. 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 among the ecotypes entailing that the stress-induced tissue damage caused could be of similar degree in the chickens. In White Leghorn laying hens, feed restriction has also been shown to cause increased liver iNOS gene expression (Kang *et al*., 2011). iNOS is involved in protecting the liver against hepatic apoptotic cell death during tissue damage by promoting the catalysis of nitric oxide, a molecule with anti-inflammatory activities (Clemens, 1999;

Surh *et al*., 2001; Zhao *et al*., 2013). However, iNOS governed nitric acid production can be either beneficial or detrimental depending on the type of stress stimulus, abundance of ROS and the cellular redox status of the liver (Chen *et al*., 2003). 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).

The current studies have further shown that feeding the chickens 55% less energy than the control compounded the effects of heat stress by the end of the study. Heat stress and low dietary energy synergistically raised serum corticosterone and caused significant up-regulations ($p<0.05$) of liver iNOS and hsp70 relative gene expression in all the ecotypes, with hsp70 levels in KU markedly higher ($p<0.05$) than in MM. It appears 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 KU and MM in the expression levels of hsp70 in the liver. The lower levels of hsp70 expression for MM than for KU may entail a better recovery by this time as the lower the stress stimulation the lower the induction of hsp70. It therefore may mean that the apparent differences in hsp70 expression are also linked to the adaptative genetic variations in the hypothalamic-pituitary-adrenal axis and cellular activation. The hsp70 is highly inducible and plays a protective role under stressful conditions, and in addition to hyperthermia, a number of stimuli are known to induce its transcription, including energy depletion (Kregel, 2002). In broiler chickens, feed restriction has been shown to induce increased hsp70 gene expression levels in the liver (Delezie*et al*., 2007; Al-Aqil and Zulkifli, 2009).

The trends in the current studies appear to show that the chickens' responses to combined heat stress and low dietary energy, with respect to serum corticosterone levels, are different for acute and lower temperatures but tended to respond similarly at higher temperatures and longer exposures. In broiler chickens, heat stress has been shown to increase serum corticosterone levels (Quinteiro-Filho *et al*., 2010; Soleimani *et al*., 2011). In addition, chronic exposure for 9 days and 8 weeks to higher temperatures has been shown not to affect plasma corticosterone concentrations (Mack *et al*., 2013; Xie *et al*., 2014). 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 (Wingfield *et al*., 1997; Kitaysky *et al*., 1999) but chronic elevation of corticosterone is known to suppress immune systems, promote wasting of muscle tissue, and cause neuronal cell death (Kitaysky *et al*., 1999).Thus, plasma corticosterone may inhibit further HPA axis activation through intracellular receptors that are widely distributed throughout the brain and peripheral tissues (Smith and Vale, 2006).

In the current studies, differential alterations in serum metabolites were evident among the chicken ecotypes and between heat treatments. The chickens showed ecotype-differences in their responses to heat stress and a combination of heat stress with low dietary energy, with respect to serum uric acid levels, after exposure to 32±1^{o}C for 7 days and to 37±1^{o}C for 10 days. Uric acid is the metabolic product of purine metabolism and is a potent plasma antioxidant in birds as it acts as a scavenger of singlet oxygen, peroxy and hydroxyl radicals, whose imbalance within a biological system can result in oxidative damage and inflammation (Settle and Klandorf, 2014). The reduction of serum uric acid in CH could be an indication that the chickens had not yet recovered and were sliding into

increased inflammation and oxidative stress (Settle and Klandorf, 2014). Conversely, the marked increase in KU 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 MM were only affected by a combination of heat stress and low dietary energy, demonstrating better tolerance better tolerance when exposed only to high temperature.However, exposure to 37±1^{o}C for 24hrs showed similar response of marked reductions ($p<0.05$) in serum uric acid levels of all ecotypes but levels were not significantly affected when the chickens fed low energy diets were subjected to similar conditions. The differences in responses between the two treatment groups may highlight the differences in metabolic rates and states at this stage, signifying protein catabolism for energy generation in energy restricted birds resulting from increased corticosterone levels (Virden *et al*., 2007). Previous research in poultry has portrayed contradictory observations, showing increases (Ozbey *et al*., 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 studies.

