

**COMPARATIVE EVALUATION OF  $^1\text{H}$  NMR SPECTROSCOPY AS A NOVEL TOOL  
FOR ASSESSING STRESS WITH TRADITIONAL METHODS OF BLOOD  
CORTICOSTERONE LEVELS AND HETEROPHIL LYMPHOCYTE RATIO IN  
BROILER CHICKENS (*GALLUS DOMESTICUS*)**

A Thesis Submitted to the College of  
Graduate Studies and Research  
In Partial Fulfillment of the Requirements  
For the Degree of Masters  
In the Department of Veterinary Biomedical Sciences  
University of Saskatchewan  
Saskatoon

By

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## ABSTRACT

Stress can be defined as any event that alters physiological homeostasis. Any event that elicits a response (either behavioral or physiological) can be considered a “stressor”. Proper response to a stressor can be beneficial in life threatening situations; however, chronic or repeated exposure can have marked deleterious effects on reproduction, behavior, metabolism and immunocompetence. Identification of stress in poultry research has been done using various physiological endpoints with the two most accepted indicators of stress being elevated corticosterone (CORT) and increased circulating heterophil/lymphocyte (H/L) ratios. The lack of consistency between studies measuring CORT and H/L ratios results in some skepticism when using them to quantify stress and animal welfare in the poultry industry. <sup>1</sup>H Nuclear Magnetic Resonance spectroscopy (NMR) may provide insightful mechanistic information on effects of stressors by simultaneously measuring a large number of low molecular weight endogenous metabolites that may potentially be used as biomarkers for stress in chickens (*Gallus domesticus*). The objectives of this thesis are to identify metabolites associated with chronic stress in broilers fed on a corticosterone containing diet using NMR spectroscopy, evaluate and compare the use of NMR in the identification of stress metabolites to more traditional methods of stress (CORT and H/L ratio), compare the use of CORT, H/L ratio, and metabolic profiles in broilers reared under 4 different lighting photoperiods to evaluate stress and welfare, and compare the use of CORT, H/L ratio and metabolic profiles in broilers reared under 4 different light intensities to evaluate stress and welfare. Chronically stressed broilers fed a CORT (30 mg/kg feed) diet for four weeks had a significant increase in both H/L ratio ( $P \leq 0.002$ ) and serum CORT levels ( $P \leq 0.001$ ) at all time points of collection (days 14, 21, 28 and 35) in comparison to the control birds. Both H/L ratio and serum CORT levels failed to identify or differentiate any stressed groups in both photoperiod (14L:10D, 17L:7D, 20L:4D and 23L:1D) and lighting intensity (1, 10, 20 and 40 lux) experiments. Behavioral, welfare and production data reported by the sister studies however showed the 23L:1D group (photoperiod) and 1Lux (light intensity) to have compromised welfare. Both H/L ratio and serum CORT levels were highly suggestive of age related changes in the lighting experiments. H/L ratio showed significant difference between the days of sampling in both photoperiod ( $P \leq 0.001$ ) and light intensity ( $P \leq 0.001$ ) experiments, while serum CORT showed a significant difference between days of sampling in the light

intensity experiment ( $P \leq 0.001$ ). Metabolomics using Proton Nuclear Magnetic Resonance (NMR) clearly differentiated the chronically stressed birds fed on a CORT diet (30 mg/kg diet) from the control birds. It helped in identifying metabolites like isoleucine, lysine, valine and tryptophan that are highly indicative of stress induced anxiety, depression and behavioral changes. Metabolites like methionine, betaine, histidine suggested compromised methylation process and oxidative stress response. Metabolites threonine, glutamine and histidine suggested reduced immune response while metabolites like carnitine, lactate, glucose and  $\beta$  hydroxybutyrate suggested the undergoing process of energy mobilization. Metabolomic analysis of the serum samples of the photoperiod groups showed the presence of amino acids valine, threonine, lysine, proline, histidine, methionine and other metabolites like creatine, carnitine, choline, glycerol, betaine and lactate. Out of the top common metabolites identified between this study and that of the chronically stressed birds fed CORT (30 mg/kg diet), 5 metabolites (valine, lysine, methionine, histidine and glucose) showed similar relative abundance and trends in lighting groups 20L:4D and 23L:1D. Metabolomic analysis of the lighting intensity groups showed the presence of amino acids isoleucine, threonine, histidine, methionine, tyrosine, serine and other metabolites including carnitine, choline, betaine, lactate, glucose and fructose. Out of the top common metabolites identified between this study and that of the chronically stressed birds fed CORT (30 mg/kg diet), 5 metabolites (isoleucine, histidine, carnitine, glucose and betaine) in lighting groups 1 lux and 40 lux showed similar relative abundance. Findings of this thesis suggest that metabolomics was able to successfully differentiate the chronically stressed birds from the control birds with valuable insights about the physiological process involved in the stress response. In the lighting experiments metabolomics was able to identify metabolites suggestive of the behavioral changes, energy homeostasis, immune response, oxidative stress and osmoregulation. Metabolomics supported evidence of compromised welfare in photoperiod (23L:1D) and lighting intensity (1 lux) while H/L ratio and serum CORT levels failed to do so.

## **ACKNOWLEDGMENTS**

My sincere thanks and gratitude to my supervisor Dr. Karen Machin for her guidance and unflinching support during my M.Sc research program. My thanks to helpful people like Dr. Susantha Gomis, Dr. Bob Brua, Dr. Alan Chicoine and Mrs. Susan Cook of the Endocrinology lab who have guided and aided me in gaining more knowledge. I would also like to thank my committee member Dr. Hank Classen and his research team for being collaborators in two of my experiments. Special thanks to the graduate chairs Dr. Linda Heibert, Dr. Jaswant Singh and Dr. Gillian Muir. I would also like to thank the faculty and staff of the Department of Veterinary Biomedical Sciences. Special thanks to Dr. Barry Blakley the Department Head and cordial people like Cathy Coghlin and Darlene Hall of the physiology lab. Thanks to all the graduate students who have been part of this wonderful graduate student life of mine at U of S. Special thanks to graduate students June Yang and Shankar Channabasappa for their help during my experiments. Thanks to Drs, Keith and Gabrielle of the Saskatchewan Structural Science Centre, U of S for their guidance and training on Nuclear Magnetic Resonance technology. Special thanks to the project funding organization Saskatchewan Chicken Industry Development Fund (SCIDF) and the Graduate Teaching Fellowship of the Research Deans office, WCVM for financing my studies at U of S. I would also like to thank my friends Vikrama Baskaran, Venu Shunmugavel and Ayyasamy Thevanam for their support in Canada. I would like to thank my parents Mr. Premkumar and Dr. Saradhambal for their support and guidance in every walk of life. I am what I am for their love, prayers and well wishes. I am also grateful to my in laws late Mr. Sankaranarayanan and Mrs. Kanchanamala for their support and guidance. I would also want to thank my sister Rachel Sasikala, brother in law Arvind Ratnagar and niece Grace Elizabeth for their love and support. Last but not least I would like to thank my near and dear ones my wife

Kadhambari and son Karthik for being my hope, inspiration and pillars of support. I would like to dedicate this work of mine to all my family members.

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## LIST OF ABBREVIATIONS

<b>14L:10D</b>	(14L)
<b>17L:7D</b>	(17L)
<b>20L:4D</b>	(20L)
<b>23L:1D</b>	(23L)
<b>AAs</b>	(AMINO ACIDS)
<b>ACTH</b>	(ADRENO CORTICOTROPIN HORMONE)
<b>ANCOVA</b>	(ANALYSIS OF COVARIANCE)
<b>ANNOVA</b>	(ANALYSIS OF VARIANCE)
<b>CBG</b>	(CORTICOSTERONE – BINDING GLOBULIN)
<b>CON</b>	(CONTROL)
<b>CORT</b>	(CORTICOSTERONE)
<b>CRH</b>	(CORTICOTROPIN RELEASING HORMONE)
<b>D</b>	(DARK)
<b>E</b>	(EPINEPHRINE)
<b>GLUT-4</b>	(GLUCOSE TRANSPORTERS – 4)
<b>GR</b>	(GLUCOCORTICOID RECEPTORS)
<b>H/L</b>	(HETEROPHIL / LYMPHOCYTE RATIO)
<b>HPA</b>	(HYPOTHALAMO-PITUITARY ADRENAL AXIS)
<b>IL</b>	(INTERLEUKIN)
<b>L</b>	(LIGHT)
<b>Lx</b>	(LUX INTENSITY)
<b>mGR</b>	(NON GENOMIC MEMBRANE – ASSOCIATED RECEPTOR)
<b>MR</b>	(MINERALOCORTICO RECEPTORS)
<b>NE</b>	(NOREPINEPHRINE)
<b>NK</b>	(NATURAL KILLER CELLS)
<b>NMR</b>	(NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY)
<b>PCA</b>	(PRINCIPLE COMPONENT ANALYSIS)
<b>PLS-DA</b>	(PARTIAL LEAST SQUARE - DISCRIMINANT ANALYSIS)
<b>PR</b>	(PERMUTATION)
<b>PSE</b>	(PALE SOFT EXUDATIVE)
<b>SAM</b>	(SYMPATHO – ADRENAL MEDULLARY AXIS)
<b>SD</b>	(STANDARD DEVIATION)
<b>TCA</b>	(CITRIC ACID CYCLE)
<b>Th</b>	(T HELPER CELLS)
<b>TRT</b>	(TREATMENT)
<b>VIP</b>	(VARIABLE IMPORTANT PROJECTION)

## CHAPTER 1

### GENERAL INTRODUCTION

Stress can be defined as any event that alters physiological homeostasis (Selye, 1951; Siegel, 1980). The “stress response”, is an evolved primary defensive, adaptive mechanism that allows an animal to deal with changes or conditions within its environment and to restore homeostasis. Any situation that elicits a response (either behavioural or physiological) can be considered a “stressor” and any combination of circumstances in the bird’s environment may potentially act as stressors. These include: climatic (i.e. extreme heat, cold or humidity), environmental (i.e. poor ventilation, light regimes, housing), nutritional (i.e. shortages of nutrients), physical (i.e. handling, transport), social (i.e. overcrowding), physiological (i.e. rapid growth), psychological (i.e. fear, noise), and pathological (i.e. disease). The purpose of physiological changes following an exposure to a stressor aids in minimizing detrimental effects of the stressor. Proper response to a stressor can be beneficial in life threatening situations; however, chronic or repeated exposure can have marked deleterious effects on reproduction, behavior, metabolism and immunocompetence (Siegel, 1980). Conditions which induce stress in an animal can reduce welfare and as well as performance (Olanrewaju, 2006; Scheele, 1997).

Response to a stressor can either be specific or non-specific (Siegel, 1980). Non-specific stressor responses are brought about by two main systems namely the sympatho-adrenal medullary axis (SAM) also called the neurogenic system and the hypothalamic pituitary axis (HPA). The initial fight or flight response is mediated by SAM (Cannon, 1929) and is typically defensive in nature lasting a first few minutes. Catecholamines, epinephrine and norepinephrine, are released causing an increase in heart rate, respiration, muscle tone and blood glucose aimed at removing the threat of the stressor and restoring homeostasis (Siegel, 1980). If the stressor persists then the hypothalamic-pituitary-adrenal (HPA) axis is activated and to induce the primary adaptive mechanism against the stressor (Siegel, 1980). Corticotropin-releasing hormone (CRH) from the hypothalamus is secreted directly into the anterior pituitary via the hypophyseal porta circulation resulting in a rapid release of adrenocorticotropin (ACTH) from the pituitary, which stimulates production and release of glucocorticoids from the adrenal

gland(Johnson et al., 1992; Sapolsky et al., 2000; Selye, 1951; Siegel, 1980). In birds, the major adrenal secretion is corticosterone (CORT) (deRoos, 1961; Holmes, 1976). Rapid increase in circulating CORT triggers a complex array of physiological, metabolic, hormonal and behavioral responses aimed at dealing with the stressor and recovering homeostasis by reallocating resources from nonessential functions to the life-saving needs. Long-term exposure to CORT during chronic stress can result in cardiovascular disease (i.e. arteriosclerosis, ascites), hypercholesterolemia, decreased weight, ulcerative gastrointestinal lesions, immune suppression and increased susceptibility to disease (Siegel, 1980; Thaxton & Puvadolpirod, 2000e). Chronic stress has also been found to induce pale soft exudative-like (PSE) lesions in broiler meat (Tankson et al., 2001) consisting of pale, dehydrated areas and with reduced water holding capacity. Feeding schedules, lighting management, vaccine administration, litter management, environmental temperature, personnel etc, may act as stressors in the poultry industry. With chicken meat topping the highest per capita consumption in Canada at 31.34 kg and the farm cash receipts of revenues and payments from poultry and egg being \$3billion CAD by December 2010 (Statistics Canada), identification and reduction of stressors in chickens is very important to the poultry industry both from a welfare and production standpoint.

Identification of stress in the poultry industry has been done using various physiological endpoints with the two most accepted indicators of stress being elevated CORT and increased circulating heterophil/lymphocyte (H/L) ratios (Puvadolpirod & Thaxton, 2000a). Lack of consistency between studies measuring CORT and H/L ratios has resulted in skepticism when using them to quantify animal welfare in the poultry industry (Maxwell, 1993; Rushen, 1991) Environmental metabolomics is an emerging approach for examining metabolic profiles in biological systems exposed to environmental stress (Lin et al., 2006). Serkova (2009), defines metabolomics as “one of the ‘omic’ sciences in systems biology, and is used for the assessment and validation of endogenous small-molecule biochemicals (metabolites) within a biologic system”. Utilization of 1-dimensional <sup>1</sup>H Nuclear Magnetic Resonance spectroscopy (NMR) can provide insightful mechanistic information on effects of stressors by simultaneously measuring a large number of low molecular weight endogenous metabolites (Viant et al.,



203). Metabolic profiles can be examined using pattern recognition algorithms to reveal subsets of metabolites, potentially identifying biomarkers (Lin et al., 2006; Viant et al., 2003). Biofluids like serum, plasma, urine, cerebrospinal fluid, saliva and sweat have been used by researchers in analyzing small molecular weight metabolites. Metabolomics has been successfully used to identify stressed and control groups in cattle (Aich et al., 2007) and Sprague-dawley rats (Teague et al., 2007). The advantage of using NMR over more traditional methods is that it provides information about an abundance of multiple metabolites that give information about the physiological changes occurring within the organism. The objectives of this thesis are to: 1) identify metabolites associated with chronic stress in broilers fed on a corticosterone containing diet using NMR spectroscopy 2); evaluate and compare the use of NMR in the identification of stress metabolites to more traditional methods of stress (CORT and H/L ratio); 3) compare the use of CORT, H/L ratio, and metabolic profiles in broilers reared under four different lighting photoperiods to evaluate stress and welfare; and 4) compare the use of CORT, H/L ratio and metabolic profiles in broilers reared under four different lighting intensities to evaluate stress and welfare.

## CHAPTER 2

### LITERATURE REVIEW

#### **2.1 Stress an Evolved Trait**

Canon (1929) was the first to use the term ‘homeostasis in describing an organism’s response to stress giving a picture of how the sympathetic system is responsible in mounting a stress response. Selye (1936) proposed the ‘General Adaptation Syndrome’ wherein he emphasized the involvement of the hypothalamo-pituitary adrenal (HPA) axis in the non specific stress response following three stages “1) alarm reaction which can also produce tissue catabolism, hypoglycemia and gastrointestinal lesions, 2) stage of resistance, where the alarm reaction disappears or reverses itself, and finally 3) stage of exhaustion, where signs of the alarm reaction reappear and death occurs” (Seyle, 1936). Sapolsky et al. (2000) define the stress response as something that “permits, stimulates or suppresses an ongoing stressor or prepares the body against a subsequent stressor”. The stress response is a combination of behavior, neuronal and endocrinal factors working in tandem ensuring bodily homeostasis (Johnson et al., 1992; Sapolsky et al., 2000)

#### **2.2 Sympathetic Adrenal Medullary Axis (SAM)**

The SAM axis, primarily neurogenic in origin, responds immediately to a stressor. The fight response (Canon, 1929) involves both the hormonal release of epinephrine (E) via the autonomic innervations of the adrenal medulla and a neuronal component involving norepinephrine (NE) secretion from the nerve synapse (Tsigos et al., 2002; Siegel, 1980) in maintaining homeostasis. Typically the SAM axis is the first to be activated in response to an acute stressor or upon perception of a stressor. Catecholamines, NE and E, are released leading to an increase in muscle tonicity, tachycardia, vasoconstriction, gluconeogenesis, lipolysis, glycogenolysis and increased respiratory rate all aimed at overcoming the immediate threat rather than accommodating it (Siegel, 1980), and all aimed at allowing the animal to fight or flee. With persistence of the stressor, excess NE secreted in the brain and adrenal medulla stimulate the release of

corticotropin releasing hormone (CRH) from the hypothalamus leading to the initiation of the HPA axis (Kvetnansky et al., 1995; Pacak et al., 1995) prolonging a stress adaptive response.

### **2.3 The Hypothalamus Pituitary Adrenal Axis (HPA)**

The HPA axis is key in allowing an organism to adapt to a stressor. It involves the release of CRH from the paraventricular nucleus of the hypothalamus which then via the hypophyseal portal system reaches the anterior pituitary stimulating the release of adrenocorticotropin hormone (ACTH). The ACTH via the blood stream stimulates the adrenal gland situated cranial to the kidneys, to secrete glucocorticoids (Holmes, 1976; Siegel, 1980). Unlike mammals, where cortisol is the major hormone released in response to stress, corticosterone (CORT) is the principle glucocorticoid in avian species (deRoos, 1961; Holmes, 1976). Following an increase in CORT plasma concentration, gluconeogenesis, glycogenolysis, lipolysis, increased cardiac output, and decreased insulin resistance are initiated and aimed at providing the organism with necessary energy and to survive the stressor (Puvadolpirod & Thaxton, 2000c; Siegel, 1980). It is to be noted that all these changes initiated following exposure to a stressor, is an adaptation to the stress response mounted by the body and not necessarily removing the stressor itself.

### **2.4 Regulation of Corticosterone Action**

#### **2.4.1 Corticosterone-Binding Globulin (CBG)**

After CORT is released from the adrenals, CORT binding globulin protein (CBG) determines bioavailability to target tissues and metabolic clearance (Breuner & Orchinik, 2002). Approximately 90% of the CORT in plasma exists in bound form while the remaining 10% exists as free CORT (Thompson, 1981), with the latter being the physiologically active form that penetrates cell membranes and is catabolized by the liver (Virden, 2009; Breuner et al., 2002). The availability of CBG for CORT-binding greatly influences the degree of stress response (Breuner & Orchinik, 2002). Malisch et al.

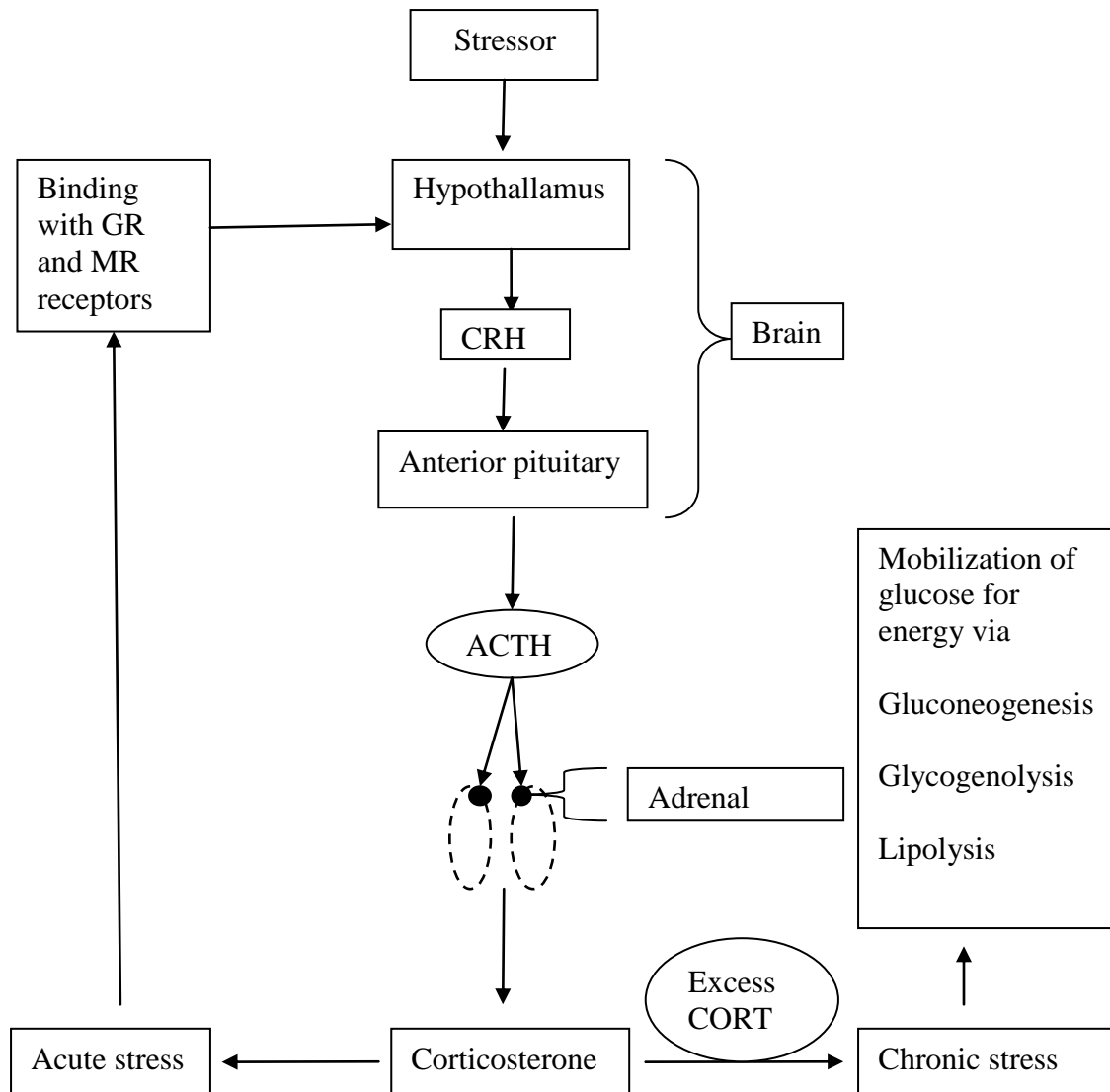
(2009) demonstrated that Japanese quail experiencing acute stress 24 hours prior to sampling had reduced CBG available for binding. This leads to excess biologically active free CORT that brings about deleterious effects on other tissues (Breuner & Orchinik, 2002). Free CORT is also responsible for initiating the negative feedback mechanism involving the HPA axis (Johnson et al., 1992).