The observed reductions in serum total protein levels in all chicken ecotypes by a combination of heat stress and low dietary energy after increasing the temperature to 37±1^{o}C for 24hrs, 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). In the current studies it appears the energy restricted birds probablyrelied more on protein catabolism for their energy needs as evidenced by marked decline in serum total protein levels for CH and MM after exposure to 37±1^{o}C for 10 days. The significant rise in KU may highlight the apparent differences among these chicken ecotypes in metabolic adjustments under stressful conditions. Previous research in

commercial exotic poultry is consistent with the reductions (Ozbey*et al*., 2004) observed in the current studies after heat challenge.

The results of the current study have shown that a combination of heat stress and low dietary energy significantly reduced serum triglyceride levels for KU and MM. Higher environmental temperatures cause severe changes in plasma metabolites thereby indicating alterations in carbohydrate and lipid metabolism (Xie *et al*., 2014). In the current study, it appears that 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. Meanwhile only KU had serum glucose levels markedly reduced by a combination of heat stress and low dietary energy after increasing temperature to 37±1^{o}C for 24hrs, which is an indication of higher metabolic activities in this ecotype when compared to other ecotypes under study. For chickens fed normal control diet, glucose levels were not affected 24hrs after raising the temperature to 37±1^{o}C but had significantly reduced glucose and triglycerides levels for KU and not CH and MM after exposure to 37±1^{o}C for 10 days. This shows that alteration of serum metabolites was closely related to the intensity of heat challenge (Xie *et al*., 2014) and that heat stress alone could not alter the metabolism of CH and MM at that stage. Conversely, exposure of chickens fed low energy diet to 37±1^{o}C for 10 days significantly increased glucose levels for KU but markedly reduced triglyceride levels for all ecotypes, with MM recording the highest drop. 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 and Jefferson, 2008). The elevated glucose levels, such as those observed in KU under heat stress and low dietary energy, might be an adaptation for the survivability and tolerance just as Bogin *et al*. (1996) showed in their

study that chickens that survived 40^{o}C heat shock had high blood glucose levels than the non-surviving.

Under high temperature conditions birds alter their behaviour and physiological homeostasis seeking thermoregulation, thereby decreasing body temperature (Lara and Rostagno, 2013). In the current study, panting and wing droop, as expected, were significantly increased ($p<0.05$) after exposure to 37±1^{o}C for 10 days both for the chickens fed control diet and those fed lowenergy diet. Ecotypes differences were only observed for chickens fed low dietary energy whereby KU showed a greater ($p<0.05$) frequency of panting than CH and MM, whilst wing droop frequencies were markedly lower in CH than in KU and MM. Panting increases water loss through evaporative cooling but also increases the risk of respiratory alkalosis (Mack *et al*., 2013). Wing droop exposes the lightly feathered apteria under the wings and helps reduce body temperature because the skin of apteria contains near-surface blood vessels that promote heat transfer to the environment (Mack *et al.*, 2013). The observed differences appear to be genetically enshrined though body size might have played a role as KU hens were slightly bigger than CH but almost at par with MM.

Heat stress induced marked reductions in resting and locomotion for KU and MM and in feeding behaviour for all ecotypes but no significant reduction was observed for drinking and preening behaviours. The reductions in locomotion and resting may be linked to exhaustion and restlessness, respectively caused by heat stress. Reduced walking is also a behavioural adaptation to heat stress to decrease body temperature (Mack *et al.*, 2013). Moreover, a negative correlation between serum corticosterone and resting behaviour, and positive correlations of corticosterone with panting and wingdroop were observed in KU and CH, making it likely that this hormone had a role in the induction of restlessness in

these chickens. Dysregulation of corticosterone concentrations has been previously associated with induction of varied changes in such behaviours under heat stress (Cheng and Jefferson, 2008). There were no notable ecotype differences in all behaviour-types except preening as a comfort behavioural indicator, which was lower ($p<0.05$) in KU than in CH. On the other hand, heat stress and its combination with low dietary energy caused a marked reduction in preening and locomotion behavioural frequencies for all chicken ecotypes. Previous research in broilers and layers has also shown decreases in preening, locomotion, feeding, and increases in drinking behaviors (Mack *et al*., 2013; Li *et al*., 2015) in consistent with some aspects of the current studies, with differences linked to genetic variability with local chickens. Reduced feeding behaviour is applied as a coping strategy to achieve thermoregulation and metabolic alterations thereby decreasing body temperature (Lara and Rostagno, 2013). Increased drinking, though waned likely because of adaptation for all chickens fed control diet and KU fed low dietary energy in the current study, ensures that birds are rehydrated in order to maintain osmolality of the extracellular fluid (Cheng and Jefferson, 2008).