#### **2.4.2 Corticosterone receptors**

Unbound CORT binds with a non genomic membrane-associated receptor (mGR) (Landys et al., 2006; Sapolsky et al., 2000), and with two types of intracellular genomic receptors (that initiate gene expression); namely mineralocorticoreceptors (MR) with high affinity, and glucocorticoid receptors (GR) with less affinity. When stressed, CORT binds in sequence to MR, GR and mGR (Landys et al., 2006). The type of receptor binding CORT determines its physiological action. The CORT bound to MR is important in osmoregulation, binding with GR influences feed intake and energy metabolism, while binding with mGR regulates locomotor activity (Landys et al., 2006). The physiological relevance of CORT action largely depends on the bioavailability of CBG, MR, GR and mGR receptors as they change according to prior stressful events, age and seasonal variation (Breuner & Orchinik, 2002; Landys et al., 2006).

#### **2.4.3 Negative Feedback Mechanism**

Excess CORT levels are constantly regulated by a negative feedback mechanism involving the HPA axis (Selye, 1951). With a sustained stress response, excess CORT will inhibit CRH release from the hypothalamus. Reduced CRH reduces ACTH secretion from the anterior pituitary with a subsequent reduction of CORT release from the adrenal cortex (Sapolsky et al., 2000; Selye, 1951; Siegel, 1980).



**Figure 2.1: Diagrammatic representation of the cascade of events initiated by HPA axis following a stressor. CRH = Corticotropin Releasing Hormone ACTH = Adrenocorticotrophic Hormone CORT = Corticosterone**

## 2.5 Role of Corticosterone in Bodily Homeostasis

### 2.5.1 Corticosterone and Metabolism

When exposed to a stressor, the main aim is to provide glucose for bodily activities and restore homeostasis. Hallmarks of a stress response are increased hepatic gluconeogenesis ensuring available glucose and insulin resistance preventing further

storage of glucose (Tsigos, 2002, Dupont et al., 1999b, and Sapolsky et al., 2000). The main function of CORT is to provide the necessary glucogenic substrates and regulate the glucogenic pathways in the liver (Exton, 1979). In addition, CORT provides enhanced action of E in liver gluconeogenesis and glycogenolysis. It also provides permissive action of glucagon in lipolysis of adipose tissue (Exton et al., 1972). Adrenalectomised rats treated with exogenous CORT show increased activity of gluconeogenic enzymes like phosphoenolpyruvate carboxykinase from the liver (Exton et al., 1972; Scanes, 2009).

Survival of an organism depends on appropriate response to a stressor through a short term fight response. Adequate protein (muscle) reserves are key to survival. Chronic stress, can lead to loss of muscle through increased proteolysis to provide energy. Proteins serve as the major non-carbohydrate source of energy during periods of stress ((Siegel & Vankampen, 1984). Muscle breakdown produces amino acids which can then be utilized for gluconeogenesis by the liver. Broilers treated with exogenous ACTH or CORT had increased non-protein nitrogenous compound excretion and reduced muscle mass indicating extensive protein catabolism (H. Lin et al., 2006; Puvadolpirod & Thaxton, 2000d) Lin et al., 2006). Similarly Odedra et al. (1983) showed an increase in protein degradation and decrease in protein synthesis following CORT administration in rats.

Under normal physiological homeostasis, insulin suppresses hormone sensitive lipase essential for lipolysis and opens up GLUT-4 glucose transporters in adipose tissues enabling uptake of glucose and storage as triacylglycerides. In chickens, the major site of lipogenesis is the liver (Leveille et al., 1968). In a stressed scenario, increased CORT accompanied by increased glucagon (an important lipolytic hormone, Goodridge, 1968) results in insulin resistance. Glucagon stimulates the release of hormone sensitive lipase thereby providing abundant plasma free fatty acids. These free fatty acids serve as source of acetyl coA which is an important substrate for energy synthesis via the citric acid (TCA) cycle.

## **2.6 Avian Immune System**

The immune system is designed to prevent entry of foreign or potentially harmful pathogens into the body (e.g. bacteria, viruses etc.) with the main aim of identifying and neutralizing the foreign invader. The principle immune organs in chickens are the spleen, thymus, bone marrow and Bursa of Fabricius (Fleischer, 1981; Glick, 1979; Sharma, 1991). The major immune cells are the lymphocytes, macrophages, dendritic cells, heterophils, and natural killer (NK) cells. With birds lacking lymph nodes and dendritic cells, macrophages become the main antigen presenting cells to the lymphocytes (Kaiser et al., 2009; Sharma, 1991). The B-lymphocytes are responsible for humoral immunity, producing antibodies against antigens. They will go on to become antibody producing plasma cells that act against infection and memory cells will respond to future infections. T-lymphocytes are responsible for cellular immunity and are found in abundance in the thymus and in an equal proportion to B-lymphocytes in the spleen (Glick, 1979; Sharma, 1991). They further divide into subsets of T helper cells (Th) 1 and 2 and are involved in the phagocytosis and lysis of the invading agent (Iwakabe et al., 1998; McEwen, 1997). Following an antigenic immune response, cytokines and chemokines produced by leukocytes regulate the inflammatory process and recruitment of appropriate immune cells towards sites of infection (Kaiser et al., 2009).

### **2.6.1 Stress and the immune response**

Stress via activation of the SAM and HPA axis modulates the immune response. The autonomic nervous system and hormones of the neuroendocrine complex directly modulate the immune system whereas the immune system sends signals to the neuroendocrine complex via cytokines (Besedovsky, 1985; Besedovsky et al., 1977; Dantzer & Kelley, 1989; Khansari et al., 1990). Almost all immune cells have receptors for various hormones like CORT, ACTH, B-endorphins, catecholamines, vasopressin, prolactin, etc. (Besedovsky & Rey, 1996; Sullivan & Wira, 1979). There are also direct innervations of lymphoid organs like the spleen and thymus by the sympathetic branch of the autonomic nervous system (Bellinger et al., 1990; Felten et al., 1987; Glaser & Kiecolt-Glaser, 2005; Khansari et al., 1990), indicating the influence of catecholamines

on lymphoid tissues. Besedovsky et al. (1986) demonstrated that an antigenic immune response could activate the HPA axis. Human monocyte derived interleukin (IL) -1 given to mice and rats stimulated the synthesis of CRH from the hypothalamus leading to an increase in plasma levels of ACTH and CORT. This overall signalling occurred through receptors, innervations and cytokines, and establishes the bi-directionality of the feedback mechanism existing between the immune system and the neuroendocrine complex.

Sustained increases in CORT during chronic stress has an anti- inflammatory and immunosuppressive effect (Johnson et al., 1992). The anti-inflammatory effect is brought about by suppression of proinflammatory cytokines like IL-1, IL-1 $\beta$ , IL-6 and interferons by CORT (Kaiser et al., 2009; Khansari et al., 1990). Stress related immunosuppression comes about through a shift towards humoral immunity (mediated by antibody producing B-lymphocytes) from cellular immunity (mediated by cytotoxic T-lymphocytes, NK cells) with a loss in the phagocytic property and memory cell production thereby increasing susceptibility towards increased risk of infection or disease (Iwakabe et al., 1998). In mice and humans, increased CORT caused a shift in the Th1/Th2 helper cells towards a dominant Th2 presence (Iwakabe et al., 1998; Yang & Glaser, 2002). Th1 helper cells are involved in the synthesis of interferon IFN- $\gamma$ , IL-2 and lymphotoxins essential in cellular immunity, whereas Th2 cells are involved in the synthesis of IL-4, IL-5, IL-6 and IL-10 involved in antibody production. This shift towards a dominant Th2 presence causes a loss in cellular immunity thereby increasing susceptibility of the organism to various immune diseases and infection. Increased CORT also causes suppression of the NK cells essential for lysis of virus infected cells and tumour cells (Iwakabe et al., 1998; McEwen et al., 1997; Yang & Glaser, 2002). Regression of lymphoid organs like spleen, bursa, and thymus has been demonstrated in chickens with increased CORT (Post et al., 2003; Puvadolpirod & Thaxton, 2000a; S. Shini et al., 2008). The amount of T cell receptors, the concentration of hormones (CORT) and the cytokines produced determine the type of immune modulation the stressor will bring about.



### **2.6.2 Stress and the Avian Heterophil**

Heterophils are granulated leukocytes and are the avian equivalent of mammalian neutrophils (Davis et al., 2008; Gross & Siegel, 1983). Structurally, they are circular in shape and have a bi-lobed nucleus with rod shaped cytoplasmic granules (Jain, 1983). They are found in abundance during acute inflammatory response, infection and stress (Harmon, 1998; Maxwell & Robertson, 1998) and are the second highest number of cells in circulation following lymphocytes, forming the first line of cellular defense (Harmon, 1998; Maxwell & Robertson, 1998). Following release of CORT, circulating heterophils rise within the first hour (Davis et al., 2008; Maxwell, 1993). Increased heterophil to lymphocyte (H/L) ratio has been reported in studies done on chickens treated with exogenous CORT (Gross & Siegel, 1983; Post et al., 2003; S. Shini et al., 2008). In addition, increased immature heterophils are seen in both bone marrow and peripheral blood circulation in chickens treated with exogenous CORT (Shaniko Shini et al., 2008). Electron microscope evaluation of these heterophils revealed increased numbers of round or abnormal cytoplasmic granules indicating a general preparedness of the immune system for elevated CORT levels (Shaniko Shini et al., 2008). This heterophilia following stress is mainly due to an increase in recruitment of heterophils from the bone marrow, decreased escape from the blood stream and an increased egress (i.e. exiting) of lymphocytes from the peripheral blood to lymphoid tissues (Bishop et al., 1968; Dhabhar, 2006; Ottaway & Husband, 1994).

### **2.6.3 Stress and the Avian Lymphocyte**

Lymphocytes are round agranular leukocytes with a centrally placed large nucleus and a comparatively small cytoplasm. Generally they are divided into three types namely B-lymphocytes, found in the bursa, T-lymphocytes, found in the thymus, and NK cells that are proficient circulating phagocytes. They are also involved in production of immunoglobulins and lymphokines (Fleischer, 1981; Glick, 1979; Sharma, 1991). Circulating catecholamines (as in acute stress) increase circulating lymphocytes within minutes. But once the HPA axis is stimulated and the stressor persists, increased levels of CORT cause lymphocytes to decrease mainly due to changes in recruitment and

adhesion (Dhabhar, 2006). Changes in recruitment are brought about by lymphocyte egression through inter-endothelial cell gaps to other compartments such as thymus, spleen, bone marrow, skin and lymph nodes (absent in birds) (Davis et al., 2008; Dhabhar, 2006; Ottaway & Husband, 1994). Increases in lymphocyte adhesion is brought about by cytokines produced following antigenic stimulation that invoke synthesis of E-selectin and ICAM-I adhesion molecules associated with endothelial cells. These molecules aid in the adhesion and subsequent migration of leuckocytes from the blood stream into tissues (Leeuwenberg et al., 1992). However, CORT inhibits synthesis of these adhesion molecules causing egression of lymphocytes from the blood vessel to other compartments (Ottaway & Husband, 1994). Exogenous CORT treated birds had no change in the ultra structure of the lymphocytes whereas bacterial lipopolysaccharide (LPS) challenged birds caused enormous destruction of lymphocytes (Shini et al., (2008). This emphasizes that CORT brings about a change in lymphocyte numbers by altering recruitment and adhesion rather than through destruction.

## **2.7 Physiological Adaptation to Chronic Stress**

Adaption to a stressor depends on the duration of the stressor, whereas removal of a stressor allows return of homeostasis. During the fight response there is energy mobilization for mainly increased locomotor activity aimed towards escape or defense (Landys et al., 2006). When the stressor persists, the acute stress response progresses to chronic stress where the HPA axis is continually activated resulting in elevated plasma CORT. During chronic stress, physiological processes are primarily aimed at maintaining a necessary source of energy with increases in gluconeogenesis, glycogenolysis, and proteolysis to provide readily available energy for bodily functions (Sapolsky et al., 2000). There is a reduction in growth due to decreased protein synthesis and increased protein catabolism, poor feathering, regression of lymphoid tissues like the spleen, thymus and bursa of Fabricius, visceral adiposity, hepatic lipidosis and ulcerative gastro intestinal lesions (Black, 1988; Odedra et al., 1983; Puvadolpirod & Thaxton, 2000a). Behaviorally there is a decline in feeding behavior, disinclination to move, suppression of reproductive function, and impaired immune response (Landys et al., 2006). Neuro-immuno-endocrinal changes include a down regulated response of the HPA axis to

subsequent acute stress, decreased secretion of growth hormone, luteinizing and gonadotropin releasing hormone, a reduction in the MR and GR, a decrease in CBG, and loss of hippocampal neurons (Sapolsky et al., 2000). A shift from cellular immunity to humoral immunity leading to increased susceptibility to infectious diseases and autoimmune diseases also accompanies the physiological changes (Iwakabe et al., 1998).

## **2.8 Stress and Poultry Welfare**

Well being of farm animals can be put forth as something conducive to a better or preferable life, and according to Fraser (2003) can be three pronged:

“(1) Animals should be raised under conditions that promote good biological functioning in the sense of health, growth and reproduction, (2) animals should be raised in ways that minimize suffering and promote contentment, and (3) animals should be allowed to lead relatively natural lives.”

Concern about stress in poultry production has long been an issue from both animal welfare and economic perspectives. These conditions include: climatic (i.e. extreme heat, cold or humidity), environmental (i.e. poor ventilation, light regimes), nutritional (i.e. shortages of nutrients), physical (i.e. catching, transport), social (i.e. overcrowding), physiological (i.e. rapid growth), psychological (i.e. fear, noise), and pathological (i.e. disease). Current trends for fast growth and increased production of broiler meat in a short duration of time has led to various management practices that may act as potential stressors (Bessei, 2006). Breeds that have been genetically modified for fast growth are increasingly being exposed to disease associated with fast growth like sudden death syndrome. Increased stocking density leading to poor litter quality with increased ammonia can produce respiratory problems, decreased growth rate and foot pad dermatitis (Bessei, 2006; Estevez, 2007; Kristensen & Wathes, 2000). Stamp et al. (2004) suggest that indirect effects of bird stocking density such as temperature, humidity, litter and air quality may affect the well being of broilers more than stocking density per se. Current assessment of welfare is mainly based on behavioral changes such as increased aggression, stereotypic movements, lethargy, disinclination to move,

physical changes including poor growth rate and feathering, leg deformities, asymmetry, and hematological changes like changes in H/L ratio, increased or decreased hormone levels (Fraser, 1993; Moneva et al., 2009). Conditions which induce stress in an animal can reduce welfare and as well as performance (Olanrewaju et al., 2006; Scheele, 1997), thus, measurements of stress can be used to evaluate and validate management techniques.

## **2.9 Measures of Stress in Poultry**

### **2.9.1 Assessment of Heterophil to Lymphocyte Ratio as a Measure of Stress.**

Evaluation of stress using the H/L ratio is based on the number of circulating heterophils in relation to the number of lymphocytes in blood. It has been used as a tool in assessing stress as reported by (Gross & Siegel, 1983), who evaluated the H/L ratio of chickens fed on a CORT in the diet. They measured both the H/L ratio and CORT levels in birds and reported an increase in circulating heterophils and a decrease in lymphocytes, therefore, suggesting this method (H/L ratio) to be a more reliable and less variable to time in assessing stress. CORT was reported to be a sensitive indicator of stress variable with sampling time. They suggest that H/L ratio values of 0.2, 0.4 and 0.8 indicate stress levels of mild, moderate and severe, respectively. Research using H/L as an indicator of stress demonstrates differences between studies with H/L ratio being influenced by factors such as age, sex, breed, social ranking and rearing method (Campo & Davila, 2002a, 2002b; Gross & Siegel, 1983; Maxwell, 1993; Maxwell & Robertson, 1998; Moneva et al., 2009). Data compiled by (Maxwell, 1993) on the avian blood leukocyte response to stress have shown that severe food restriction causes a heteropenia, whereas climate, environmental, transport and heat stress cause heterophilia. In life threatening situations heteropenia and basophilia are seen and H/L ratio cannot be used as measure of stress in such conditions (Maxwell, 1993). Thus, H/L ratios may not indicate the magnitude of stress experienced (Moneva et al., 2009). Campo et al. (2002a) found that birds reared under a photoperiod of 23L:1D do not have discernible alterations of H/L ratio suggest a lack of stress, but do display increased duration of tonic immobility, which may suggest an enhanced fear response. Even though H/L ratios show

less variation to time, they cannot on their own be used as a measure of stress (Campo & Davila, 2002a, 2002b; Gross & Siegel, 1983; Maxwell, 1993; Maxwell & Robertson, 1998; Moneva et al., 2009).

### **2.9.2 Plasma Corticosterone as a Measure of Stress.**

The most immediate noticeable change following exposure to a stressor is the spike in plasma CORT concentrations. CORT has been effectively used as the most sensitive indicator of stressful events (Post et al., 2003; Puvadolpirod & Thaxton, 2000a, 2000b, 2000c). However, CORT is highly variable and can be influenced by handling time and sampling methods. The half life of CORT in the chicken is 8 minutes (Carsia et al., 1988). Studies conducted by (Romero & Reed, 2005; Wingfield et al., 1982) have demonstrated that blood samples collected within 2 to 3 minutes of handling time have CORT concentrations which better represent an unstressed state (i.e. baseline). Given that the effect of CORT depends on a number of other factors such as receptor availability, sensitivity and binding protein availability (Landys et al., 2006) and that these factors can differ between species and prior stressful events, the use of CORT as a sole indicator of stress can be questioned (Chloupek et al., 2009; Romero, 2004). Also there is decreased basal CORT in chronic stress, either due to habituation to the stressor, adrenal exhaustion or because of down regulation in the synthesis and release of CORT to prevent its deleterious effects (Rich & Romero, 2005). Studies on Sprague-Dawley rats (Martí et al., 2001) and free living blue birds (*Sialia sialis*, (Lynn et al., 2010) have shown that a single exposure to a stressor can cause long lasting effects on the HPA axis such that the response to subsequent stressors is attenuated. This casts doubt on the reliability of the baseline CORT for evaluation of the “stressed state”. Broilers between 2 to 7 weeks of age showed decreasing levels of CORT as they acclimatized to their environment (Thaxton et al., 2005). Data compiled by Rushen (1991) demonstrated varying plasma CORT concentrations between experiments studied with similar conditions. For example, plasma CORT can be increased (Gibson et al., 1986), decreased (Koelkebeck et al., 1987) or the same (Craig et al., 1986) in laying hens reared in battery cages compared with those reared in pens. To overcome the inconsistency,

researchers have resorted to using a combination of plasma H/L ratio and CORT in the assessment of stress and welfare (Moneva et al., 2009).

### **2.9.3 Tonic Immobility as a Measure of Stress**

Tonic immobility is a disassociated state observed in birds, frogs, lizards, rats, rabbits, and other species characterised by unresponsiveness and increased flexibility. It can be induced by physical restraint and procedures known to induce fear. Tonic immobility is characterized by a catonic-like state with temporary loss of righting reflex, reduced vocalization, intermittent eye closure, rigidity, altered electroencephalographic patterns, and changes in heart rate, respiratory rate and core body temperature (Gallup, 1977). It is thought to be a defense mechanism which may allow for escape from predators (Jones, 1986; Jones & Faure, 1981; Maser & Gallup, 1974). Tonic immobility is influenced by strain, sex, age, and the method of induction in chickens (Jones & Faure, 1981). This measure of fear has its own controversies aptly put forth by (Archer, 1979):

“1) there are low correlations between different measures of fear, 2) the nature of behavioural fear responses are complex and 3) several behavioural indices of fear may be influenced by factors other than fear-evoking stimuli.”

Thus contradictory results between various fear and tonic immobility have led to skepticism in the use of these methods as stress assessment.

### **2.10 Lighting as a Potential Stressor in Broiler Management**

Light is an essential management technique in broiler production and is capable of affecting various physiological and behavioral processes in birds (Olanrewaju, 2006). It consists of three components namely: intensity, color or wavelength and duration each capable of altering physiological processes. Varying photoperiods and light intensity may act as stressors in broiler farms leading to alteration of physiology and production. Therefore it is important to assess lighting as a stressor in broiler management.

### **2.10.1 Sleep and the HPA Axis**

Sleep is an essential physiological process which allows for recuperation of the body in terms of energy conservation, tissue restoration and growth (Adam, 1980; Adam & Oswald, 1977; Drucker-Colín & Spanis, 1976; Malleau et al., 2007). It is suggested that adult chickens require a minimum of 6 to 8 hours of scotophase (darkness) for rest/sleep and optimum production (Prescott et al., 2003). The circadian rhythm exists in CORT levels with a decrease before sleep initiation, followed by a plateau in deep sleep and then increasing levels before awakening (Buckley, 2005; Tobler et al., 1983). Sleep deprivation causes activation of the HPA axis and subsequent elevation in CORT (Meerlo et al., 2002; Tobler et al., 1983). Chronic sleep deprivation in rats dampened the HPA axis response to subsequent stressors with a decrease in ACTH (Meerlo et al., 2002). In stressful situations, excess CORT binds with GR in the amygdala causing increased CRH secretion. This increased CRH causes a decrease in the slow wave sleep cycle leading to wakefulness (Buckley, 2005). Thus, continuous lighting and some light intensity management strategies may affect normal circadian variation of the HPA axis, emphasizing the importance of rest/sleep in chickens.

### **2.10.2 Photoperiod as a Potential Stressor**

It is believed that continuous lighting allows broilers more time to feed and thereby increased growth rate (North and Bell, 1990). Thus, broilers are kept on a continuous or nearly continuous lighting schedule to maximize growth rate (Rozemboin et al., 1999). However, this practice of continuous lighting has been linked to emergence of the “disease of fast growth” such as ascites, sudden death syndrome, skeletal abnormalities and deposition of fat. All of these are known to reduce bird welfare and decrease profit margins (Ononiwu et al., 1979). Continuous lighting can also cause buphtalmia (enlarged eyes) and blindness in birds (Whitley et al., 1984). Kirby and Froman (1991) reported immune system impairment with constant lighting in White Leghorn cockerels; similarly impairment of the immune system with continuous lighting was reported in Japanese quail (*Coturnix japonica*) (Moore & Siopes, 2000). Continuous lighting also caused sleep deprivation resulting in pathological stress-like symptoms (Rechtschaffen et al., 1983).

### **2.10.3 Light Intensity as a Potential Stressor**

Increasing or decreasing lighting intensities can result in altered behavior and compromised welfare of birds. Recent work by Blatchford et al. (2008) showed altered behavior and well being of birds reared under an low light intensity of 5 lux (Lx) with no effect on body weight and immunocompetence. Extended periods of dim lighting or darkness cause a decrease in corneal thickness (Jenkins et al., 1979) and heavier eyes (Blatchford et al., 2008) in chickens. Birds exposed to 5 Lx light intensity were lethargic and showed less change in activity between day and night than birds under 50 or 200 Lx lighting (Blatchford et al., 2009). Extended lighting photoperiods confounded with low lighting intensities had a negative effect on breast meat weight production of broilers in spite of improving the broiler live performance (Lien et al., 2009). In contrast, increased light intensity has little effect on broiler health but is associated with behavioral changes. Birds subjected to 200 Lx of light have increased activity with more hock and footpad bruising but less erosion of the hock joints than birds grown at 5 or 50 Lx (Blatchford et al., 2008). Similar results were reported by Prayitno et al (1997), where birds showed improved gait scores and less angular deformity of the legs when reared under high intensity red light early in the growth period when compared to blue light intensity.

### **2.11 Metabolomics**

Metabolomics is the study of detectable small molecular weight metabolites approximately < 1000 daltons present in the cells, bio fluids, tissues and organs involved in primary or secondary metabolism (Kim et al., 2008; Shanaiah et al., 2008; Viant, 2008). Metabolomics is the end point of the “omics” cascade of studies involving in sequence, genomics-transcriptomics-proteomics (Dettmer & Hammock, 2004; Serkova & Glunde, 2009). While genomics is the study of gene function, transcriptomics that of gene expression and proteomics the study of proteins translation, metabolomics is the study of metabolites at the cellular level(Dettmer & Hammock, 2004). It gives insight into genetic alterations, disease and environmental influence on the organism as metabolites are more predictive of the ongoing physiological expression (Dettmer &



Hammock, 2004). As gene and protein expression determine what happens at the cellular level, the cellular environment by itself can be affected by exogenous factors like toxins, drugs, hormones and existing nutritive state (Schmidt, 2004), and metabolites in such scenarios are highly representative of the current homeostatic state present in an organism. Studies by Ter Kuile et al., (2001) with parasitic protists grown in a glucose environment demonstrated that the glycolytic flux of enzymes not necessarily follows the classical hierarchical regulation of information flow from DNA to RNA to proteins. Enzymes glycerol-3-phosphate dehydrogenase in *Typanosoma brucei* and phosphoglucoseisomerase in *Leishmania donovani* were completely under metabolic regulation rather than genomic regulation. The transcribed levels of mRNA did not correlate to functional levels of these enzymes casting doubt on whether transcriptome and proteome analysis alone are sufficient to understand biological functions. In turn this suggests that metabolomics must also be considered in functional genomics (Ter Kuile & Westerhoff, 2001). Raamsdonk et al., (2001) in their study on *Saccharomyces cerevisiae* showed that the metabolites reveal the functions of silent mutant genes. These two studies indicate the informative role provided by metabolomics in understanding biological processes. However, in terms of systems biology, an integration of all the “omics” studies is warranted for an in depth analysis of the underlying regulative process.

### **2.11.1 Environmental Metabolomics**

Environmental metabolomics is an emerging approach for examining metabolic profiles in biological systems exposed to environmental changes (C. Y. Lin et al., 2006). It has gained importance within the last decade with the unavoidable influence of anthropogenic factors on the environment. Environmental metabolomics involves the study of the organism and its interaction with the environment thereby assessing the function and health of the organism at a molecular level (Bundy et al., 2009). As metabolomics data are highly suggestive of the existing homeostatic state of an organism, it provides valuable insight of the functional and regulatory state of the organism *vis a vis* its environment (Bundy et al., 2009; Shanaiah et al., 2008). Eco physiological factors may include climatic, environmental, nutritional, social,

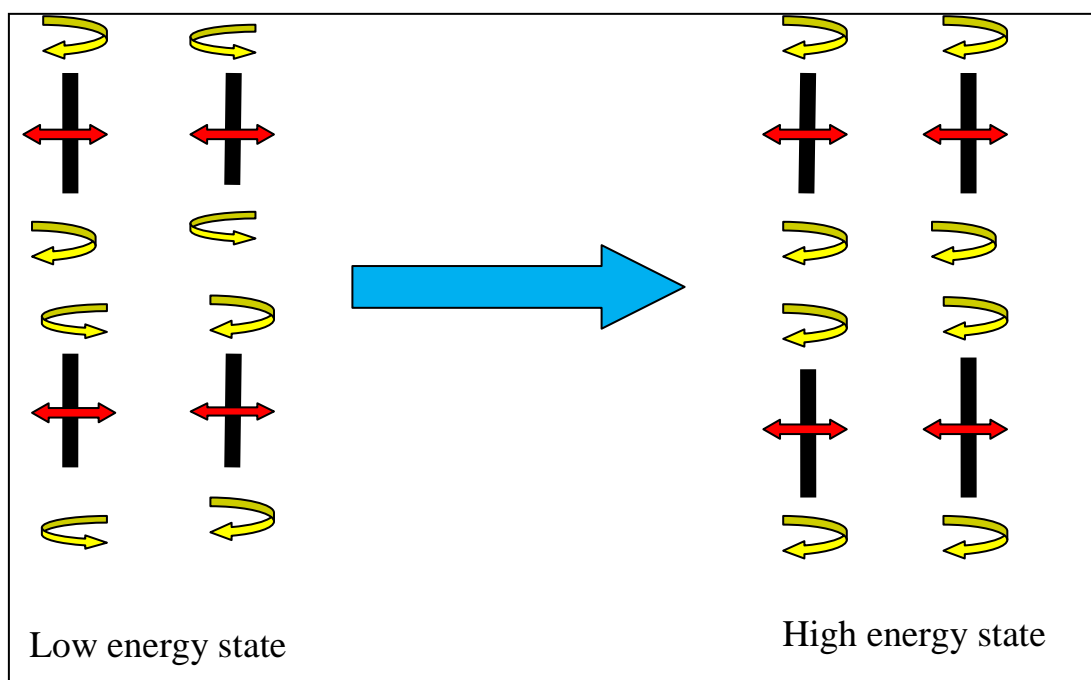
physiological, psychological and pathological states. Viant et al (2003) successfully separated healthy, diseased and food deprived groups in red abalones (*Haliotis rufescens*) based on their metabolites using NMR spectroscopy, thereby identifying metabolic fingerprints of biological stressors. Similar NMR metabolomic studies on eco physiological factors like heat stress homeostasis in *Drosophila melanogaster* (Malmendal et al., 2006) and soil metal contamination at different habitats in earthworms (*Lumbricus rubellus*) (Bundy et al., 2007) demonstrate the potential use of metabolomics in ecological studies, with clear indications of significant influence of local environment on the metabolome of an organism. Environmental metabolomics sheds light on the mechanism of an organism's functional homeostatic response to its surroundings (Viant, 2008).

### **2.11.2 Nuclear Magnetic Resonance Based Metabolomics**

Mass spectroscopy and nuclear magnetic resonance (NMR) are the two most popular analytical tools used in metabolomics (Dettmer & Hammock, 2004; Greef et al., 2004; Serkova & Glunde, 2009). Mass spectroscopy is highly sensitive and for the most part has to be combined with other chromatography techniques like gas or liquid chromatography for the best results as variable ionization efficiency causes a non uniform detection of metabolites (Shanaiah et al., 2008). However, it is an excellent tool in quantitative metabolomics. On the other hand NMR based metabolomics is an excellent tool in identifying abundance of metabolites with less sample preparation, high reproducibility, nondestructive, nonselective metabolite detection, and ability to quantify multiple metabolites (Serkova & Glunde, 2009). Nuclear magnetic resonance has been successfully used to identify metabolites from bio fluids (Lindon et al., 1999) and intact tissues (Cheng et al., 1998). Use of NMR aids in detection of hundreds of metabolites simultaneously, providing information on altered biochemistry and thereby diagnostic information (Shanaiah et al., 2008). The success of NMR metabolomics is in the identification of subtle changes of metabolites due to underlying conditions prior to the phenotypic expression of the condition by itself (Kim et al., 2008).

### 2.11.3 Principles of $^1\text{H}$ Nuclear Magnetic Resonance

The nucleus of an atom contains both protons and neutrons. Their numbers determine the type of spin the nucleus carries. Nuclei from hydrogen ( $^1\text{H}$ ) and Carbon-13 ( $^{13}\text{C}$ ) atoms have odd numbers of protons and neutrons and thereby carry a  $\frac{1}{2}$  spin whereas in nuclei with even number of protons and neutrons like carbon-12 ( $^{12}\text{C}$ ), carbon-16 ( $^{16}\text{C}$ ), and carbon-32 ( $^{32}\text{C}$ ) the charges cancel out each other and hence carry zero or no spin (Lindon et al., 2006; Silverstein, 2005). Odd numbered nuclei behave like small magnetic bars carrying a magnetic field. In the presence of an external magnetic field they align themselves along the magnetic field and are in a low energy spin state. In NMR spectrometry, electromagnetic radiations are applied to flip the low energy level spin aligned nuclei to a high energy spin opposed state (Figure 2.2). These applied electromagnetic radiation frequency are later Fourier transformed to give the actual spectra. Several such electromagnetic radiation pulses or scans are done on the samples to differentiate signals from the background noise. Proton NMR ( $^1\text{H}$  NMR) spectra are derived by exciting the odd atomic numbered hydrogen atoms present with a natural abundance of 99.98% in biological compounds. The resultant spectrum is phased and baseline corrected and then normalized to an internal standard for further spectral processing and multivariate statistical analysis.



**Figure 2.2: In NMR spectrometry, electromagnetic radiations are applied to flip the low energy level spin aligned nuclei to a high energy spin opposed state. Applied electromagnetic radiation frequency are later Fourier transformed to give the actual spectra.**

#### **2.11.4 Nuclear Magnetic Resonance Spectroscopy of Biofluids - Blood**

Metabolites in biofluids are in a dynamic equilibrium with cells and tissues, and therefore any alteration due to abnormal cellular processes will result in altered metabolite composition of the biofluid (Lindon et al., 2000). Metabolomics has been used successfully in biofluids such as urine, cerebrospinal fluid, seminal fluids, bile, saliva, blood plasma and serum for identifying metabolites (Lindon et al., 1999).

Vertebrate blood functions involve the transport of oxygen, carbon dioxide, metabolites and hormones and any pathophysiological change is reflected by an altered blood composition (Lindon et al., 2000). NMR analysis of serum or plasma measures the homeostatic levels of metabolites present in the organism (Shanaiah et al., 2008). As blood clotting affects the analysis of small molecule composition, serum is preferred for most metabolomic studies (Tiziani et al., 2008). Catalytically active proteins and

enzymatic processes in blood plasma and serum tend to bring constant metabolic flux that can be eliminated by deproteinization before spectroscopic analysis (Tiziani et al., 2008). Deproteinization by ultrafiltration of the serum or plasma has been found to be supportive in retaining metabolite concentrations and removing the broad resonances associated with the proteins with a high degree of reproducibility (de Graaf & Behar, 2003; Lindon et al., 2000; Tiziani et al., 2008).

## **2.12 Use of $^1\text{H}$ Nuclear Magnetic Resonance in Poultry Welfare Assessment**

Traditionally assessment of poultry welfare has been heavily based on blood CORT levels, H/L ratio, measure of tonic immobility and other parameters like mortality rate, body weight, fluctuating asymmetry, morbidity (e.g. ascites, lameness), behavioral changes and production statistics (Bryan Jones & Mills, 1983; Campo et al., 2007; Davis et al., 2008; Gross & Siegel, 1983; Jones & Faure, 1981; Puvadolpirod & Thaxton, 2000c; Scahaw, 2000; Thaxton et al., 2005). In spite of their prevalent use in poultry welfare assessment, they by themselves do not provide enough information of the magnitude and stage of the underlying homeostatic response (Campo & Davila, 2002a; Maxwell, 1993; Moneva et al., 2009; Rushen, 1991). Environment plays a vital role in the well being of broilers (Bessei, 2006; Dawkins et al., 2004) and with stringent welfare regulations being implemented by the European Union and subsequent impact on world poultry trade (Scahaw, 2000; Van Horne & Achterbosch, 2008) it is imperative to have appropriate assessment tools that aid in proper interpretation of poultry welfare and interaction with their environment.

Evaluation of stress using NMR has been done in cattle (Aich et al., 2007) and Sprague-Dawley rats (Teague et al., 2007). Aich et al (2007) was able to identify metabolic fingerprints between calves that were abruptly weaned and preconditionally weaned with a subsequent challenge of bovine herpes virus-1. Abruptly weaned calves showed increased serum levels of amino acids, lipids and  $\beta$ -glucose suggestive of psychological (weaning) stress altering the physiological response to a viral challenge. Teague et al. (2007) using physiological stress (e.g. shaking and restraint) on male Sprague-Dawley rats was able to differentiate metabolite profiles between acute and chronically stressed groups. Animals in the acute stressed group had increased levels of

glucose, ketone bodies and decreased levels of acetate, lactate, leucine, isoleucine and valine whereas the chronically stressed group had increased levels of leucine, valine, choline and decreased levels of acetate and lipoproteins. NMR studies in poultry have mostly been limited to understanding cellular metabolites and their structure. Quirk et al (1989) used NMR in determining the cellular component of metabolites in chicken enterocytes while Li et al (2000) employed NMR in understanding the properties of water in chicken breast marinated with different phosphates. Other studies include, understanding the structure of kringle 4 domain of the chicken plasminogen using NMR (Petros et al., 1988). Birds in a commercial poultry setting are continuously being exposed to various environmental perturbations both psychological and physical (Bessei, 2006). To date there has been no work using NMR based metabolomics to study the environmental impacts (stressor) on physiological homeostasis in poultry. Therefore, I investigated the effect of chronic stress, mimicked by feeding broilers with CORT (30 mg/kg diet) on the metabolite profile and compared it with more traditional measures of stress (H/L ratio and serum CORT). I also utilized NMR to examine the metabolite profile in broilers exposed to different lighting managements (photoperiod and light intensity) and again compared it with H/L ratio and CORT.

## CHAPTER 3

### COMPARATIVE EVALUATION OF BLOOD CORTICOSTERONE, HETEROPHIL: LYMPHOCYTE RATIO AND $^1\text{H}$ NMR SPECTROSCOPY AS MEASURES OF STRESS IN BROILER CHICKENS (*GALLUS DOMESTICUS*)

#### 3.1 Introduction

Stress can be defined as any event that alters physiological homeostasis. Any event that elicits a response (either behavioral or physiological) can be considered a “stressor.” The “stress response”, is an evolved trait that allows an animal to deal with changes or conditions within its environment. Proper response to a stressor can be beneficial in life threatening situations; however, chronic or repeated exposure can have marked deleterious effects on reproduction, behavior, metabolism and immunocompetence.(Selye, 1951; Siegel, 1980). Stressors can be events or environmental conditions. These include: climatic (i.e. extreme heat, cold or humidity), environmental (i.e. poor ventilation, light regimes, housing), nutritional (i.e. shortages of nutrients), physical (i.e. handling, transport), social (i.e. overcrowding), physiological (i.e. rapid growth), psychological (i.e. fear, noise), and pathological (i.e. disease). Conditions which induce stress in an animal can reduce welfare and as well as performance (Olanrewaju, 2006; Scheele, 1997).

Response to a stressor can either be specific or non-specific (Siegel, 1980). The response to a non-specific stressor is brought about by two main systems namely the sympathoadrenal medullary axis (SAM) or neurogenic system, and the hypothalamic pituitary axis (HPA). The initial response to a stressor comes about via SAM and is also known as the fight or flight response (Cannon, 1929). It is typically defensive in nature against the stressor lasting for the first few minutes. It involves the release of catecholamines: epinephrine and norepinephrine and thereby an increase in heart rate, respiration, muscle tonicity and blood glucose levels aimed at restoring homeostasis (Siegel, 1980). If the stressor persists, the hypothalamic-pituitary-adrenal (HPA) axis becomes activated (Siegel, 1980). Corticotropin-releasing hormone (CRH) from the hypothalamus is secreted directly into the anterior pituitary via the hypophyseal portal circulation resulting in a rapid release of adrenocorticotropin (ACTH) from the pituitary,

which stimulates production and release of glucocorticoids such as corticosterone (CORT) from the adrenal gland (Johnson et al., 1992; Sapolsky et al., 2000; Selye, 1951; Siegel, 1980).

In poultry, conditions such as varying temperature, humidity, and litter and air quality (Dawkins et al., 2004), stocking density (Bessei, 2006) and varying lighting schedules (Olanrewaju, 2006) have been found to be potential stressors compromising the well being of the birds. Long-term exposure to CORT can result in cardiovascular disease (i.e. arteriosclerosis, ascites), hypercholesterolemia, decreased weight, gastrointestinal lesions, immune suppression and increased susceptibility to disease (Puvadolpirod & Thaxton, 2000a, 2000c; Siegel, 1980). Chronic stress can produce pale, dehydrated meat in broilers (Tankson et al., 2001) suggestive of pale soft exudative lesions. Various physiological endpoints have been used to identify stress in the poultry industry, but the two most accepted indicators of stress are elevated CORT and increased circulating heterophil/lymphocyte (H/L) ratios (Puvadolpirod & Thaxton, 2000a). However, the length of time taken to obtain the blood sample can influence both estimates, with CORT reacting as early as 3 minutes (Voslarova et al., 2008) and H/L ratio within the first 15-20 minutes (Moneva et al., 2009) after handling for sample collection. These errors may lead to problematic interpretation of the data. A lack of consistency between studies has resulted in skepticism when using these procedures to quantify animal welfare in poultry research (Maxwell, 1993; Rushen, 1991).

I propose the use of metabolomics as an alternative method of assessing stress in broilers. Serkova et al. (2009) define metabolomics as “an ‘omic’ science in systems biology, is the global assessment and validation of endogenous small-molecule biochemicals (metabolites) within a biologic system”. Environmental metabolomics is an emerging approach for examining metabolic profiles in biological systems exposed to environmental stress (Lin et al., 2006). Utilization of 1-dimensional <sup>1</sup>H Nuclear Magnetic Resonance spectroscopy (NMR) can provide insightful mechanistic information on effects of stressors by simultaneously measuring a large number of low molecular weight endogenous metabolites (Viant et al., 2003). Metabolic profiles can be examined using pattern recognition algorithms to reveal subsets of metabolites, potentially identifying biomarkers (Lin et al., 2006; Viant et al., 2003). The advantage of using NMR over more



traditional methods is that it provides information about multiple metabolites, many that are not influenced by sampling handling time and may potentially identify biomarkers for stress. The objective of this study is to evaluate and compare the use of NMR to more traditional methods of stress assessment (CORT and H/L ratio) in broilers. In addition, I will utilize the metabolite profile to identify potential biomarkers of chronic stress.

### **3.2 Materials and Methods**

#### **3.2.1 Animals**

One day old Ross x Ross 308 broilers were obtained from Lilydale in Wynyard, Saskatchewan, Canada. Fifty birds were randomly assigned to one of two rooms (n = 25 each) at the Animal Care Unit, University of Saskatchewan with a target stocking density 30 kg/m<sup>2</sup> per room. Feed was obtained from a commercial source and provided on an *ad libitum* basis. Major ingredients in the diets were corn and soybean meal and diet specification and feeding amounts were according to Aviagen recommendations (Aviagen, 2007a). Starter (0 to 10 days) and grower (10 to 25 days) diets were fed in a crumble form and the finisher (25 to 35 days) diet in a pellet form. Feed did not contain a coccidiostat or a growth promotant. A single feeder was placed in the pen with ad libitum feeding, water was provided through nipple drinkers and wood shavings were used as litter material. During the acclimation period (first 7 days) lighting was 23L:1D with a minimum of 20 Lx intensity. At day 7, lighting was changed to 17L:7D at 10 Lx intensity. Feed containing corticosterone (Sigma Aldrich, St Louis USA) dissolved in 100% ethanol for a concentration of 30 mg/kg of diet (Gross & Siegel, 1983; H. Lin et al., 2006) was fed to chickens in one room treatment (TRT), while the other room control (CON) had nothing added to the diet. Animals were treated in accordance with the Canadian Council on Animal Care guidelines and protocols were approved by the University of Saskatchewan's Animal Care Committee.

#### **3.2.2 Sample Collection**

Twelve randomly selected birds from each room were blood sampled via the right jugular vein on days 14, 21, 28 and 35. Day 7 blood samples were obtained from six randomly selected birds from each room that were sacrificed using a guillotine. Blood sampling occurred between 7 and 10 am. The time elapsed from the handler entering the

room to the time the blood sample was obtained was recorded as sampling time for each bird in seconds. After blood collection, pressure was gently applied until the bleeding stopped and birds were returned to their respective rooms. A time interval of 20 minutes was maintained between entry into any given room to minimize stress and allow for blood CORT to return to baseline. Upon obtaining the blood sample, blood smears were made immediately and the remainder of the sample was transferred to sterile vacutainers and stored in ice water until centrifugation. After centrifugation at 3000 rpm for 15 minutes serum was harvested and frozen at -80°C for further analysis. Blood smears were fixed in 100% methanol and stained using May-Grunwald-Giemsa stain (Campo & Davila, 2002b; Gross & Siegel, 1983). A total of hundred cells including both granular (heterophils, eosinophils and basophils) and agranular (lymphocytes and monocytes) leukocytes were counted for each slide under oil immersion at 100 X. The H/L ratio was reported as the number of heterophils per hundred leukocytes to the number of lymphocytes per hundred leukocytes (Campo & Davila, 2002b; Jones et al., 1988). Serum CORT concentration was determined using a Double Antibody Rat Corticosterone Kit (ICN Pharmaceuticals Inc., Orangeburg, NY) (Sorenson et al., 1997). Average body weight of birds in each group (total weight of all birds per room/total number of birds per room) was recorded for every week of sample collection.

### **3.2.3 Preparation of Samples for NMR Analysis**

Serum samples were prepared by diluting 150 uL of serum with sodium phosphate buffer at pH 7.0 made in Deuterium oxide (D<sub>2</sub>O, 99.9% Cambridge Isotope Inc., Andover, M.A, U.S.A, 1:4 by volume) which included 0.5 mM TMSP (sodium-3-trimethylsilyl -[2, 2, 3, 3-d<sub>4</sub>]propionate , 98%, Cambridge Isotope Inc., Andover, M.A, U.S.A) added for referencing and locking (Viant, 2007). Ultrafiltration of samples was done using 10 kda Nanosep centrifugal filters (Pall Life Sciences, Ontario, Canada). The filtration membranes were prepared by washing each filter 10 times with distilled water to remove glycerol (Tiziani et al., (2008). Diluted samples were filtered at 15,000 rpm for 15 minutes at 4°C to remove high molecular weight components. The filtrate was transferred into modified 3 mm NMR tubes (New Era Enterprises Inc, Vineland, NJ, U.S.A.) and conventional NMR spectra obtained. The tubes were washed 5 times in distilled water and soaked in Extran (EMD chemicals Inc, Gibbstown, NJ, U.S.A) for 1

hour to remove them of biological debris. The tubes were once again washed 5 times in distilled water to clear the Extran and then rinsed with acetone (BHA Inc, Toronto, Ontario, Canada) to remove any moisture adhering to the tubes before new samples were added.