5.4 Conclusion

The results of the current study have demonstrated that there were ecotype differences and similarities in local chicken ecotypes' responses to heat stress and a combination of heat stress and low dietary energy with respect toblood indices, behavioural responses, liver hsp70 and iNOS gene expressions.MM had greater tolerance to heat stress and its combination with low dietary energy than KU and CH but similar to CH when only heat stress was applied. The chickens' responses to heat stress and low dietary energy, with respect to serum corticosterone levels, were different for acute and lower temperatures but tended to respond similarly as the stress intensity was 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. Preening was one of the key behavoural traits that showed ecotype-differences under stressful conditions. This study therefore has provided possible avenues for future research to devise programs that include physiological, biochemical and behavioral traits that would enhance selection for heat and low dietary energy tolerance among the local chicken stocks.

5.5 Acknowledgements

The authors acknowledge financial support from Intra-ACP Mobility Scholarship Program through the Education, Audiovisual and Culture Agency of the EU. Chicken flocks utilized in this study were generated through the support by the US Agency for International Development on Feed the Future Innovation Lab for Genomics to Improve Poultry.

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CHAPTER SIX

7.0CONCLUSIONS AND/OR RECOMMENDATIONS

7.1 Conclusions

Feed containing lower energy levels induced stress in all the chicken ecotypes studied. Ecotype-specific effects and tolerance of this stress were manifested mainly through differences in theliver HSP70 and iNOS relative gene expression levels, serum corticosterone concentrations, growth rates, mean percent weight gains, feed utilization efficiencies, mortality indicators and behavioural responses.These parameters as identified are useful biomarkers for future selection for improved resilience to heat stress, suboptimal nutrition and even other stressors. MM and to a lesser extent CH ecotypes,were shown to be better tolerant at the lowest energy levels used in this study, whilst KU appeared to be the least tolerant(Chapterstwo and three). The MM ecotype's better performance at very low energy levels could be an evolutionary adaptation to how these chickens have been bred in localities they originate from. These findings imply that MM can better withstand periods and seasons of the year when feed supply is limiting or scarce. It can therefore be said that MM can readily be promoted and reared in many parts or regions of Tanzania including those that are without a stable all-year round supply of scavengeable feed stuff. On the other hand the KU ecotype could perform better when dietary energy levels in the feed are optimum and therefore it's production is of greater value to farmers in areas enjoying an all-year round availability of scavengeable feed stuff or for farmers that provide feed supplements to their chickens.

The liver HSP70 but not iNOS gene expressions (Chapter five) have shown ecotype differences under both heat stress and a combination of heat stress and low dietary energy, which shows that only stress response leading to adaptation and tolerance, and not degree

of tissue damage is ecotype-dependant. MM had greater resistance and tolerance to a synergistic effect of heat stress and low dietary energy but similar to CH when only heat stress was applied,with respect to liver HSP70 relative gene expression and serum corticosterone concentration (Chapters five).These findings imply that the MM ecotype can thrive not only in areas or regions with good climatic conditions but also in those regions with seasonal high ambient temperatures and seasonal scarcity of scavengeable feed stuffs or in both. The indications are that MM is posed for wide distribution across Tanzaniaas it seems to have adapted better to a wide range of environments over the years.The similar resistance and tolerance indications between MM and CH when only heat stress was applied show that CH also has a good potential to thrive in regions or areas with higher ambient temperatures but with all-round optimal nutrition.While MM ecotypedemonstrated better percent weight gainat moderately high a temperature, KU and CH had better percent gainsata higher temperature (Chapter four). This contradiction with results in Chapters five and six could be as a result of tradeoffs between accelerated growth and physiological adaptation to stress conditions.

The findings presented in this dissertation are the first on Tanzanian local chickens andprovide important additions to the current body of scientific knowledge. The study has further provided possible avenues for future research to devise programs that include physiological, biochemical and behavioral traits that would enhance selection for heat and low dietary energy tolerance among the local chicken stocks.