### **3.2.4 $^1\text{H}$ -NMR Spectroscopy**

Conventional NMR spectra were recorded at a resonance frequency of 600.17 MHz on a Bruker Avance-600 spectrometer (Rheinstetten, Germany) equipped with an inverse triple resonance probe (TXI 5mm) at the Saskatchewan Structural Science Centre, University of Saskatchewan, SK, Canada. Water suppression using excitation sculpting with gradients was used to accomplish efficient suppression of the water resonance in the spectral data (Bollard et al., 2005). For each sample, 32 free induction decays (FID) were accumulated over a spectral width of 7183.91 Hz with an acquisition time of 2.28 seconds and relaxation delay of 5 seconds at a temperature of 298°K. The experimental time for each sample was 4.18 minutes. The resultant FID was Fourier transformed into one-dimensional  $^1\text{H}$ -NMR for further analysis. The resulting spectra were phased manually; baseline corrected, and calibrated (TMSP at 0.0 ppm) using XWIN-NMR (version 3.5, Bruker). Spectra were converted to an appropriate format for analysis using custom-written *Prometab* running within MATLAB (Version R2009a; The MathWorks, Natick, MA, USA). Spectra were segmented into equidistant 0.04-ppm bins between 0.2 and 10.0 ppm and the total spectra area within each bin was integrated, as described previously (Viant, 2003). Bins between 4.4 ppm and 6.0 ppm containing the suppressed water resonance were excluded (Aich et al., 2007). After the spectral bin (2.22 ppm) corresponding to acetone a common laboratory impurity in NMR experiments (Fulmer et al., 2010; Gottlieb et al., 1997) was removed from the model, a PCA was done to determine the trend and outliers. A total of 2 birds (day 14), 6 birds (day 21), 3 birds (day 28) and 2 birds (day 35) were identified as outliers and excluded from the model.

### **3.2.5 Data Analysis**

Sampling time was analyzed using simple regression, H/L ratio and CORT data were analyzed using an analysis of variance (ANOVA) using SPSS version 14.5. Since sampling time was a factor that influences the level of H/L ratio and CORT, residuals of

the both H/L and CORT to time were used to determine the levels of significance of the treatment. Moneva et al (2009) has reported the H/L ratio levels to increase in as early as 15-20 minutes post handling for sample collection in normal chickens. Studies (Romero & Reed, 2005; Wingfield et al., 1982) have demonstrated that blood samples collected within 2 to 3 minutes of handling time have CORT concentrations which better represent an unstressed state (i.e. baseline). To control for sampling time, H/L ratio and CORT were regressed against time, and the residuals were used in subsequent analyses (Wayland et al., 2003).

For multivariate analysis, data were imported into the MetaboAnalyst software (Xia et al., 2009). Spectral resonance 2.22 ppm corresponding to acetone was removed before further spectral analysis because it was added to remove moisture from the NMR tubes. Spectra were baseline corrected for noise, normalised and Pareto-scaled. An unsupervised pattern recognition method (principal component analysis (PCA)) was used to examine intrinsic variation in the NMR data set and to identify and exclude outliers. A supervised partial least square-discriminant analysis (PLS-DA) was performed to identify the discriminating significant features between treatments. Permutation (Pr) by randomly assigning samples to different groups was done to assess the strength of the model (Bijlsma et al., 2005; Rubingh et al., 2006). Metabolites were assigned to peaks based on their resonance frequency using peer reviewed metabolite databases (Lindon et al., 1999; Lindon et al., 2000; D. S. Wishart et al., 2009). Regression analyses and *a priori* contrasts were used as appropriate. Differences were considered significant when  $P \leq 0.05$  and noted when  $P \leq 0.10$ . Values are reported as mean +/- standard deviation.

### **3.3 Results**

Seven treatment and two control birds died. The two birds in CON died during the acclimation (brooding) period. Two mortalities in TRT occurred between days 7 to 14, three between days 14 to 21 and two died between days 28 and the end of the experiment on day 35. Of the seven birds six were found dead in their pens while one died during blood sampling. On day 35, only 11 TRT birds were available for sampling due to lameness sample from the 12<sup>th</sup> bird were not obtained, thus n=53 TRT for CORT and metabolomic analysis. On day 7, owing to a small blood volume and rapid clotting, blood smears were not prepared for two birds (CON) n=52 for the H/L ratio.

Average body weight of birds was taken during every week of sampling. Table 3.1 shows a clear difference of average body weight between the TRT and CON groups. At day 7 they both were of almost the same average weight of 135g and 136g respectively. On day 14 the treatment birds had an average body weight of 0.371kg whereas the control group had an average body weight of 0.579 kg. By day 35 the TRT birds reached a maximum weight of 0.798 kg whereas the CON birds were 2.265 kg.

Sampling time was determined as the time from entering the room until blood samples were obtained. Mean sampling time was  $82 \pm 36$  and  $75 \pm 33$  seconds for treatment and control groups, respectively. There was a significant relationship between sampling time and the H/L ratio in the treatment group ( $F_{1, 53} = 9.83$ ,  $P = 0.002$ , Figure 3.1A) whereas there was a significant relationship with sampling time and CORT in the control group ( $F_{1, 54} = 6.51$ ,  $P = 0.01$  Figure 3.2B).

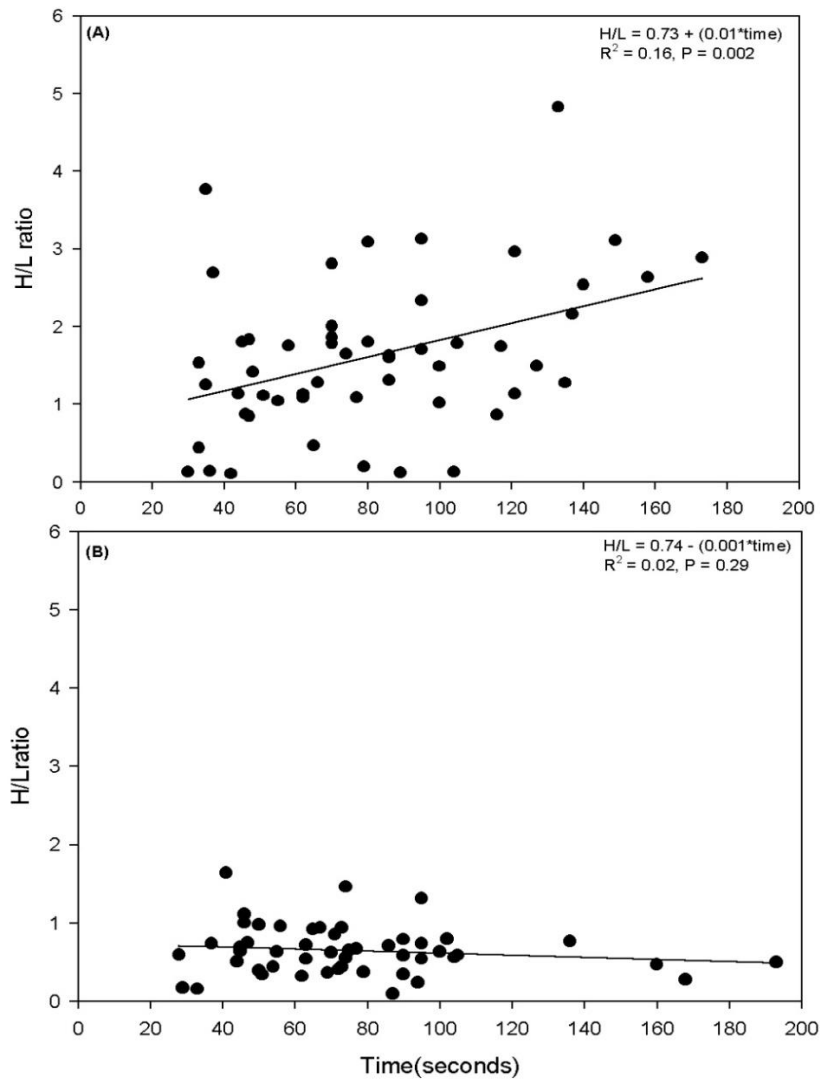
The H/L ratio was significantly greater in TRT (CORT fed) birds at each sampling period after addition of CORT to the feed compared with CON ( $F_{1, 105} = 20.128$ ,  $n=105$ ,  $P=0.002$ , Figure 3.3A). Serum CORT was elevated in TRT at each sampling period after addition of CORT to the feed ( $F_{1, 107} = 32.632$ ,  $n=107$ ,  $P=0.0001$ , Figure 3.3A). Although not significant, CORT was highest at day 14 (Figure 3.3B) and then declined through day 35.

Treatment and control groups (day 14, Figure 3.4) could be distinguished using two dimensional (2D) score plots from PLS-DA analysis of metabolites. Permutation (Pr) by randomly assigning samples to different groups was done to assess the strength of the model (Bijlsma et al., 2005; Rubingh et al., 2006). Principal component 1 of PLS-DA accounted for 37.2 % of the variation between the groups in day14  $Pr = 0.002$ . Similar 2D score plots of PLS-DA showed separation of metabolites between treatment and control on day 21 along component 2 with 44.6 % separation ( $Pr = 0.006$ , Figure3.5), day 28 along component 1 with 76.7 % separation ( $Pr=0.001$ , Figure3.6) and day 35 with component 1 accounting for 52.5 % separation ( $Pr=0.001$ , Figure3.7). Significant features differentiating treatment and control groups were identified based on the variable importance in projections (VIP) scores of the PLS-DA. All metabolic data analysis and generation of graph were done using MetaboAnalyst (Xia et al., 2009).

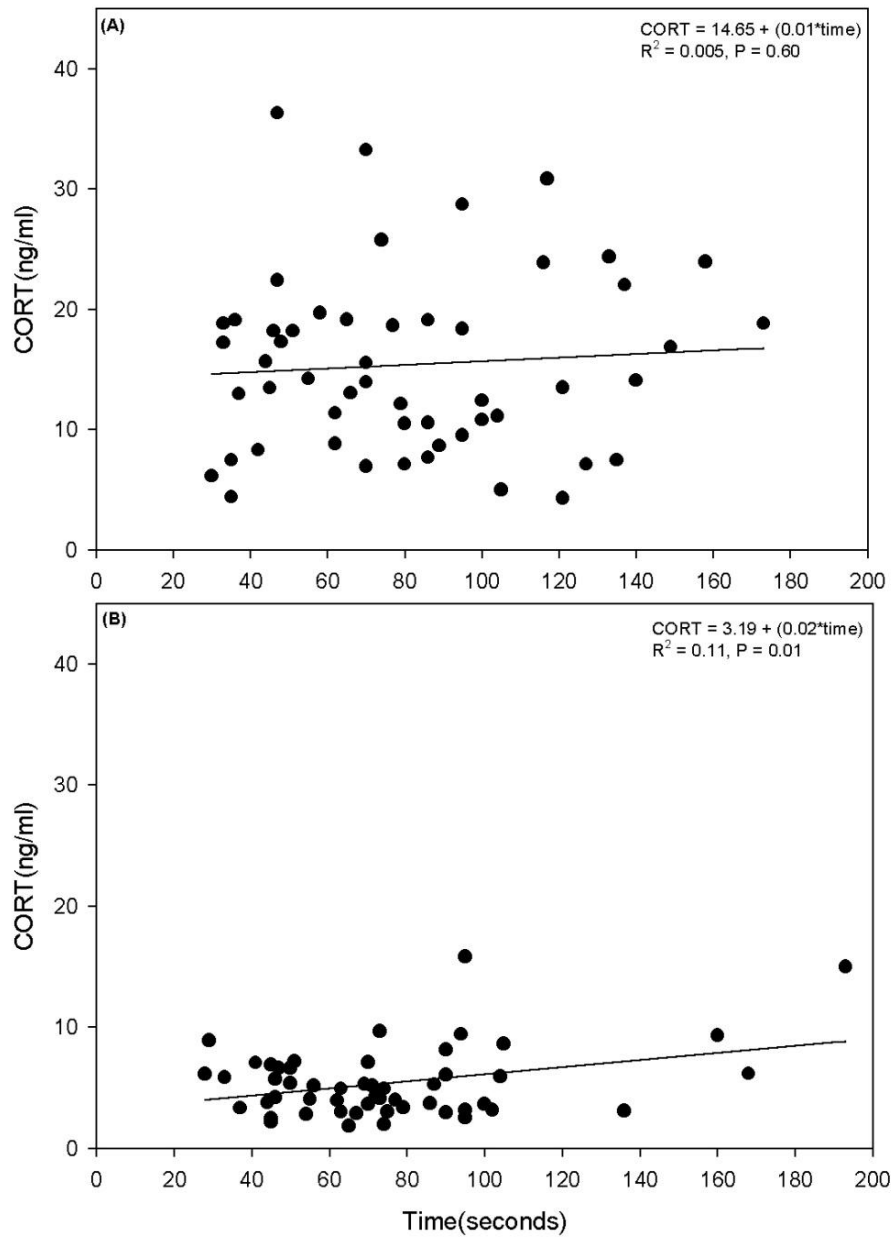
**Table 3.1 Average body weight in kilograms of the treatment birds (fed on a CORT diet from day 7) and control birds (no feed additive added). Sampling was done on day 7 (treatment n=19, control n=17) and day 14 (treatment n=17, control n=17), day 21 (treatment n=14, control n=17), day 28 (treatment n=14, control n=17) and day 35 (treatment n=12, control n=17).**

<b>Sampling days</b>	<b>Aviagen standards Ross 308 body weight (kg)*</b>	<b>Control body weight (kg)</b>	<b>Treatment body weight (kg)</b>
Day 7	0.182	0.136	0.135
Day 14	0.455	0.579	0.371
Day 21	0.874	0.906	0.516
Day28	1.142	1.342	0.683
Day35	2.021	2.265	0.798

\* Data adapted from Aviagen Ross 308 broiler performance objectives (Aviagen, 2007b)

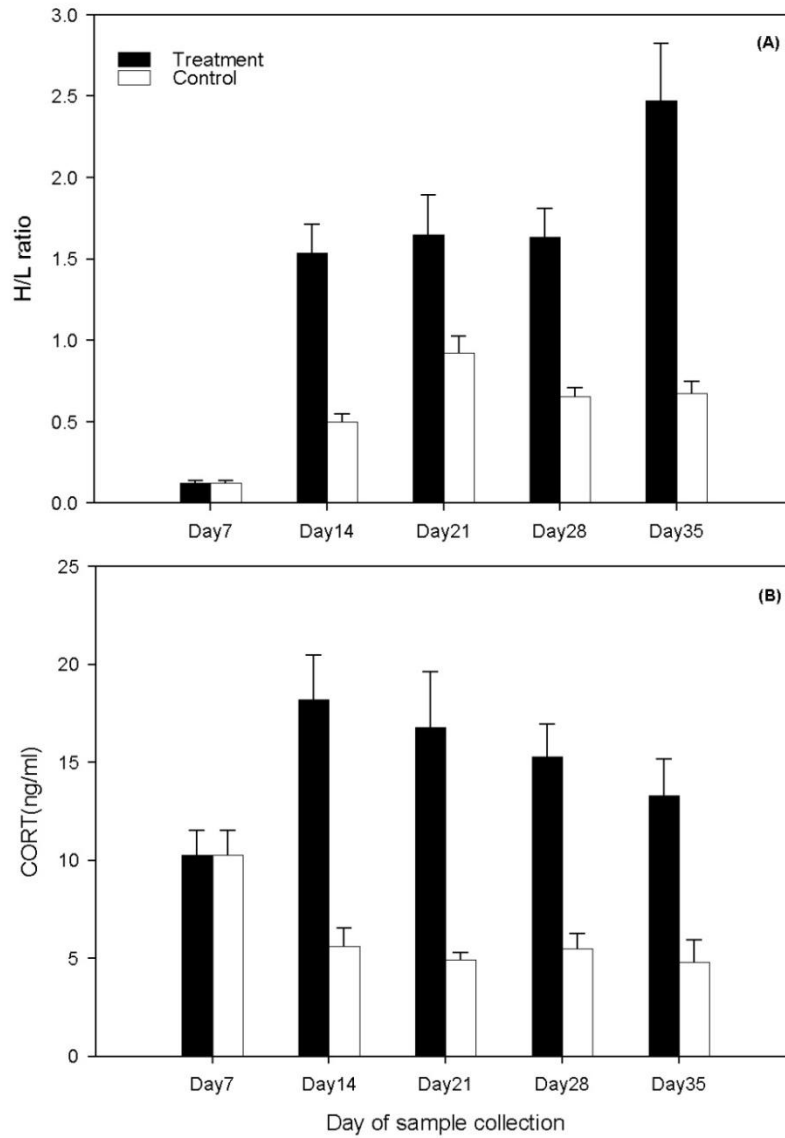


**Figure 3.1 Relationship between H/L ratio and sampling time (seconds, time from entering the room to obtaining the blood sample) in broiler chickens sampled on days 7 (prior to treatment), 14, 21, 28 and day 35 (following CORT 30 mg/kg addition to feed (treatment (A), n=53) or no feed additive (control (B), n=52).**

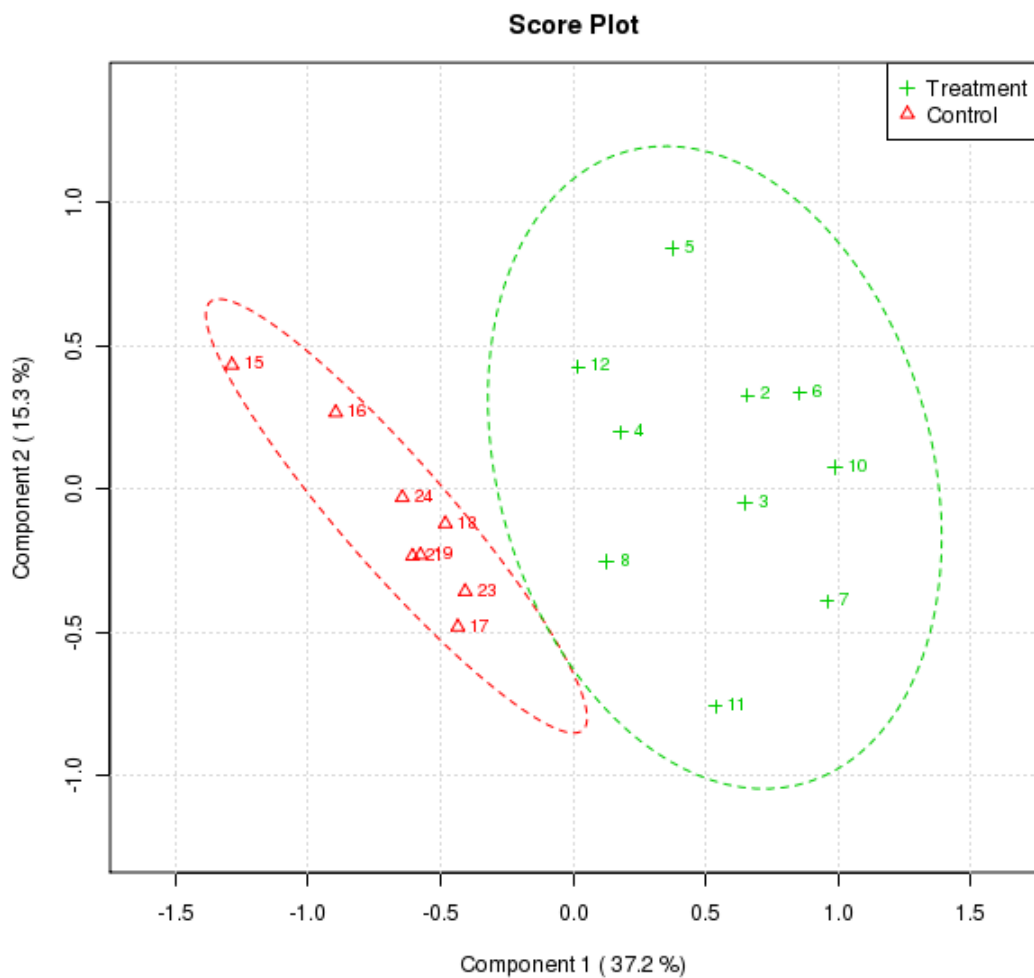


**Figure 3.2 Relationship between serum CORT (ng/ml) and sampling time (seconds, time from entering room to obtaining the blood sample) in broiler chickens sampled on days 7 (prior to treatment), 14, 21, 28 and day 35 (following CORT 30mg/kg addition to feed (treatment (A), n=53) or no feed additive (control (B), n=54).**

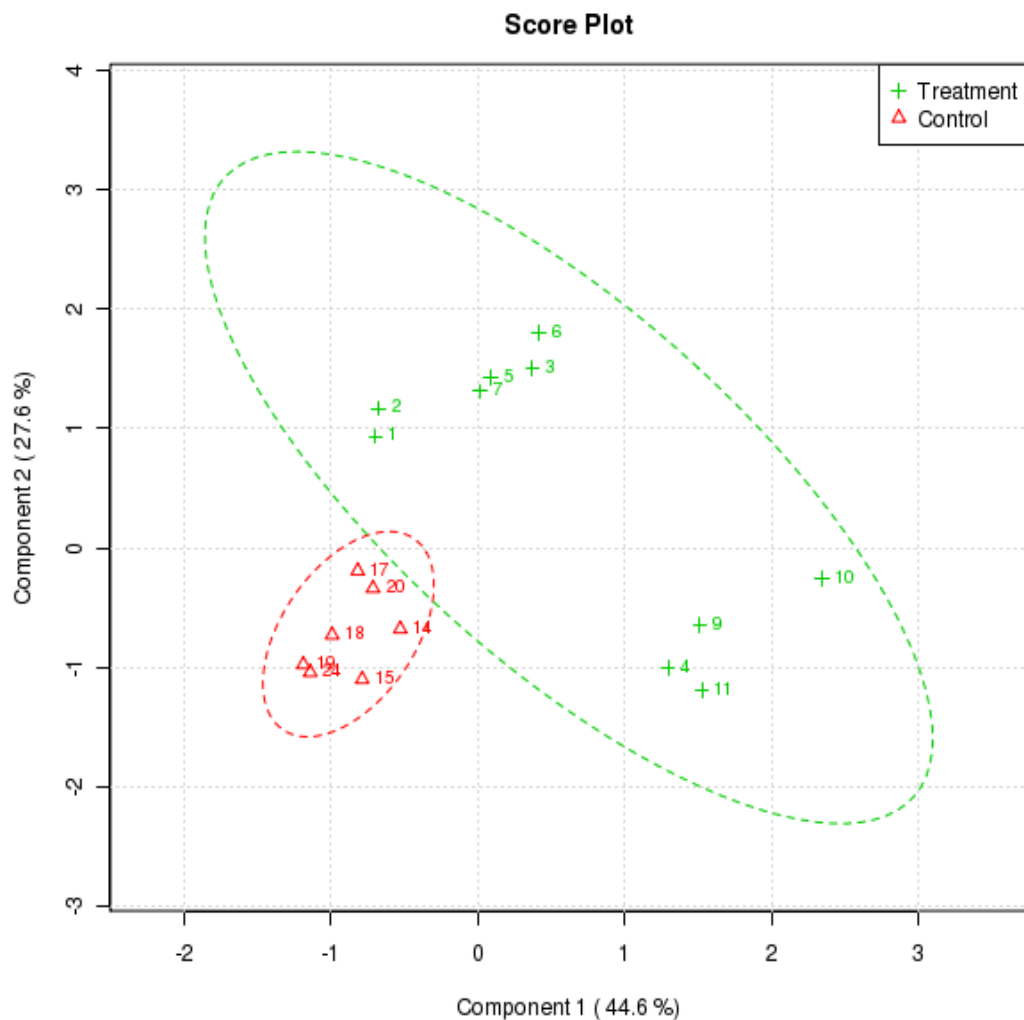




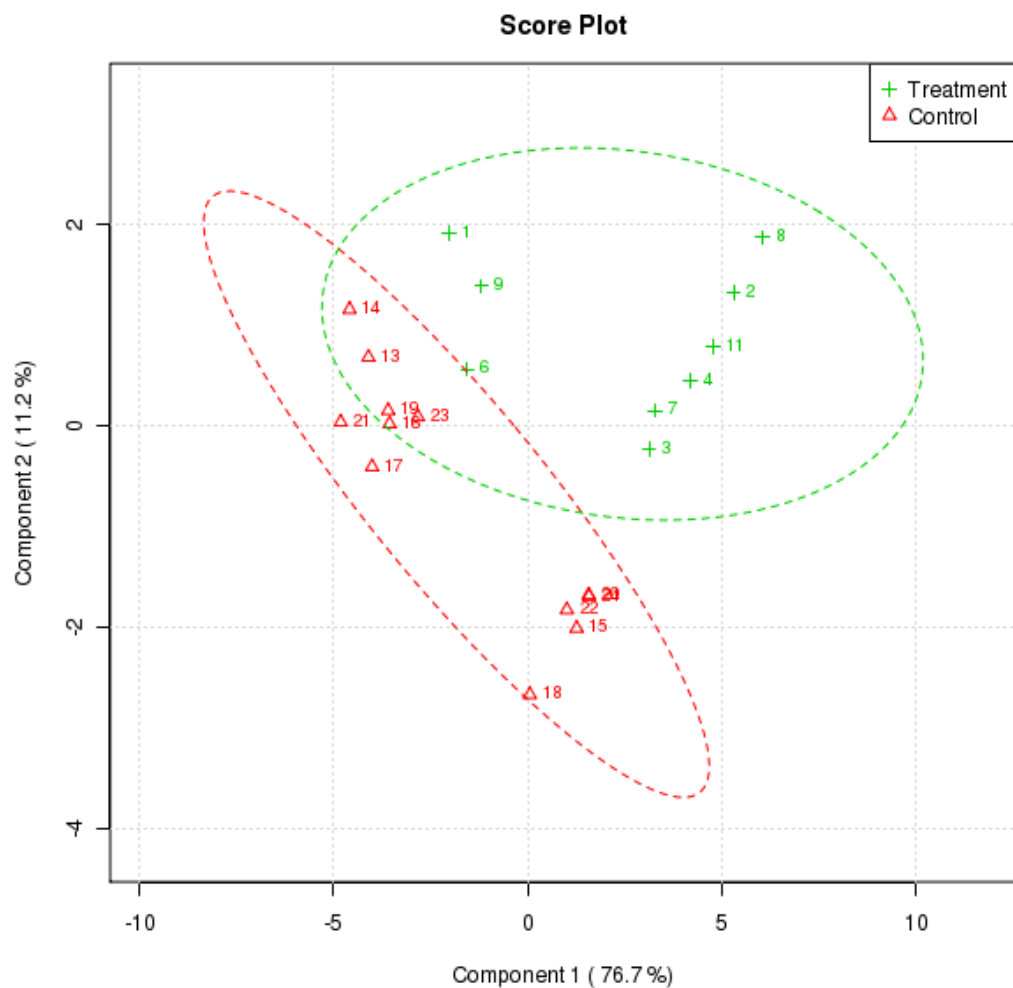
**Figure 3.3 Heterophil/lymphocyte (H/L) ratio (A) in broiler chickens sampled on days 7 (prior to treatment, treatment n=6 and control n=4), 14, 21, 28 (following CORT 30 mg/kg in feed, treatment n=12 and control n=12) and day 35 (treatment n=11 and control n=12) ( $F_{1, 105}=20.128$ ,  $P=0.002$ ). Serum corticosterone (CORT) ng/ml, (B) in broiler chickens sampled on days 7 (prior to treatment, treatment n=6 and control n=6), 14, 21, 28 (following CORT 30mg/kg in feed, treatment n=12 and control n=12) and day 35 (treatment n=11 and control n=12) ( $F_{1, 107}=32.632$ ,  $P=0.0001$ ). Error bars represent  $\pm$  Standard deviation(SD).**



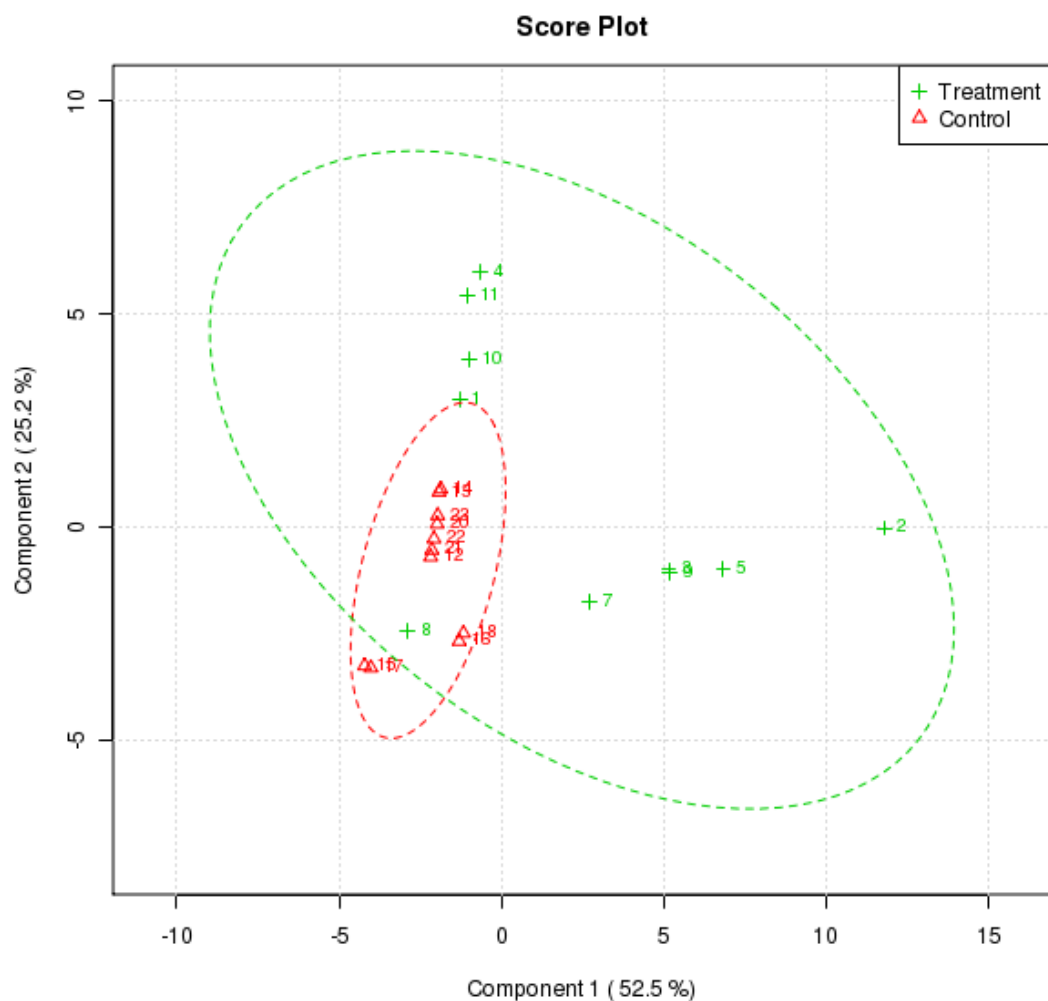
**Figure 3.4** Scores for the first two principal components of a PLS-DA model based on  $^1\text{H}$  NMR spectra of broiler chicken serum day 14.  $\text{Pr} \leq 0.002$ . Treatment birds fed CORT (30mg/kg diet,  $n=10$ ) and control ( $n=12$ ) with 6 outliers (treatment=2 and control=4) removed. Plot generated using MetaboAnalyst (Xia et al., 2009).



**Figure 3.5** Scores for the first two principal components of a PLS-DA model based on  $^1\text{H}$  NMR spectra of broiler chicken serum day 21. Permutation  $\text{Pr} \leq 0.01$ . Treatment birds fed CORT (30 mg/kg diet,  $n=10$ ) and control ( $n=8$ ) with 6 outliers (2 treatment, 4 control) removed. Plot generated using MetaboAnalyst (Xia et al., 2009).



**Figure 3.6 Scores for the first two principal components of a PLS-DA model based on  $^1\text{H}$  NMR spectra of broiler chicken serum day 28. Permutation  $\text{Pr} \leq 0.002$ . Treatment birds fed CORT (30 mg/kg diet,  $n=9$ ) and control ( $n=12$ ) with 3 outliers (treatment) removed. Plot generated using MetaboAnalyst (Xia et al., 2009).**



**Figure 3.7** Scores for the first two principal components of a PLS-DA model based on  $^1\text{H}$  NMR spectra of broiler chicken serum day 35. Permutation  $\text{Pr} \leq 0.01$ . Treatment birds fed CORT (30 mg/kg diet,  $n=10$ ) and control ( $n=11$ ) with 2 outliers (1 treatment, 1 control) removed. Plot generated using MetaboAnalyst (Xia et al., 2009).

**Table 3.2 Relative abundance of the significant metabolites based on the Variable importance in projection (VIP) scores of PLS-DA on respective sampling days. Treatment group were fed on CORT @ 30 mg/kg of diet from day 7, control group birds received no feed additive. A ↑ or ↓ arrow indicates high or low relative abundance respectively of the metabolite between the groups.**

Spectral ppm	Metabolites	Relative abundance	
		Treatment	Control
<b>0.86</b>	2-hydroxybutyric acid	Day 14- ↑	Day 14- ↓
		Day 28- ↑	Day 28- ↓
<b>1.22</b>	β-hydroxybutyrate	Day 14- ↑	Day 14- ↓
		Day 21- ↓	Day 21- ↑
		Day 28- ↑	Day 28- ↓
<b>1.26</b>	Isoleucine	Day 14- ↑	Day 14- ↓
		Day 21- ↓	Day 21- ↑
		Day 28- ↑	Day 28- ↓
		Day 35- ↑	Day 35- ↓

Spectral ppm	Metabolites	Relative abundance	
		Treatment	Control
<b>1.3</b>	Lactate	Day 14- ↑	Day 14- ↓
		Day 21- ↓	Day 21- ↑
		Day 28- ↑	Day 28- ↓
		Day 35- ↑	Day 35- ↓
<b>1.34</b>	Threonine	Day 14- ↓	Day 14- ↑
		Day 21- ↓	Day 21- ↑
		Day 35- ↓	Day 35- ↑
<b>1.5</b>	Lysine	Day 35- ↓	Day 35- ↑
<b>2.1</b>	Glutamine	Day 35- ↓	Day 35- ↑
<b>2.18</b>	Methionine	Day 35- ↓	Day 35- ↑
<b>2.26</b>	Valine	Day 21- ↑	Day 21- ↓
		Day 35- ↑	Day 35- ↓
<b>2.3</b>	β-hydroxybutyrate	Day 35- ↑	Day 35- ↓
<b>3.26</b>	Histidine	Day 14- ↑	Day 14- ↓
		Day 21- ↓	Day 21- ↑
		Day 35- ↓	Day 35- ↑

Spectral ppm	Metabolites	Relative abundance	
		Treatment	Control
3.3	Tryptophan	Day 28- ↓	Day 28- ↑
3.42	Carnitine	Day 14 -↑ Day 21- ↓	Day 14- ↓ Day 21- ↑
3.46	β – glucose	Day 14- ↑ Day 21- ↑ Day 28- ↓	Day 14- ↓ Day 21- ↓ Day 28- ↑
3.9	Betaine	Day 14- ↓ Day 21- ↓ Day 28- ↓	Day 14- ↑ Day 21- ↑ Day 28- ↑
4.14	β-hydroxybutyrate	Day 28- ↑	Day 28- ↓

### 3.4 Discussion

Seven birds in the TRT died. Two birds were found dead in their pen during their first week of acclimatisation. Three birds died between days 7 to 14 immediately after



starting the treatment diet containing CORT at 30mg/kg diet. Of the three, two were found dead in their pen while one collapsed during blood sampling and died subsequently. Handling stress in broilers has been reported to cause an increased incidence of mortality (Bayliss and Hinton, 1990). Even though utmost care was taken during collection, inexperienced handling might have caused the reported mortality. In addition, two birds were found dead in their pen between days 28 and 35. In comparison, CON experienced only two mortalities (first 7 days) during the entire experiment. This stark contrast in mortality between TRT (7 birds) and CON (2 birds) groups is suggestive that high stress levels and/or other metabolic effects associated with CORT treatment played a major contributing factor in the increased mortality. No post-mortems were done.

Simple regression showed a positive correlation between sampling time and the H/L ratio across all weeks in the TRT group (Figure 3.1A). With our treatment birds already in a stressed condition with high H/L values, it was expected to have a positive correlation between H/L ratio and sampling time. Moneva et al. (2009) reported that the H/L ratio increases as early as 15-20 minutes post handling for sample collection in normal chickens. On the other hand the H/L ratio of the control birds (Figure 3.1B) which were least stressed showed a negative correlation to sampling time. There was a positive correlation between CORT concentrations and sampling time TRT (Figure 3.2A,  $P \leq 0.60$ ) and CON (Figure 3.2B,  $P \leq 0.01$ ). This is similar to other research in avian species that suggests CORT can start increasing in as little as 1.5 to 2 minutes of sampling time (Chloupek et al., 2009; Romero & Reed, 2005; Voslarova et al., 2008). The sampling time here was  $82 \pm 36$  seconds and  $75 \pm 33$  seconds for TRT and CON, respectively. However the TRT showed an attenuated response to sampling time. Post et al. (2003) reported an attenuated response of the adrenal glands in the synthesis and release of CORT to an ACTH challenge in 21 day old broiler chickens receiving CORT via drinking water. In this study, altered adrenal response or exhaustion caused by excessive CORT may have resulted in an attenuated response to short term handling or sampling stress in TRT (Figure 3.2A).

The H/L ratio has been used as tool in assessing stress (Gross & Siegel, 1983). They evaluated the H/L ratio of chickens fed CORT and observed an increase in

circulating heterophils and a decrease in lymphocytes, therefore, suggesting this method to be a more reliable and less time variable tool of assessing stress than blood CORT levels. The H/L ratio was greater in the treatment group at all time points (Figure 3.3A) except for day 7 (prior to treatment). In addition, it appears that there is an increase in H/L ratio at day 35 in the TRT. High H/L ratio in TRT is similar to other research showing increased H/L ratio following a stressor (Gross & Siegel, 1983; Maxwell & Robertson, 1998; Puvadolpirod & Thaxton, 2000a; Shaniko Shini et al., 2008). However, H/L values in the treatment group were higher than most of the reported findings except for Jones et al (1988) who reported a H/L ratio of  $1.75 \pm 0.23$  after 11 days of stress, caused by an osmotic mini pump infusion of CORT in 16 week old chickens. Reference values of 0.2, 0.5 and 0.8 of H/L ratio have been reported to indicate low, moderate and high stress levels in chickens (Campo & Davila, 2002b; Gross & Siegel, 1983). On day 21, the H/L ratio of CON ( $0.92 \pm 0.35$  Figure 3.3A) birds was suggestive of stress even though the average body weight, an indicator of growth for the same period of the group (0.906 kg/bird) suggesting normal growth in comparison to the average body weight of TRT 0.516 kg/bird (Table 3.1). Chickens fed on a CORT diet have been reported to have retardation in growth due to decreased muscle protein synthesis and increased catabolism of the existing protein reserves to serve the energy needs (Puvadolpirod & Thaxton, 2000a; Scanes, 2009; Virden et al., 2007). Elevated CORT generally increases H/L ratio through recruitment of heterophils from bone marrow and decreases exit into the surrounding tissues. Alternatively there is increased exit of lymphocytes from the circulation into lymphoid tissue (Ottaway & Husband, 1994).

Research using H/L as an indicator of stress demonstrates differences between studies with H/L ratio being influenced by age, sex, breed, social ranking, rearing methods, etc. (Campo & Davila, 2002a, 2002b; Gross & Siegel, 1983; Maxwell, 1993; Maxwell & Robertson, 1998; Moneva et al., 2009). Data compiled by (Maxwell, 1993) on the avian blood leukocyte response to stress have shown that severe food restriction causes a heteropenia, whereas climate, environmental, transport and heat stress causes heterophilia. In life threatening situations heteropenia and basophilia are seen and H/L ratio cannot be used as measure of stress in such conditions (Maxwell, 1993). Campo et al (2002a) have shown that birds reared under a photoperiod of 23L:1D do not have

discernible alterations of H/L ratio to suggest stress, but they do display increased duration of tonic immobility which may suggest an enhanced fear response. My findings add to the discrepancy in the use of H/L ratio as a reliable indicator of stress as control birds had increased H/L ratio of 0.92 on day 21. My findings suggest that H/L ratio is less variable with time as it stayed consistently over 1.5 H/L ratio for 4 weeks, indicating chronic stress. No other studies have examined growing broilers fed CORT (30mg/kg diet) for 4 weeks. A study by Gross and Siegel (1983) showed similar findings of H/L ratio of  $1.58 \pm 0.26$  in 12 week old chickens fed on CORT (30 mg/kg diet) for two days. Although H/L ratios by themselves may suggest of a stress response (Gross & Siegel, 1983) they do not indicate the magnitude of stress experienced and hence cannot be used as the sole indicator of stress (Maxwell, 1993; Maxwell & Robertson, 1998; Moneva et al., 2009).

The most immediate noticeable change following exposure to a stressor is the spike in plasma CORT concentrations. Studies (Romero & Reed, 2005; Wingfield et al., 1982) have demonstrated that blood samples collected within 2 to 3 minutes of handling time have CORT concentrations which better represent an unstressed state (i.e. baseline). The half life of CORT in chickens is 8 minutes (Carsia et al., 1988). CORT has been successfully used for assessment of stressful events (Post et al., 2003; Puvadolpirod & Thaxton, 2000a, 2000b, 2000c), but there is controversy regarding its usage as a reliable indicator of stress (Rushen, 1991). In this study, CORT measured at day 14 in TRT showed a dramatic increase compared to samples taken at 7 days (prior to treatment) and to CON levels. Levels remained elevated in comparison to CON, although there was a decline at day 35 (Figure 3.3A). This decline may have been because of down regulation of CORT to physiological levels, adrenal atrophy, or decreased feeding behavior leading to less dietary intake of CORT. During chronic stress birds can experience decreased basal CORT related to habituation to the stressor, adrenal exhaustion or down regulation in the synthesis and release of CORT to prevent its deleterious effects (Rich & Romero, 2005)

In comparison, CORT in the CON (Figure 3.3B) were well within the physiological limits. Broilers between 2 to 7 weeks of age showed decreasing levels of CORT as they get acclimatized to their environment (Thaxton et al., 2005). This was

evident with CON which had a baseline CORT level of  $10.26 \pm 4.72$  ng/ml on day 7 and an average serum CORT level of  $5.20 \pm 0.39$  ng/ml between days 14 to day 35. Data compiled by (Rushen, 1991) demonstrated varying plasma CORT concentrations between experiments studied with similar conditions. For example, plasma CORT can be increased (Gibson et al., 1986), decreased (Koelkebeck et al., 1987) or the same (Craig et al., 1986) in laying hens reared in battery cages compared with those reared in pens. Given that the effect of CORT depends on a number of other factors such as receptor availability, sensitivity and binding protein availability (Landys et al., 2006), and as these factors can differ between species and prior stressful events, it becomes more difficult to compare or use CORT as a sole indicator of stress (Chloupek et al., 2009; Romero, 2004). To overcome the inconsistency, researchers have resorted to using a combination of blood H/L ratio and CORT in the assessment of stress and welfare (Moneva et al., 2009).

Metabolomic analysis of the serum samples showed the presence of amino acids (AAs) isoleucine, valine, threonine, lysine, glutamine, histidine, methionine and tryptophan, ketone body  $\beta$  hydroxybutyrate and other metabolites like carnitine, betaine, lactate and sugars like glucose differentiated TRT birds from CON birds. Out of the top common metabolites identified metabolites like isoleucine, lysine, valine and tryptophan were highly suggestive of stress induced anxiety, depression and behavioral changes while metabolites like methionine, betaine, histidine suggested a compromised methylation process and oxidative stress response. Metabolites threonine, glutamine and histidine suggested reduced immune response while metabolites like lactate, glucose and  $\beta$  hydroxybutyrate suggested the on-going process of providing energy for the physiological needs of the body.

When exposed to a stressor, energy is required to fuel bodily activities that will deal with the stressor and restore homeostasis (Sapolsky et al., 2000). A stress response involves increased gluconeogenesis ensuring availability of glucose and insulin resistance preventing storage of glucose (Tsigos, 2002; Dupont et al., 1999b, Sapolsky et al., 2000). The brain and erythrocytes are always in need of readily available glucose. The brain derives about 20% of its energy from ketone bodies and the remainder 80%

form glucose, whereas, erythrocytes lacking mitochondria are solely dependent on glucose as the primary source of energy (Murray et al., 2006). Because available carbohydrate sources of glucose are being used up by the brain and erythrocytes, others tissues must meet their energy requirements via gluconeogenesis (synthesis of glucose from non carbohydrate sources). This is achieved through a variety of routes including glycolysis using lactate, amino acids (AAs) from protein degradation of muscle reserves, ketone bodies synthesized from acetyl CoA in liver and glycerol from adipose tissue. In response to a stressor, the main function of CORT is to provide the necessary glucogenic substrates and regulate the glucogenic pathways in the liver (Exton et al., 1972; Scanes, 2009).