7.2 Research Limitations

This study had its own limitations just like many other research works that are of experimental nature and therefore the findings should be taken with caution. The experiments that constituted this study required the chickens to be confined, which is not

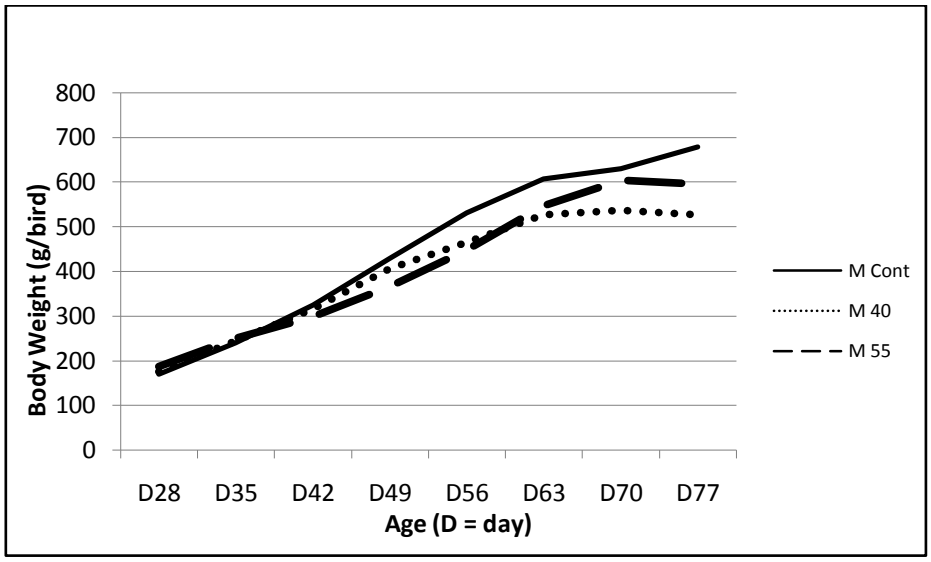
exactly the case in their natural environments. However, confinement could not be avoided as it was the only reliable way controlled temperatures could be assigned. This study was done using female chickens in the early growth phase period between four and eleven weeks old and findings may strictly apply to this category of birds. Only female chickens were used to minimize sex-induced response differences that could have complicated interpretation of the findings. In the places where they are reared, the chickens are generally exposed to environmental stressors seasonally over longer periods of time than the time they were exposed in this study and as a result progression of adaptation and tolerance could not be exactly depicted.

7.3 Recommendations

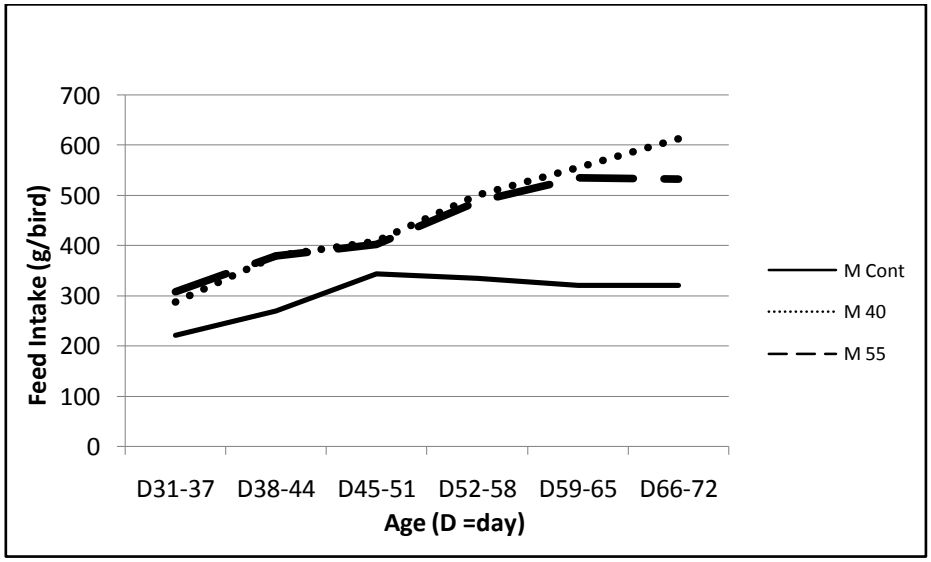
There is need to promote the MM ecotype across the country as it has shown ability to cope better under harsh environmental manipulation. Since Tanzania is endowed with a diversity of local chicken ecotypes, it is recommended to subject other ecotypes to similar studies to ascertain their potential in coping with specific environmental stressors including high ambient temperature. Future studies should also consider including chickens at productive ages so that the impact and degree of tolerance at that critical age can be compared. This study has provided starting points for future research to devise programs that include physiological, biochemical and behavioral traits that would enhance selection for heat and low dietary energy tolerance among the local chicken stocks.By partly using the research information on the adaptation and tolerance levels of these chickens generated through this study, genotyping technologies should be used to enhance selection for better adaptation to high ambient temperatures among the indigenous chicken stocks.