Sugars including glucose are readily available sources of energy for bodily function. Stress has been shown to lead to gluconeogenesis and thereby increased plasma glucose with the intent of providing energy to all bodily cells and tissues (Sapolsky et al., 2000; Selye, 1951). In this study, glucose was of high relative abundance in the TRT birds on days 14 and 21 with subsequent low relative levels by day 28 (Table 3.2). The increased levels seen during day 14 and 21 are expected as chronic stress leads to hyperglycaemia in provisioning of energy. The low levels seen by day 28 might suggest of the depletion in energy reserves as the birds were already under chronic stress for three weeks. Body weight data (Table 3.1) for the corresponding period also supports this fact as TRT birds (0.683 kg) had reduced growth in comparison to the CON birds (1.342 kg).

Lactate was found in all of sampling days in high relative abundance on days 14, 28 and 35 and in low relative abundance on day 21 in the TRT group (Table 3.2). Similar findings of the presence of lactate was reported by Teague et al (2007) in low abundance in acutely stressed (day 1- 9) Sprague-Dawley rats followed by an increase in abundance in chronically stressed rats (day 9-35). Lactate is synthesised anaerobically during glycolysis in the skeletal muscles and erythrocytes and is used as a glucogenic substrate by the liver in the production of glucose via the lactic acid cycle (Murray et al., 2006). Increased glucagon during stress also aids in the gluconeogenic process utilising AA and lactate. Gluconeogenesis is a highly oxygen dependant process with consumption of one oxygen molecule for every ATP formed. This leads to an imbalance in the oxygen

leading to reversible adaptative process of anaerobic glycolysis producing lactate rather than the normal aerobic glycolysis of pyruvate production by the skeletal muscles (Wishart et al., 2009). Increased levels of CORT have been reported to exhaust aerobic cellular respiration and enhance anaerobic respiration leading to high levels of plasma lactate (Nijdam et al., 2005). Olanrewaju et al (2006) reported an increase in erythropoiesis in stressed chickens. This provides evidence that more erythrocytes with increased haemoglobin levels are needed to meet increased oxygen demand during stress. Increased lactate in this study may be attributed to the provision of gluconeogenic substrates for energy production during stress and to compensate for the excessive oxygen demand created by gluconeogenesis.

Insulin suppresses hormone sensitive lipase essential for lipolysis and opens up GLUT-4 glucose transporters in adipose tissues enabling uptake of glucose and storage as triacylglycerides. In chickens, the liver is the major site of lipogenesis (Leveille et al., 1968). In a stressed scenario, insulin resistance due to increased CORT levels accompanied by increased glucagon (Exton et al., 1972), stimulates the release of hormone sensitive lipase thereby providing abundant plasma free fatty acids. These free fatty acids serve as source of acetyl CoA which is an important substrate for energy synthesis via the TCA cycle. Apart from entering the TCA cycle for energy production the acetyl CoA formed from the fatty acid oxidation are converted into ketone bodies, namely acetone, acetoacetate and 3 hydroxybutyrate in the liver and serve as substrates for the gluconeogenic process (Murray et al., 2006). In this study, 3 hydroxybutyrate was found in relatively high abundance in the TRT birds in sampling days 14, 28 and 35 and low relative abundance in day 21 (Table 3.2). This variation between days might be suggestive of the undergoing homeostatic process in the TRT birds. However quantification in future experiments would help us in understanding the regulatory process. Ketone bodies are known to be acidic and when accumulated in large amounts for long periods of time lead to ketoacidosis (Murray et al., 2006).

In the stress scenario, where hyperglycaemia is the norm with an enhanced gluconeogenesis (Dupont et al., 1999, Sapolsky et al., 2000), increased carnitine aids in production of acetyl CoA and ketone bodies from fatty acids for the provision of energy. Fat metabolism, in this study, was altered as indicated by an increased relative abundance

of carnitine in birds fed CORT (30 mg/kg diet, chapter 3) had a high relative abundance of carnitine on day 14 with low relative abundance by day 21. Carnitine is synthesised endogenously by the body in muscles, kidney and liver (Murray et al., 2006; Wishart et al., 2009). Broilers supplemented with dietary carnitine had reduced abdominal fat accumulation (Rabie & Szilagyi, 1998; Xu et al., 2003). Carnitine has been reported to enhance the fatty acid oxidation process and decrease adiposity (Janssens et al., 1998; Murray et al., 2006; Xu et al., 2003). Conversely, reduced plasma carnitine has shown to decrease the  $\beta$  oxidation process of fatty acids leading to hypoglycaemia and increased adiposity (Murray et al., 2006).

Proteins serve as the major non-carbohydrate source of energy during periods of stress (Siegel & Vankampen, 1984). Muscle breakdown produces amino acids which can then be utilized for gluconeogenesis in the liver. Under conditions of stress or starvation the carbon skeleton derived after deamination (removal of amino group) from AAs is utilized by the citric acid cycle for energy, used for the synthesis of ketone bodies or used in the synthesis of glucose by gluconeogenesis (Murray et al., 2006). In situations of starvation and stress amino acids form the main glucogenic substrates (Brosnan, 2003). Studies have shown that under chronic stress, CORT apart from decreasing protein synthesis also causes the degradation of protein reserves to AA, in order to provide energy (Scanes, 2009). Broilers treated with exogenous ACTH or CORT excrete more non-protein nitrogenous compounds and have reduced muscle mass indicating extensive protein catabolism (H. Lin et al., 2006; Puvadolpirod & Thaxton, 2000d). Odedra et al (1983) reported an increase in protein degradation and decrease in protein synthesis following CORT administration in rats.

Of the three branched chain amino acids (BCAA (i.e., isoleucine, leucine and valine), isoleucine and valine were found in the top metabolites (Table 3.2). Isoleucine, leucine and valine have been found to be involved in stress, and energy and muscle metabolism (Wishart et al., 2009). Branched chain amino acids are primarily metabolized in the skeletal muscles with tissue aminotransferases reversibly interconverting all the three BCAA to  $\alpha$  keto acid and thereby maintaining a balance between them. Stress, trauma, starvation and fever increase requirements of BCAA (Li et al., 2007). Endogenous synthesis of glutamine from skeletal muscles (principal AA involved in

ammonia excretion) is from BCAA. The BCAA enhance antibody and cytokine production in the body, inhibit cell apoptosis and are essentially important in immune stimulation. With enhanced gluconeogenesis during stress (Dupont et al., 1999; Sapolsky et al., 2000), isoleucine and leucine are converted to acetyl CoA and enter the citric acid cycle (TCA) to produce energy (ATP), while valine is converted to succinyl CoA and enters the TCA cycle. Acetyl CoA is also used in the synthesis of ketone bodies in the liver, which in turn serve as energy fuel during prolonged fasting and chronic stress (Murray et al., 2006). Branched chain amino acids are important sources of non carbohydrate energy.

Teague et al (2007) reported a decrease in the BCAA following an acute stressor in Sprague-Dawley rats on day 1 of the experiment. They reported an increase in BCAA leucine and valine during chronic stress lasting 44 days. In this study, isoleucine was found at all sampling points and in relatively high abundance in the TRT group compared to the CON group (Table 3.2). With stress induced hyperglycaemia and ketoacidosis, an increase isoleucine is expected (McCowen et al., 2001; Soriano et al., 1967). Valine was found in relatively high abundance in TRT on day 21 and day 35 in comparison with CON. Leucine another BCAA did not rank in the top metabolites in all our sampling days. Despite leucine not being in the top metabolites, it is worth mentioning that leucine aids in the process of protein synthesis and prevents protein degradation (Harper et al., 1984; Wu, 2009). It is essential to have a balance of all three BCAA for leucine to function appropriately. An excess of leucine and/or isoleucine or valine by themselves does not contribute to protein synthesis (Harper et al., 1984). Excessive leucine can cause increased depletion of isoleucine and valine leading to accelerated protein degradation. Excessive leucine and valine also compete for tryptophan receptors in the brain, thereby preventing tryptophan entry into brain leading to inadequate synthesis of serotonin and dopamine. As tryptophan is essential for the synthesis of serotonin (Murray et al., 2006; Yogman & Zeisel, 1983), decreased levels of tryptophan in turn reduce serotonin levels leading to depressive symptoms often associated with stress (Russo et al., 2003). Further quantification and examination of the balance between the three BCAA under chronic stress conditions requires further investigation.



Tryptophan is an essential AA and is also the precursor of serotonin a brain neurotransmitter and a potent vasoconstrictor. Tryptophan levels will also influence melatonin as it is biosynthesised from serotonin (Murray et al., 2006). Tryptophan also enters the TCA cycle as a source of energy by getting converted to acetyl CoA (Murray et al., 2006). In this study, tryptophan was of low relative abundance in the TRT birds on day 35 (Table 3.2). Sprague-Dawley rats subjected to a variety of stressors had a decrease in tryptophan (Wang et al., 2009). As tryptophan is essential for the synthesis of serotonin (Murray et al., 2006), decreased levels of tryptophan in turn reduce serotonin levels leading to depressive symptoms often associated with stress (Russo et al., 2003). Elevated CORT increases the activity of the enzyme tryptophan oxygenase in the liver thereby leading to excessive oxidation of tryptophan resulting in decreased plasma levels. This may explain the low relative abundance of tryptophan seen in the TRT group

Lysine is converted to crotonyl CoA and further into acetyl CoA which enters the TCA cycle for energy production or is converted to ketone bodies in the liver (Murray et al., 2006). Lysine was seen only on day 35 in this study and had low relative abundance in TRT in comparison with CON (Table 3.2) birds. Similar NMR experiments on Sprague-Dawley rats subjected to restraint stress showed decreased levels of lysine (Teague et al., 2007). Lysine deficiency caused a reduction in cytokine synthesis and proliferation of lymphocytes with an impaired immune response in chickens (Chen et al., 2003; Konashi et al., 2000; Li et al., 2007). In humans, requirements of lysine are reported to increase during stress and low plasma levels have been associated with depression (Wishart et al., 2009). The low levels seen in the TRT birds might suggest impaired immunity along with stress induced depression.

Glutamine is the most abundant plasma amino acid found in the body (Li et al., 2007; Wu, 2009). Being the most important precursor of urinary ammonia (excreted as uric acid), renal catabolism of glutamine plays a vital role in the acid-balance of the body (Brosnan, 2003; Murray et al., 2006). Glutamine is synthesized in the skeletal muscles with BCAA being the major precursors. Glutamine and glutamate are also the major AAs that replenish other AA reserves of the body (Wishart et al., 2009). Glutamine becomes a conditionally essential AA in conditions of stress and trauma (Lacey & Wilmore, 1990;

Wu, 2009). Studies have shown that a diet rich in glutamine increases protein synthesis or muscle accretion (Wu, 2009). Glutamine is the major source of energy in rapidly dividing cells like intestinal enterocytes and cells of the immune system. Glutamine enhances the proliferation of lymphocytes; cytokines and antibody formation and also inhibits cell apoptosis (Li et al., 2007). In this study, glutamine did not rank among the top metabolites in the TRT on day 14, 21 and 28 but was seen on day 35 and had lower relative abundance in comparison with CON (Table 3.2). This might be due to the depletion of glutamine during stress and illness. In humans glutamine decreases during periods of severe illness (Lacey & Wilmore, 1990) trauma, surgery and chronic stress (Li et al., 2007; Wilmore, 1991; Wu, 2009). During periods of protein catabolism, glutamine along with alanine account for approximately 50% of the AA produced. Glutamine is mainly synthesized intramuscularly from glutamate, valine, isoleucine, and asparagine (Brosnan, 2003). With glutamine being important in immune cell proliferation and aiding in protein synthesis, it would be interesting to know how glutamine supplementation might alleviate chronic stress in birds.

Threonine is an essential AA, found in abundance in blood plasma. It is converted to acetyl CoA entering the TCA cycle as a source for energy production (Murray et al., 2006). It stimulates the growth of the thymus (Murray et al., 2006; Wishart et al., 2009) and is involved in the synthesis of intestinal mucin and maintaining intestinal integrity (Wu, 2009). Dietary sources of threonine have been shown to increase antibody production and inhibit cell apoptosis (Li et al., 2007). In this study, threonine was lower in relative abundance in the TRT birds on day 14, 21 and 35 (Table 3.2). This might indicate a compromised immune response in the TRT birds.

Histidine is converted to  $\alpha$  ketoglutarate which then enters into the TCA cycle for energy production (Murray et al., 2006). It was found in relatively high abundance in the TRT group on day 14 and subsequently in low relative abundance on days 21 and 35 in comparison to CON (Table 3.2). Histidine undergoes decarboxylation and yields histamine which is an important mediator of inflammatory reactions (Murray et al., 2006). Histidine is also an important component of haemoglobin and in its function (Wu, 2009). The increased levels of histidine on day 14 seen in this study may have been

sequelae to ketoacidosis. Diabetic ketoacidic children have increased excretion of urinary histidine (Szabo et al., 1991). Insulin deficiency or excess levels of CORT has also been reported to be principle causes of ketoacidosis in human subjects (Schade & Eaton, 1979). Olanrewaju et al (2006) reported an increase in erythropoiesis in stressed chickens. This provides evidence that more erythrocytes with increased haemoglobin levels are needed to meet increased oxygen demand during stress. However subsequent low relative abundance of histidine by day 21 and 35 in the TRT might indicate a compromised response to anti oxidative, anti inflammatory and erythropoetic processes.

Methionine is also known to enter the TCA cycle for energy production as succinyl CoA (Murray et al., 2006). Methionine is an indispensable dietary AA (supplemented through diet) ranked within the top metabolites in the TRT group. On day 35, methionine was lower in relative abundance compared with CON (Table 3.2). Dietary supplementation of methionine has shown improve the body weight gain, feed conversion ratio (Garcia Neto et al., 2000) and improved production of antibodies and the immunoglobulin IgG in broilers (Tsiagbe et al., 1987). Increased levels of plasma methionine causes an increase in levels of its metabolite homocysteine (Murray et al., 2006). Raised levels of homocysteine have been associated with increased incidence of cardiovascular diseases in humans (Boushey et al., 1995). Methionine toxicity has been linked to depression, spleen and liver hemosiderosis (excess hemosiderin built up leading to organ damage) and reduced feed utilization in chickens (Ekperigin & Vohra, 1981; Harter & Baker, 1978). Deficient methionine levels has been associated with fatty liver (steatohepatitis) degeneration in rats (Oz et al., 2008). Autistic children had low levels of plasma methionine with increased vulnerability to oxidative stress (James et al., 2004a). Methionine deficient diet in chickens has been associated with poor body weight gain and reduced thyroid hormone and insulin like growth factor-2 levels (Carew et al., 2003). A decreased methionine level in the TRT group indicates a compromised methionine - homocysteine metabolism.

Betaine is an organic osmolyte protecting cells and enzymes (Craig, 2004). It is found in animal tissues and is reversibly synthesized as an intermediate product from choline (Murray et al., 2006). Being a methyl donor (methyl groups are important for carnitine and creatine synthesis), betaine supplementation in poultry and pigs has been

found to increase body weight gain and enhance intestinal cell activity (Eklund et al., 2005). Betaine is also important in the methionine – homocysteine metabolism as it aids in the remethylation of homocysteine to methionine. It is essential to have adequate levels of methionine as it gets converted to S-adenosyl methionine an important metabolite involved in the maintenance of DNA structure, synthesis of epinephrine and choline. It was found in low relative abundance on days 14, 21 and day 28 and absent in the top metabolites on day 35 in our TRT birds (Table 3.2). Being an osmolyte involved in cell protection and having a methyl sparing action on choline and methionine (Kidd et al., 1997) low levels in the treatment group might suggest altered methionine – homocysteine metabolism and compromised response to oxidative stress. Betaine supplementation has been reported to alleviate the compromised methylation process and poor oxidative stress response in autistic children (James et al., 2004b).

### **3.5 Conclusion**

Analysis of the H/L ratio showed that it was greater in the treatment group at all time points except for day 7 (prior to treatment). High H/L ratio in the TRT is similar to other research showing increased H/L ratio following a stressor (Gross & Siegel, 1983; Maxwell & Robertson, 1998; Puvadolpirod & Thaxton, 2000a; Shaniko Shini et al., 2008). However, H/L values in the treatment group were higher than most of the reported findings and this might be due to the high level of stress induced by feeding CORT at 30 mg/kg diet for four weeks in the TRT birds. In comparison the change in H/L ratio seen in the CON birds was largely due to age related changes with levels decreasing with age. In this study, CORT increased dramatically at day 14 in the treatment group following the addition of CORT (30 mg/kg feed) to the diet. While levels remain elevated in comparison to the control group, there was a gradual decline in CORT to day 35 in the TRT birds. This decline may be suggestive of a down regulation process in bringing the serum CORT levels to physiological levels. During chronic stress, after the initial high CORT levels, birds can demonstrate decreased concentrations related to habituation to the stressor, adrenal exhaustion or down regulation in the synthesis and release of CORT to prevent its deleterious effects (Rich & Romero, 2005). In comparison CORT in the control birds were well within the physiological limits with age related changes. Broilers between 2 to 7 weeks of age showed decreasing levels of CORT as they get acclimatized

to their environment (Thaxton et al., 2005). Metabolomics was able to successfully differentiate the TRT birds from the CON birds. Analysis of the serum samples showed the presence of amino acids isoleucine, valine, threonine, lysine, glutamine, histidine, methionine, and tryptophan, ketone body  $\beta$  hydroxybutyrate and other metabolites like carnitine, betaine, lactate and sugars like glucose. Out of the top common metabolites identified, isoleucine, lysine, valine and tryptophan were highly suggestive of stress induced anxiety, depression, behavioral changes and ketoacidosis while metabolites like methionine, betaine, histidine suggested compromised methylation process and oxidative stress response. Metabolites threonine, glutamine and histidine suggested reduced immune response while metabolites like lactate, glucose and  $\beta$  hydroxybutyrate suggested the under going process of providing energy to the physiological needs of the body.

## CHAPTER 4

### COMPARING $^1\text{H}$ NMR METABOLOMICS AS A NOVEL TOOL OF ASSESSING STRESS WITH CORTICOSTERONE AND HETEROPHIL: LYMPHOCYTE RATIO IN BROILER CHICKENS (*GALLUS DOMESTICUS*) REARED UNDER 4 DIFFERENT PHOTOPERIODS.

#### 4.1 Introduction

Light is an important management technique in broiler production. Artificial lighting can be manipulated for broilers in three ways: photoperiod, wavelength, and light intensity (Lewis & Morris, 2000; Prescott & Wathes, 1999). Lighting regimes have the potential to modulate various behavioral and physiological pathways and therefore may have significant effects on broiler production and welfare (Olanrewaju, 2006). Retinal photoreceptors (rods and cones) are important in growth and behavioral changes, whereas, the hypothalamic photoreceptors influence sexual maturation (Lewis & Morris, 2000). In birds, light acting upon the extraretinal photoreceptors (Menaker & Underwood, 1976) present in the pineal gland and hypothalamus are important in entraining circadian rhythms and also influence sexual maturation (Lewis & Morris, 2000; Zawilska et al., 2004). Trends in the broiler chicken industry is to rear them under continuous or near continuous lighting for their lives. Continuous lighting is thought to be necessary for maximal feed intake and therefore fast growth (North and Bell, 1990). However, this practice in broilers has been linked to emergence of the “diseases of fast growth” such as ascities, sudden death syndrome, and skeletal disorders (Malleau et al., 2007), all of which reduce bird welfare and decrease profit margins to producers. There is also evidence that sleep allows important metabolic processes to occur including general tissue restoration and that disruption of sleep can result in pathological stress-like symptoms (Rechtschaffen et al., 1983).

It is believed that continuous lighting allows broilers more time to feed and thereby increased growth rate (North and Bell, 1990), however, many studies have demonstrated negative effects associated with this practice. Classen et al. (1989) reported that very long days lead to sub-optimal bird performance as circadian rhythms of metabolic functions and hormone concentrations are disrupted. Immune system impairment was demonstrated with constant lighting in White Leghorn cockerels (Kirby

and Froman, 1991) and increased susceptibility to disease in Japanese quail (*Coturnix japonica*) (Moore & Siopes, 2000). Continuous lighting may also cause buphtalmia (enlarged eyes) and blindness in birds (Whitley et al., 1984). Extended lighting photoperiods confounded with low lighting intensities had a negative effect on breast meat weight production of broilers in spite of improving the broiler live performance (Lien et al., 2009). Campo et al. (2007) demonstrated stress-like symptoms in 11 breeds of chicken exposed to photoperiod of 24L:0D in comparison with 14L:10D had increased duration of tonic immobility (a complex behavioral reaction to a painful and/or fear-inducing stimulus) producing a catatonic-like state sometimes used to measure stress (Maser & Gallup, 1974; Nash et al., 1976) and increased heterophil/lymphocyte (H/L) ratio. Variations in photoperiod may act as stressors in broilers leading to alteration of physiology and reduction in production. Therefore, it is important to assess lighting regimes and its potential impact on broiler welfare. Concern about stress in poultry production has long been an issue from both an animal welfare and production standpoint. The “stress response” is an evolved trait that allows an animal to deal with changes or conditions within its environment. Proper response to stress can be beneficial in life threatening situations; however, chronic stress can have marked deleterious effects on reproduction, behavior, metabolism and immunocompetence (Siegel, 1980). Any situation that elicits a response (either psychological (i.e. fear, noise), pathological (i.e. disease) or physiological (i.e. overcrowding) can be considered a “stressor”. Also conditions within the environment including: climate (i.e. extreme heat, cold or humidity), environmental (i.e. poor ventilation, light regimes), nutritional (i.e. shortages of nutrients), physical (i.e. handling, transport), can act as stressors. Conditions which induce stress in an animal can reduce welfare and as well as performance (Scheele, 1997; Olanrewaju et al., 2006) thus measurement of stress can be used to evaluate and validate management techniques.