APPENDICES

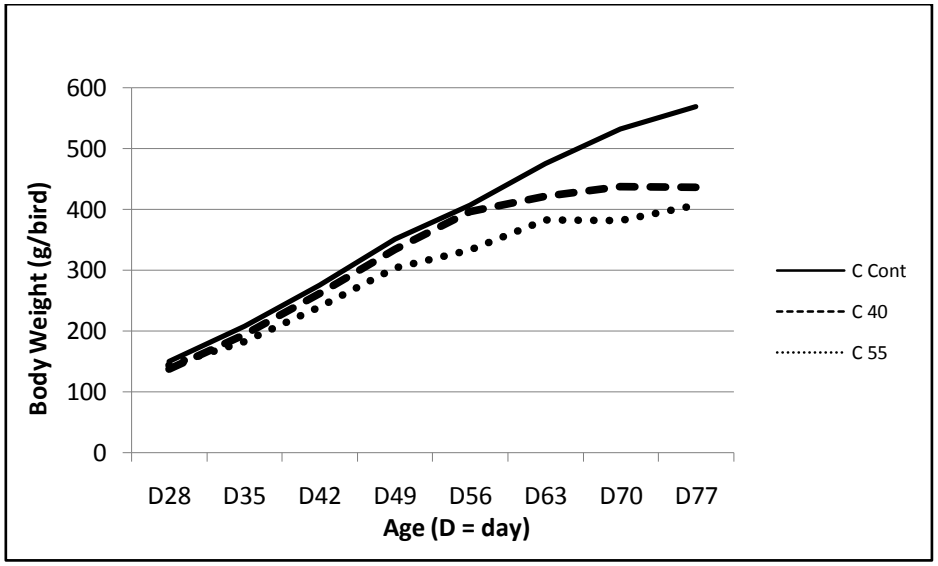
Appendix 1: Growth curves of MM chickens during 7 weeks of energy restriction (Chapters two and three).



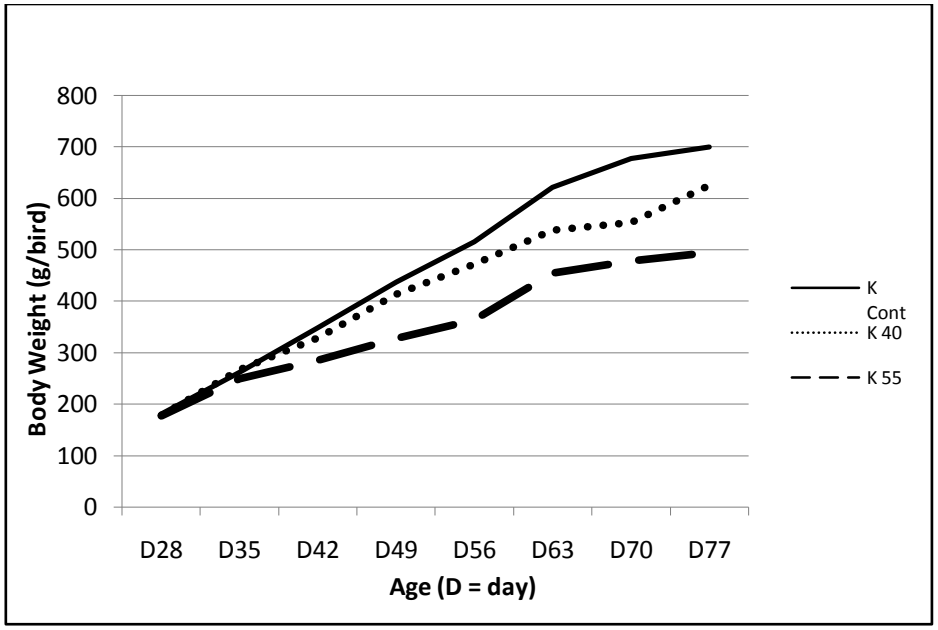
Appendix 2: Weekly feed intake for MM chickens duringenergy restriction (Chapters two and three).



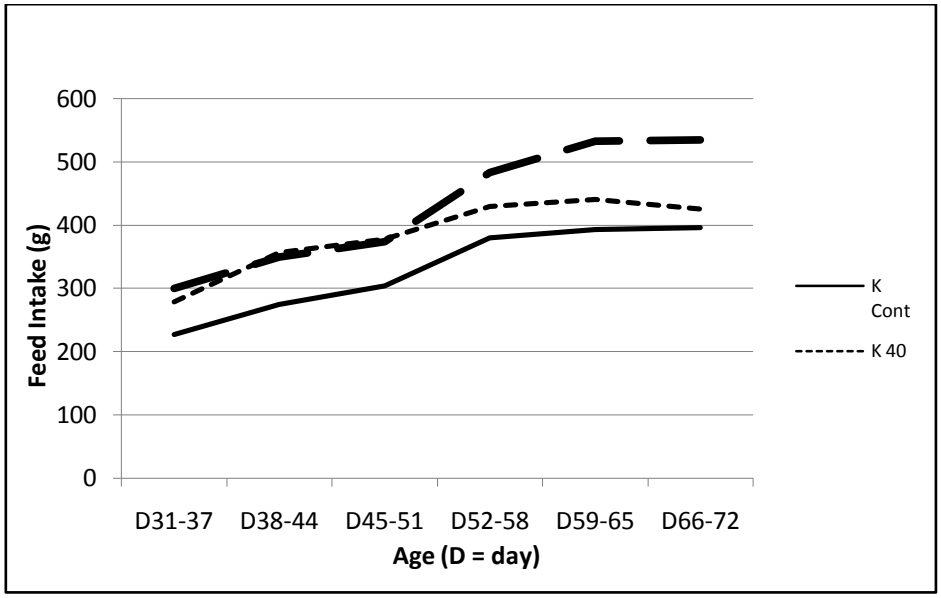
Appendix 3: **Growth Curves (during 7 wks Feed Restriction) for CH chickens duringenergy restriction (Chapters two and three).**



Appendix 4: **Growth Curves (during 7 weeks feed restriction)forKU chickens duringenergy restriction (Chapters two and three).**



Appendix 5: **Weekly feed intake for KU chickens during energy restriction (Chapters two and three).**



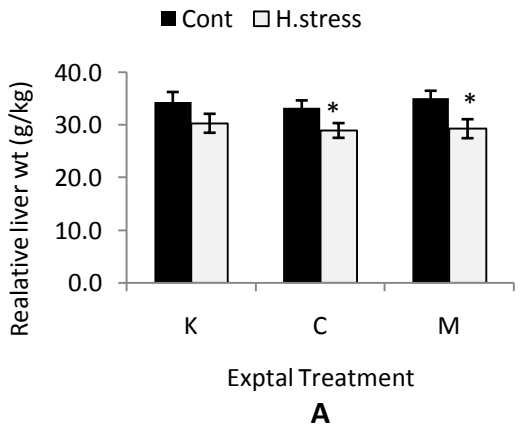
Appendix 6:Eleven weeks old chickens, a: KU, b: MM, c: CH

Appendix 7: Mortality summary (%) during seven weeks of dietary energy restriction

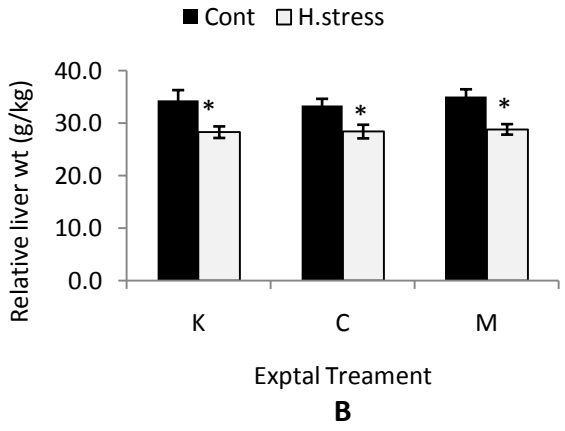
Ecotype	Control	40% Restriction	55% Restriction
K	7.7	45.1	25.7
C	11.7	39.1	46.4
M	5	15	5

K: kuchi; C: ching'wekwe; M: Morogoro medium.

Appendix 8: Relative liver weights results for chapter 5 (A) Study one (B) Study two; recorded in g/kg of chicken weight; *significantly lower than the control



A



B