Identification of stress in the poultry industry has been done using various physiological endpoints but the two most accepted indicators of stress are elevated corticosterone (CORT) and increased circulating H/L ratios (Puvadolpirod and Thaxton, 2000). However, the length of time taken to obtain the blood sample can influence both CORT as early as 3 minutes (Voslarova et al., 2008) and H/L ratio within the first 15-20

minutes (Moneva et al., 2009). These errors may lead to problematic interpretation of data. Lack of consistency between studies has resulted in skepticism when using them to quantify animal welfare in the poultry industry. Serkova et al. (2009), defines metabolomics as ‘omic’ sciences in systems biology, and is used for the assessment and validation of endogenous small-molecule biochemicals (metabolites) within a biologic system”. Environmental metabolomics is an emerging approach for examining metabolic profiles in biological systems exposed to environmental stress (Lin et al., 2006). Utilization of 1-dimensional  $^1\text{H}$  Nuclear Magnetic Resonance spectroscopy (NMR) can provide insightful mechanistic information on effects of stressors by simultaneously measuring a large number of low molecular weight endogenous metabolites (Viant et al., 2003). Metabolic profiles can be examined using pattern recognition algorithms to reveal subsets of metabolites, potentially identifying biomarkers (Lin et al., 2006; Viant et al., 2003). The advantage of using NMR over more traditional methods is that it provides information about multiple metabolites, many that are not influenced by sampling handling time and may potentially identify biomarkers for stress. The objective of this study is to compare the use of NMR to more traditional methods of stress assessment (CORT and H/L ratio) to assess the impact of photoperiod on stress in broilers. In addition, I will compare the metabolite profile identified from this study with potential biomarkers of chronic stress identified from the experiment discussed in Chapter 3 of this thesis to identify photoperiod length management that is least stressful to the birds.

## **4.2 Materials and methodology**

### **4.2.1 Animals**

A total of eight rooms at the University of Saskatchewan Poultry Centre were used to house Ross x Ross 308 broilers sourced from Lilydale (Calgary) at a target stocking density of  $30\text{kg/m}^2$ . Chicks were vaccinated with Coccivac® at the hatchery prior to delivery. The minimum ventilation curve was to match bird numbers and bird weight and ammonia concentrations did not exceed 25 ppm. For complete experimental details please see (Schwean-Lardner et al., 2012a). Commercial feed was provided on an *ad libitum* basis with the major ingredients being corn and soybean meal and diet specifications and feeding amounts were according to Aviagen recommendations (Aviagen, 2007a). Feed did not contain a coccidiostat or a growth promotant. Chicks



were placed without sex differentiation into each room and lighting to 7 days of age was set at 23 hours light (L): 1 hour dark (D) with a minimum of 20 lux intensity. At 7 days, two rooms were randomly assigned to each of four experimental photoperiod treatments: (a) 23L:1D (**23L**), (b) 20L:4D (**20L**), (c) 17L:7D (**17L**), and (d) 14L:10D (**14L**). Light treatments were in place for the remainder of the study (28 days) and light was provided by incandescent bulbs at a minimum light intensity of 8 lux. Animals were treated in accordance with the Canadian Council on Animal Care guidelines and protocols were approved by the University of Saskatchewan's Animal Care Committee.

#### **4.2.2 Sample collection**

Blood samples from birds were taken on days 7 (before lighting change), 14, 21 and 28. Birds were randomly selected from each room and a minimum time interval of 20 minutes was maintained between entering each room and taking of samples. Blood samples were obtained from the right jugular vein (n= 6 birds/room). Blood smears were made immediately and the remainder of the sample was transferred to sterile vacutainers and stored in ice water until centrifugation. After centrifugation at 3000 rpm for 15 minutes serum samples were harvested and frozen at -80° C for sample analysis. Blood smears were fixed in 100% methanol and stained using May-Grunwald-Giemsa stain (Campo & Davila, 2002b; Gross & Siegel, 1983). A total of hundred cells including both granular (heterophils, eosinophils and basophils) and agranular (lymphocytes and monocytes) leukocytes were counted for each slide under oil immersion at 100 X. The H/L ratio was reported as the ratio of the number of heterophils per hundred leukocytes to the number of lymphocytes per hundred leukocytes (Campo & Davila, 2002b; Jones et al., 1988). Serum CORT concentration was determined using a double antibody rat corticosterone kit (ICN Pharmaceuticals Inc., Orangeburg, NY, U.S.A (Sorenson et al., 1997) .

#### **4.2.3 Preparation of samples for NMR analysis**

Serum samples were prepared by diluting 150 uL of serum with sodium phosphate buffer at pH 7.0 made in deuterium oxide (D<sub>2</sub>O, 99.9%, Cambridge Isotope Inc., Andover, MA, USA, 1:4 by volume). 0.5mM of TMSP (sodium-3-trimethylsilyl – [2, 2, 3, 3-d<sub>4</sub>]propionate , 98%, Cambridge Isotope Inc., Andover, M.A, U.S.A) was added to the buffer for referencing and locking (Viant, 2007). Ultrafiltration of samples

was done using 10kda Nanosep centrifugal filters (Pall Life Sciences, Ontario, Canada). The filtration membranes were prepared by washing each filter 10 times with distilled water to remove glycerol (Tiziani et al., 2008). Diluted samples were filtered at 15,000 rpm for 15 minutes at 4 °C to remove high molecular weight components. The filtrate was transferred into modified 3mm NMR tubes (New Era Enterprises Inc, Vineland, NJ, U.S.A.) and conventional NMR spectra obtained. The tubes were washed 5 times in distilled water and soaked in Extran (EMD chemicals Inc, Gibbstown, NJ, U.S.A) for 1 hour to remove them of biological debris. The tubes were once again washed 5 times in distilled water to clear the Extran and then rinsed with acetone (BHA Inc, Toronto, Ontario, Canada) to remove any moisture adhering to the tubes before new samples were added. Owing to small serum sample size (less than 100ul) in day 14 of this experiment, samples were diluted at 1:4 ratios with the buffer and analyzed for NMR spectra without ultra filtration.

#### **4.2.4 <sup>1</sup>H-NMR spectroscopy**

Conventional NMR spectra were recorded at a resonance frequency of 600.17 MHz on a Bruker Avance-600 spectrometer (Rheinstetten, Germany) equipped with an inverse triple resonance probe (TXI 5mm) at the Saskatchewan Structural Science Centre, University of Saskatchewan, SK, Canada. Water suppression using excitation sculpting with gradients was used to accomplish efficient suppression of the water resonance in the spectral data (Bollard et al., 2005). For each sample, 32 free induction decays (FID) were accumulated over a spectral width of 7183.91Hz with an acquisition time of 2.28 seconds and relaxation delay of 5seconds at a temperature of 298°K. The experimental time for each sample was 4.18 min. The resultant FID was fourier transformed into one-dimensional <sup>1</sup>H-NMR for further analysis. The resulting spectra were phased manually; baseline corrected, and calibrated (TMSP at 0.0 ppm) using XWIN-NMR (version 3.5, Bruker). Spectra were converted to an appropriate format for analysis using *Prometab* running within MATLAB (Version R2009a; The MathWorks, Natick, MA, USA. Spectra were segmented into equidistant 0.04-ppm bins between 0.2 and 10.0 ppm and the total spectra area within each bin was integrated, as described previously (Viant, 2003). Bins between 4.4ppm and 6.0ppm containing the suppressed water resonance were excluded (Aich et al., 2007).

#### 4.2.5 Statistical Analysis

A Shapiro Wilk normality test was done on H/L ratio and CORT to determine normality and data was log transformed where required and an analysis of covariance (ANCOVA) with time as the covariate was done to determine statistical significance. Post hoc analysis was done using Fishers Least significant difference (LSD). Linear regression was used to examine the effect of sampling time on H/L ratio and CORT concentrations.

Spectral resonance 2.22 ppm corresponding to acetone used to dry the NMR tubes was removed before further spectral analysis (Gottlieb et al., 1997). The NMR data was imported into the MetaboAnalyst software (Xia et al., 2009) for multivariate analyses. Spectra were baseline corrected for noise, normalized and Pareto scaled. An unsupervised pattern recognition method (principal component analysis (PCA)) was used to examine intrinsic variation in the NMR data set and to identify and exclude outliers. Eight birds on day 14 (14L=3, 17L=1, 20L=2, and 23L=2), 11 birds on day 21 (14L=3, 17L=2, 20L=3, and 23L=3) and 14 birds on day 28 (14L=3, 17L=4, 20L=4, and 23L=3) were identified as outliers and excluded from the model.

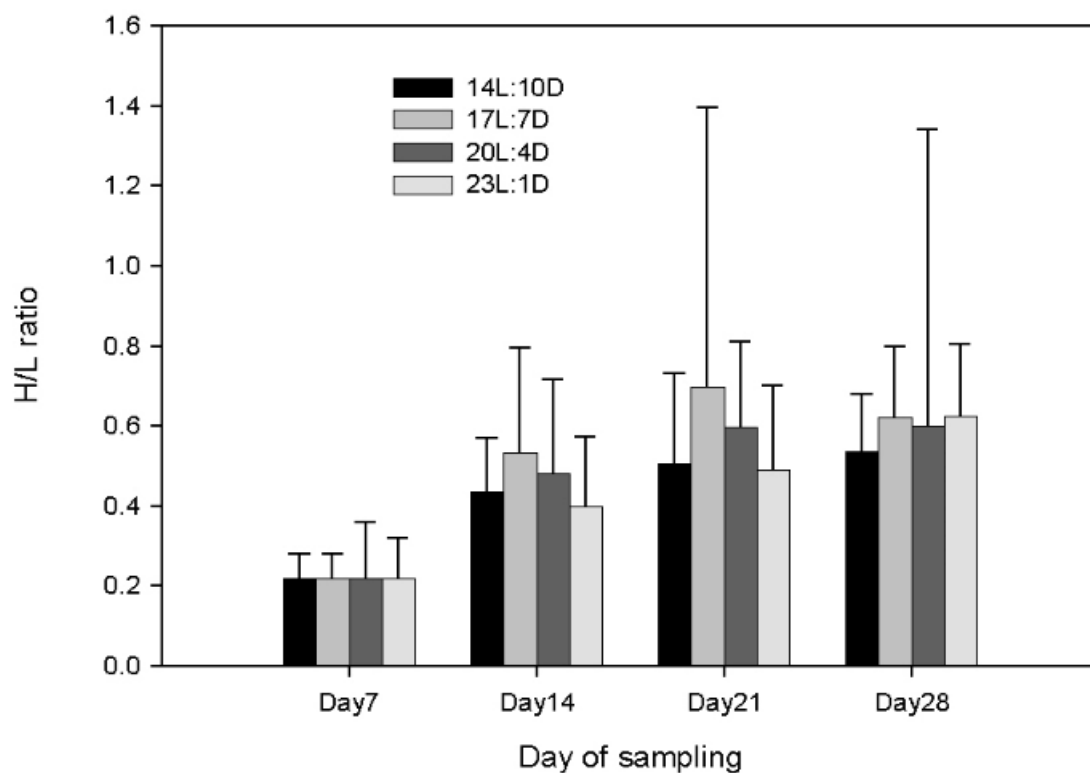
A supervised partial least square-discriminant analysis (PLS-DA) was performed to identify the discriminating significant features between treatments. Permutation (Pr) by randomly assigning samples to different groups was done to assess the strength of the model (Bijlsma et al., 2005; Rubingh et al., 2006). Metabolites were assigned to peaks based on their resonance frequency using peer reviewed metabolite databases (Lindon et al., 1999; Lindon et al., 2000; D. S. Wishart et al., 2009). Regression analyses and *a priori* contrasts were used as appropriate. All metabolic data analyses were done using MetaboAnalyst (Xia et al., 2009). Differences were considered significant when  $P \leq 0.05$  and noted when  $P \leq 0.10$ .

#### 4.3 Results

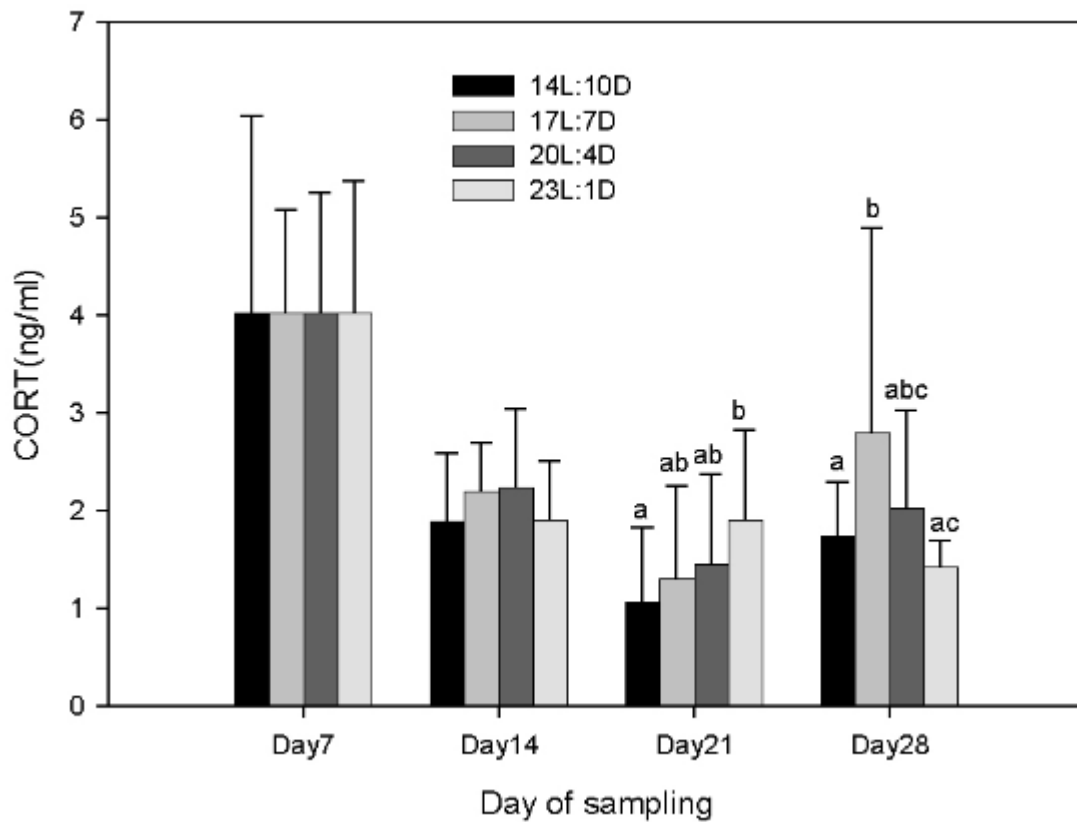
This study was done concurrently with other research on the impact of photoperiod length on the production and welfare of broilers. Results for the concurrent study are reported elsewhere (Schwean-Lardner et al., 2012a; Schwean-Lardner et al., 2012b).

Mean sampling time was  $53 \pm 23$  (seconds) for all treatments across sampling days and no significant effect of time on the H/L ratio or the CORT concentration was seen. There was a significant day effect on H/L ratio (Figure 4.1,  $F_{3, 143} = 10.398$ ,  $P \leq 0.001$ ) with H/L increasing with age. Post hoc analysis of H/L ratios indicated that days 7, 14 and 28 were significantly different from each other while that of day 21 was only different from day 7. The H/L ratio increased from day 14 to day 28 in 20L and 23L birds with the 20L birds having an H/L ratio of  $0.81 \pm 0.73$  on day 28 (Figure 4.1). The 17L group had a maximum of  $0.70 \pm 0.39$  H/L ratio on day 21 followed by a decline to  $0.62 \pm 0.17$  H/L ratio by day 28 (Figure 4.1). The 14L group had a steady H/L ratio of  $0.43 \pm 0.13$  on day 14 and  $0.50 \pm 0.22$  H/L ratio on day 21 and  $0.53 \pm 0.14$  on day 28, respectively (Figure 4.1). A significant sampling day\*treatment interaction (Figure 4.2,  $F_{(9,143)} = 2.929$ ,  $P \leq 0.001$ ) was noted for CORT levels (Figure 4.2). CORT concentrations on days 14, 21 and 28 were all lower than day 7.

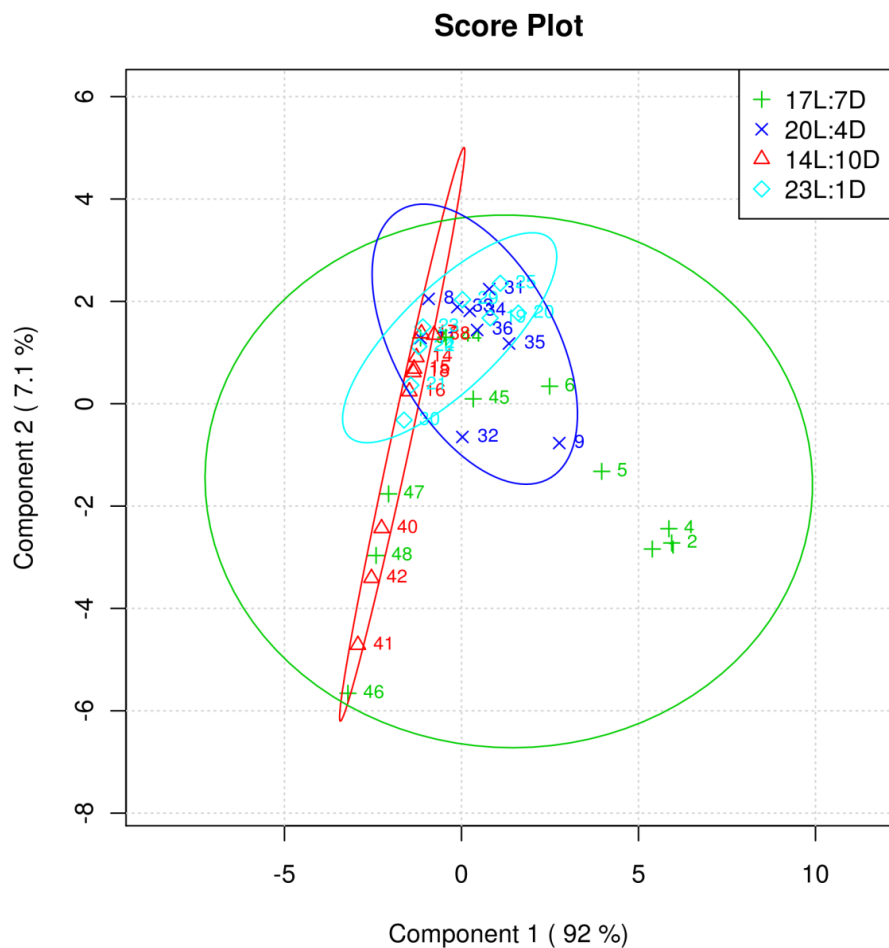
Two dimensional (2D) score plots of PLS-DA analysis of the data showed separation of metabolites between the various lighting treatments on day 14 (Figure 4.3) with a permutation (Pr) value of  $Pr < 0.01$ . Similar 2D score plots of PLS-DA showed separation of metabolites between the lighting treatments on day 21 ( $Pr = 0.01$ , Figure 4.4), day 28 ( $Pr = 0.02$ , Figure 4.5). Metabolomic analysis of the serum samples of the lighting treatments showed the presence of amino acids valine, threonine, lysine, proline, histidine, methionine and other metabolites like creatine, carnitine, choline, glycerol, betaine and lactate. Out of the top common metabolites identified between this study and that of the birds fed CORT (30mg/kg) diet in chapter 3 of this thesis, 5 metabolites (valine, lysine, methionine, histidine and glucose) showed similar relative abundance and trends in lighting groups 20L and 23L at all ages. On day 21 threonine and carnitine in lighting groups 14L and 17L followed a similar trend to the birds fed CORT (30mg/kg diet) in chapter 3 of this thesis. Betaine in lighting groups 17L and 20L followed trends of the treatment and control groups respectively of the chapter 3 study of this thesis.



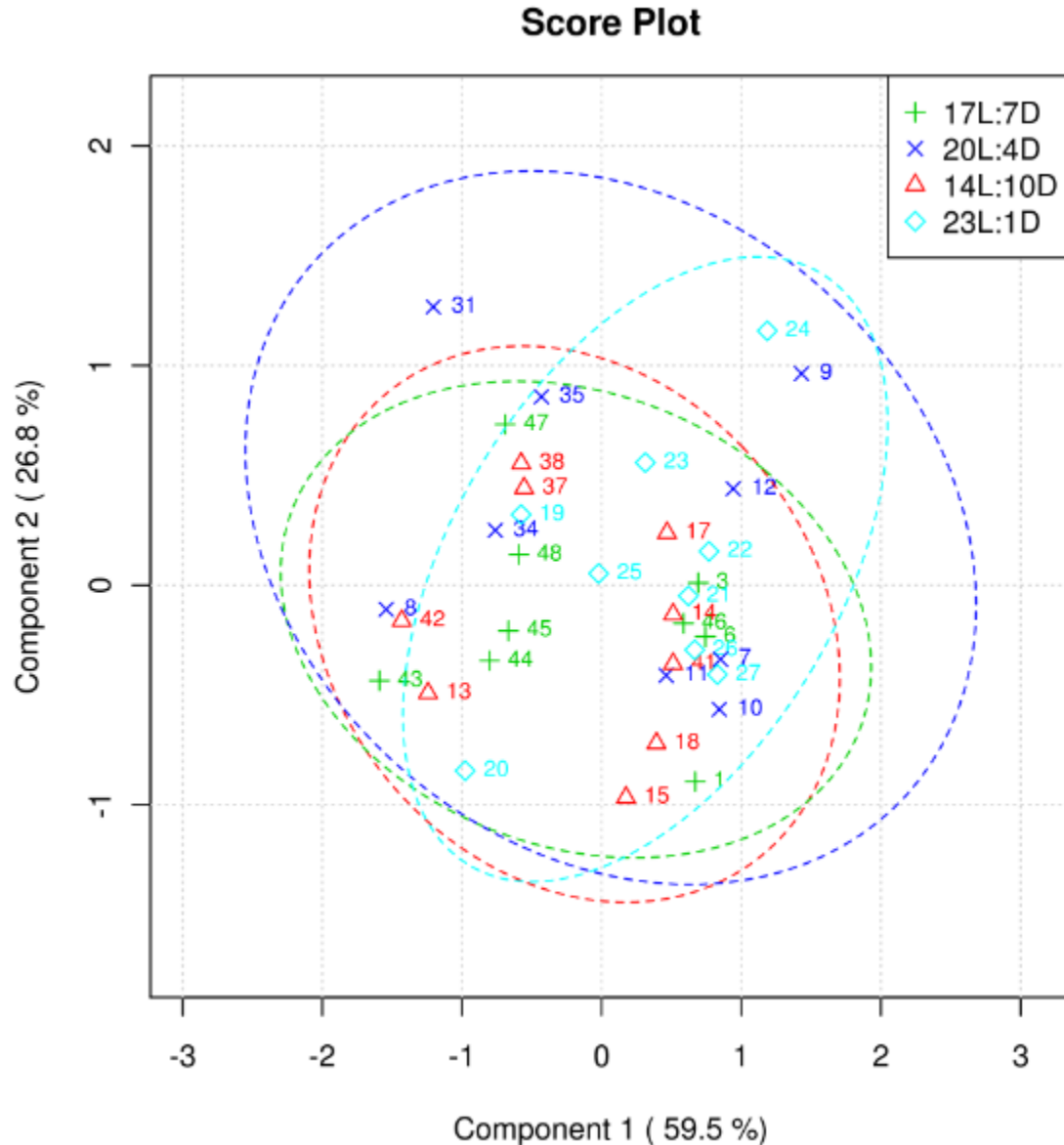
**Figure 4.1 Heterophil/Lymphocyte (H/L) ratio in broiler chickens sampled on day 7 (prior to lighting treatment, n=16), and 14, 21, 28 days (following start of lighting treatments at day 7, 14L:10D, 17L:7D, 20L:4D and 23L:1D, n=12 for each group). Significant sampling day effect ( $F_{3, 143}=10.398$ ,  $P<0.001$ ). Error bars represent  $\pm$  SD.**



**Figure 4.2 Serum corticosterone (CORT) ng/ml, in broiler chickens aged 7 (prior to lighting treatment, n=16), 14, 21, 28 days (following start of lighting treatments at day 7 of 14L:10D, 17L:7D, 20L:4D and 23L:1D 7, n=12 for each group). Significant sampling day\*treatment effect ( $F_{9,143} = 2.929$ ,  $P \leq 0.001$ ). Superscripts ‘a’, ‘b’ and ‘c’ represent means within a day with different superscripts differ significantly  $P \leq 0.05$ . Error bars represent  $\pm$  SD.**

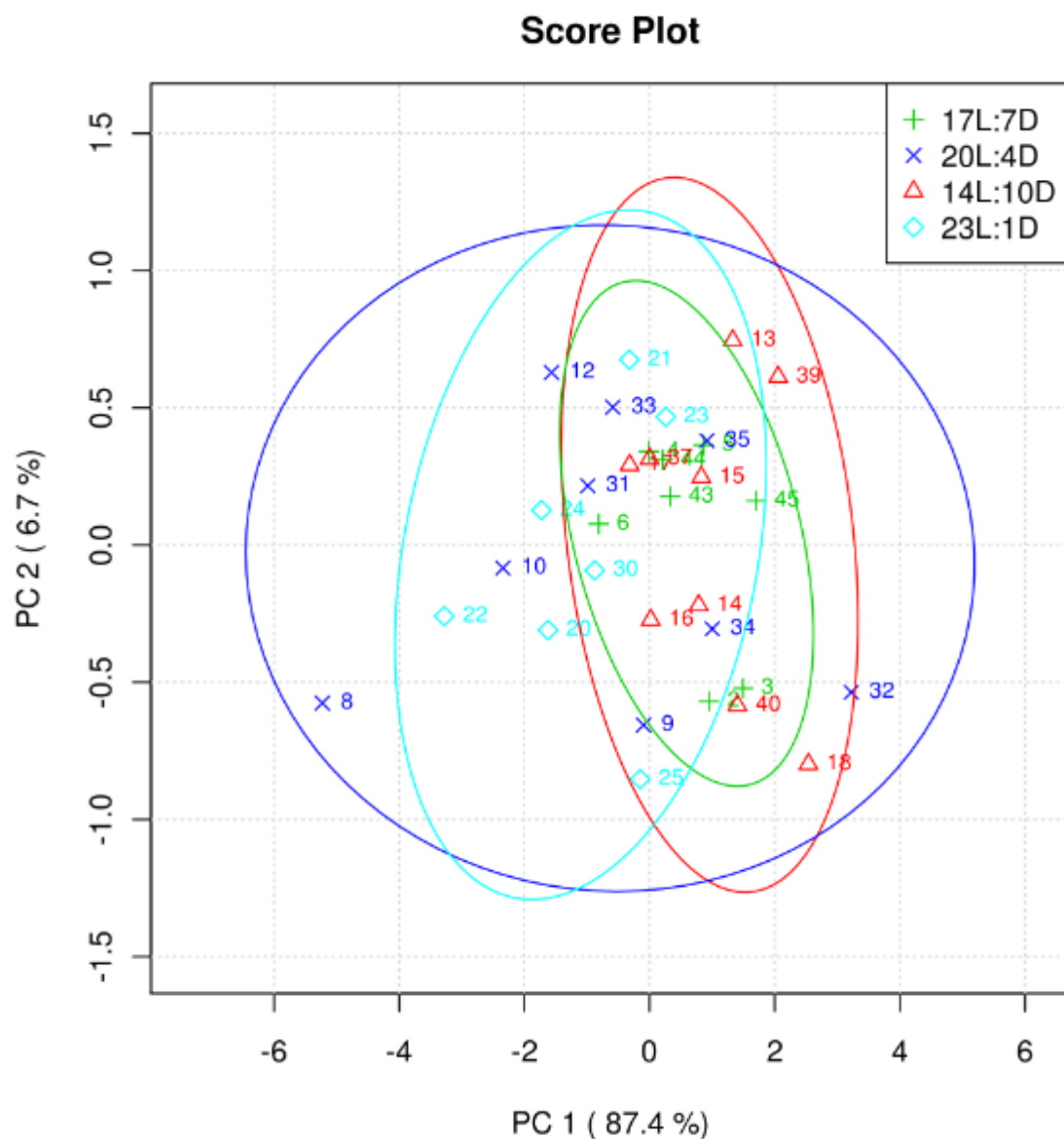


**Figure 4.3 PLS-DA score plot of broiler chickens aged 14 days following start of lighting treatments at day 7 of 14L:10D, 17L:7D, 20L:4D and 23L:1D 7, n=12 for each group showing separation of metabolites between photoperiods. PCA Component 1 accounted for 92% of the separation between the groups with  $P < 0.01$ . Three birds from 14L, one from 17L, two birds from 20L and two birds from 23L were identified as outliers and excluded from the model. Generated using MetaboAnalyst (Xia et al., 2009)**



**Figure 4.4 PLS-DA score plot of broiler chickens aged 21 days following start of lighting treatments at day 7 of 14L:10D, 17L:7D, 20L:4D and 23L:1D 7, n=12 for each group showing a separation of metabolites between photoperiod .Component 1 accounted for 62.1% of the separation between the groups with P=0.01 Four birds two each from 14L and 17L and six birds three each from 20L and 23L were identified as outliers and excluded from the model. Generated using MetaboAnalyst (Xia et al., 2009)**





**Figure 4.5 PLS-DA score plot of broiler chickens aged 28 days following start of lighting treatments at day 7 of 14L:10D, 17L:7D, 20L:4D and 23L:1D 7, n=12 for each group showing separation of metabolites between photoperiods. Component 1 accounted for 93.1% of the separation between the groups with  $P=0.02$  Three birds each from 14L, 20L and 23L and one from 17L were identified as outliers and excluded from the model. Generated using MetaboAnalyst (Xia et al., 2009)**

**Table 4.1 Relative abundance of the significant metabolites identified from broiler chickens aged 21 and 28 days following start of lighting treatments at day 7 of 14L:10D, 17L:7D, 20L:4D and 23L:1D, n=12 for each group based on the variable importance in projection (VIP) scores of PLS-DA on respective sampling days. Arrows ↑ and ↓ respectively indicate an increased or decreased relative abundance within different treatments while ↔<sup>++</sup> and ↔<sup>+</sup> represent moderately high and moderately low relative abundance respectively of metabolites. Generated using MetaboAnalyst (Xia et al., 2009)**

Spectral ppm	Metabolites	Relative abundance			
		14L	17L	20L	23L
0.98	Valine	Day28↔ <sup>+</sup>	Day28↓	Day28↔ <sup>++</sup>	Day28↑
1.34	Threonine	Day21↓	Day21↔ <sup>+</sup>	Day21↑	Day21↔ <sup>++</sup>
1.5	Lysine	Day21↔ <sup>++</sup>	Day21↑	Day21↔ <sup>+</sup>	Day21↓
2.02	Proline	Day28↔ <sup>+</sup>	Day28↑	Day28↓	Day28↔ <sup>++</sup>
3.02	Creatine	Day21↔ <sup>++</sup>	Day21↔ <sup>+</sup>	Day21↑	Day21↓
3.26	Histidine	Day21↑	Day21↔ <sup>+</sup>	Day21↔ <sup>++</sup>	Day21↓
3.42	Carnitine	Day21↔ <sup>+</sup> Day28↔ <sup>+</sup>	Day21↓ Day28↓	Day21↔ <sup>++</sup> Day28↑	Day21↑ Day28↔ <sup>++</sup>
3.46	Glucose	Day21↔ <sup>+</sup>	Day21↓	Day21↔ <sup>++</sup>	Day21↑

Spectral ppm	Metabolites	Relative abundance			
		14L	17L	20L	23L
3.5	Choline		Day28↓	Day28↑	Day28↔ <sup>++</sup>
3.78	Glycerol	Day28↔ <sup>+</sup>	Day28↓	Day28↑	Day28↔ <sup>++</sup>
3.86	Methionine	Day28↔ <sup>+</sup>	Day21↔ <sup>++</sup>	Day21↑	Day21↔ <sup>+</sup>
3.9	Betaine	Day21↔	Day28↓	Day28↑	Day28↔ <sup>++</sup>
4.1	Lactic acid/Lactate	Day28↔ <sup>+</sup>	Day28↓	Day28↔ <sup>++</sup>	Day28↔ <sup>+</sup>
		Day28↑			

#### 4.4 Discussion

In this experiment, neither H/L ratio nor CORT levels were affected by lighting (23L, 20L, 17L, and 14L) treatments indicating no stress. Metabolic profiles (metabolomics) were able to differentiate groups and indicate that birds raised in near continuous lighting (23L) are physiologically different from birds raised in other lighting regimes. The metabolomics also supports a stress component related to 23L. This study supports the sister study (Schwean Lardner & Classen, 2010) where 23L exposed broilers had compromised welfare (i.e. enlarged eyes, increased mortality, poor gait scores) in comparison to other treatments. In comparison, 17L and 14L birds were more indicative of acceptable welfare lighting regimes with reduced mortality, good gait scores, behavioral synchrony, normal eye physiology and melatonin cycles while 20L birds were intermediate in many factors with the exception of eye health where it resembled the 14L and 17L groups.

Evaluation of the H/L ratio has been used as tool in assessing stress (Gross & Siegel, 1983). Data compiled by (Maxwell, 1993) on the avian leukocyte response to stress has demonstrated that severe food restriction causes a heteropenia, whereas climate, environmental, transport and heat stress causes heterophilia. In life threatening situations heteropenia and basophilia are seen and H/L ratio cannot be used as measure of stress in such conditions (Maxwell, 1993). Increased circulating heterophils with a decrease in lymphocytes (high H/L ratio), was observed in chickens fed CORT (Gross & Siegel, 1983). High H/L ratio have also been reported following exposure to a stressor (Gross & Siegel, 1983; Maxwell & Robertson, 1998; Puvadolpirod & Thaxton, 2000a; Shaniko Shini et al., 2008). Reference values of 0.2, 0.5 and 0.8 of H/L ratio indicate low, moderate and high stress levels, respectively, in chickens (Gross, 1993). Elevated CORT seen in response to a stressor, generally increases H/L ratio (Post et al., 2003) through recruitment of heterophils from bone marrow and decreased exit into the surrounding tissues or alternatively increased exit of lymphocytes from the circulation into lymphoid tissue (Athens et al., 1968; Ottaway and Husband, 1994; Dhabhar, 2006). As the CORT levels for this study were well within physiological limits and sampling time was short, there was no discernible increase in H/L ratio. In chapter 3, birds fed CORT (30mg/kg diet) showed an increase in the H/L ratio.

In this study, H/L ratio was not a predictor of stress. In another study, birds reared in 23L:1D in comparison to birds reared under 14L:10D did not have discernible alterations of H/L ratio to suggest stress but they did display increased duration of tonic immobility which may suggest an enhanced fear response (Campo & Davila, 2002a). Studies investigating lighting management in chickens have used H/L ratio as a tool of welfare assessment but with contradicting results. Lien et al. (2007), investigating the effect of photoperiods (23L:1D and 18L:6D at 1/0.1 foot candle light intensity approximately equivalent to 10lux/5 lux, respectively) on H/L ratio in broilers reported an average 0.45 H/L ratio that was not affected by the lighting treatments. Similar results were reported by other researchers using 23L:1D in comparison with increasing photoperiod with unaffected H/L ratios in broilers (Blair et al., 1993) and in three breeds of layers (Campo & Davila, 2002a). However Campo et al. (2007) in 11 breeds of

chickens showed that a near continuous lighting regime of 24L:0D in comparison to 14L:10D caused an increased H/L ratio.

Studies on White Leghorn hens (Davis et al., 2000) and Spanish Castellana breed chickens (Campo & Davila, 2002b) demonstrated that H/L ratio increases with age. In this study, H/L ratio rose with age in all treatment groups. The increase, shown here was not as drastic as seen in birds treated with exogenous CORT (Jones et al., 1988; Shaniko Shini et al., 2008). While birds in 23L group had compromised welfare (Schwean Lardner & Classen, 2010) the H/L ratio did not indicate a higher stress level than other lighting treatments. My findings add to the discrepancy as H/L ratio did not indicate stress or compromised welfare and hence H/L ratio is likely not a good indicator of welfare.

Corticosterone is often used to assess and is considered to be a sensitive indicator of stress (Post et al., 2003; Puvadolpirod & Thaxton, 2000a, 2000b, 2000c). The most immediate noticeable change following exposure to a stressor is an increase in CORT concentration. In chickens, peak CORT is reached as quickly as 3 minutes (Voslarova et al., 2008). The half life of CORT in chickens is 8 minutes (Carsia et al., 1988) following which levels are actively reduced through down regulation. Studies conducted by (Romero & Reed, 2005; Wingfield et al., 1982) have demonstrated that blood samples collected within 2-3 minutes of handling time have CORT concentrations which better represent an unstressed state (i.e. baseline). In this study the average time of sample collection across sampling days was  $53 \pm 23$  seconds which is well within the 2-3 minutes of sampling indicative of baseline state. In chapter 3, birds fed CORT (30 mg/kg diet from day 7 on) showed a profound increase in serum CORT levels (day 14, first blood sample following treatment) with a gradual decline by day 35. During chronic stress, birds can experience decreased basal CORT related to habituation to the stressor, adrenal exhaustion or down regulation in the synthesis and release of CORT to prevent its deleterious effects (Rich & Romero, 2005). Studies in broilers have also shown that there is an age related decrease in circulating CORT levels after 2 weeks of age (Thaxton et al., 2005). The CORT concentrations in this study are not indicative of severe stress.

In this study, CORT concentration among treatments was well within the physiological limits despite reduced welfare seen by (Schwean Lardner & Classen, 2010)

in the 23L group. Release of CORT depends on a number of factors such as receptor availability, sensitivity and binding protein availability (Landys et al., 2006), and as these factors can differ between species and prior stressful events. It is difficult to compare or use CORT as a sole indicator of stress (Chloupek et al., 2009; Romero, 2004). Data compiled by (Rushen, 1991) demonstrated varying plasma CORT concentrations between experiments conducted in similar conditions. For example, plasma CORT can be increased (Gibson et al., 1986), decreased (Koelkebeck et al., 1987) or the same (Craig et al., 1986) in laying hens reared in battery cages compared with those reared in pens. This reiterates the fact that CORT cannot be used uniformly as an indicator of stress across experiments. To overcome the inconsistency, Moneva et al. (2009) suggested utilizing both H/L ratio and CORT in the assessment of stress and welfare. Low H/L ratio and CORT seen in this study demonstrate that physiological and behavioural adaptations are not necessarily measured as stress responses.

Metabolomic analysis of the serum samples of the lighting groups showed the presence of amino acids, osmolytes and sugars. Out of the top metabolites identified metabolites, lysine and valine are highly suggestive of stress induced anxiety, depression and behavioral changes while metabolites like methionine, choline, betaine, histidine, proline are indicative of a methylation process (methyl groups are important for carnitine, creatine, polyamines and spermidine synthesis), osmoprotection and oxidative stress response. Creatine is highly indicative of the ongoing protein accretion process. Metabolites threonine and histidine relate to the immune response while metabolites like glucose, fructose, glycerol and carnitine provide evidence for the process of providing energy to meet the physiological needs of the body (Murray et al., 2006).

In response to a stressor, the main function of CORT is to provide the necessary gluconeogenic substrates and regulate the glucogenic pathways in the liver (Exton et al., 1972; Scanes, 2009). However concomitant changes in other catabolic hormones like glucagon and catecholamines and anabolic hormones like insulin, growth hormone and insulin-like growth factor 1 during stress also play substantial role in the provision of energy (Carrasco & Van de Kar, 2003; Umpleby & Russell-Jones, 1996). In this study the amino acids (AAs) seen are highly unlikely to be a resultant of muscle protein breakdown as the CORT levels were within physiological limits. Their presence could be

suggestive of the regular process of muscle metabolism. The other possible mechanism for the AAs to be seen might also be as a resultant of the redox homeostasis occurring at the cellular level. Studies (Häussinger et al., 1993; Haussinger et al., 1994; Häussinger & Schliess, 1995) have shown that cellular hydration leads to protein synthesis and cellular dehydration leads to proteolysis. Hormones like insulin promote cell hydration and thereby protein synthesis while hormones like glucagon and catecholamines promote cell shrinkage and proteolysis. The cellular shrinkage leads to protein phosphorylation resulting in protein and glycogen breakdown along with an increase in expression of gluconeogenic enzymes (PEPCK) (Häussinger et al., 1993; Haussinger et al., 1994). Other factors that contribute to cellular shrinkage include stress, inadequate AAs, uraemia and oxidative stress (Häussinger et al., 1993). Oxidative stress can induce insulin resistance influencing glucose transport and glycogen synthesis (Dokken et al., 2008). Environmental factors like exposure to pollutants in humans (Hong et al., 2008), heat stress in chickens (Altan et al., 2003; Hai Lin et al., 2006) have known to contribute to oxidative stress altering the redox homeostasis at the cell level. As the CORT levels were low and the birds were being fed adequate dietary AAs, environmental factors may have contributed to oxidative stress resulting in cellular dehydration and proteolysis. However further research is warranted.

Energy is required to fuel bodily activities that will deal with a stressor and restore homeostasis (Sapolsky et al., 2000; Selye, 1951). The stress response results in increased gluconeogenesis (synthesis of glucose from noncarbohydrate sources) ensuring availability of glucose and insulin resistance preventing storage of glucose (Tsigos, 2002; Dupont et al., 1999b; Sapolsky et al., 2000). The brain and erythrocytes require a readily available source of glucose. The brain derives about 20 % of its energy from ketone bodies and the remainder 80% from glucose, whereas, erythrocytes lacking mitochondria are solely dependent on glucose as the primary source of energy (Murray et al., 2006). Because available carbohydrate sources of glucose are being used up by the brain and erythrocytes, other tissues must meet their energy requirements via gluconeogenesis. This is achieved through a variety of routes including glycolysis using lactate from glycogen, AAs from protein degradation of muscle reserves, ketone bodies synthesized from acetyl CoA in liver and glycerol from adipose tissue. Proteins and fat serve as the major non-

carbohydrate source of energy during periods of high energy requirements as evinced during stress (Siegel and Van Kampen, 1984). Hepatic proteolysis and muscle breakdown produces amino acids which can then be utilized for gluconeogenesis by the liver and kidney (Murray et al., 2006).

In the stress scenario where hyperglycaemia is the norm with enhanced gluconeogenesis (Dupont et al., 1999, Sapolsky et al., 2000), increased carnitine aids in production of acetyl CoA and ketone bodies from fatty acids. Increased relative abundance of carnitine was seen with 20L and 23L on day 21 and 28 (table 4.1) supporting my hypothesis that these birds are experiencing stress. Carnitine is synthesised endogenously by the body in muscles, kidney and liver (Murray et al., 2006; Wishart et al., 2009). Carnitine has been reported to enhance the fatty acid oxidation process and decrease adiposity (Janssens et al., 1998; Murray et al., 2006; Xu et al., 2003). The acyl coA of long chain fatty acids can penetrate the inner mitochondrial layer and gain access to the  $\beta$  oxidation process only in the presence of carnitine by getting converted to acylcarnitine. Within the mitochondria acyl coA is formed from acylcarnitine with the release of carnitine. The  $\beta$  oxidation of acyl coA leads to the production of acetyl coA which then enters the citric acid cycle or is converted to ketone bodies to fuel the gluconeogenetic process (Murray et al., 2006). Carnitine prevents the occurrence of intestinal lesions by utilising the free radicals of oxygen arising due to oxidative stress in rats subjected to cold restraint stress (Nimet et al., 2001). The presence of carnitine in 20L and 23L groups might indicate provisioning of energy and to reduce oxidative stress.

Sugars glucose and fructose are sources of energy and both can be derived from dietary sucrose. Stress induces increased plasma glucose via gluconeogenesis with the intent of providing energy to cells and tissues (Sapolsky et al., 2000; Selye, 1951). In this study, glucose was of high relative abundance in the 20L and 23L lighting groups and of relatively low abundance in the 14L and 17L lighting groups on day 21 (Table 4.1). Similar trends in glucose regulation were seen in the CORT (30mg/kg diet, chapter 3) fed birds on day 21. Fructose, an isomer of glucose, was also found in high relative abundance on day 28 in 14L and 20L lighting groups. The presence of sugars might potentially indicate an energy demanding situation.



In this study, glycerol was found in increased abundance in the 20L and 23L on day 28 (Table 4.1). The presence of glycerol may be due to glycerol being used either as substrate for gluconeogenesis as a result of lipolysis or due to glycogenesis or lipogenesis. It should be noted that both acute and chronic stress have been shown to lead to gluconeogenesis with the intent of providing energy to all bodily cells and tissue (Sapolsky et al., 2000).

Of the three branched chain amino acids (BCAA, i.e, isoleucine, leucine and valine), valine was found in the top metabolites while isoleucine and leucine were not among the top metabolites (Table 4.1). Despite the absence of isoleucine and leucine from the top metabolites its worth mentioning them as the three BCAA are integral to each other's physiological function (Harper et al., 1984). Branched chain amino acids, isoleucine, leucine and valine have been found to be involved in stress, energy and muscle metabolism (Wishart et al., 2009). They are primarily metabolized in the skeletal muscles with tissue aminotransferases reversibly interconverting all the three BCAA to  $\alpha$  ketoacids and thereby maintaining a balance between them. Stress, trauma, starvation and fever increase requirements of these BCAA (Li et al., 2007). Endogenous synthesis of glutamine from skeletal muscles (principal AA involved in ammonia excretion) is from BCAA. They enhance the antibody and cytokine production in the body, inhibit cell apoptosis and are essentially important in the immune stimulation. With enhanced gluconeogenesis during stress (Dupont et al., 1999; Sapolsky et al., 2000), isoleucine and leucine are converted to acetyl CoA to enter the citric acid cycle primarily involved in the production of energy (ATP) while valine gets converted to succinyl coA to enter the citric acid cycle. Acetyl CoA is also used in the synthesis of ketone bodies in the liver, which in turn serve as energy fuel during prolonged fasting and chronic stress (Murray et al., 2006).

Teague et al (2007) reported a decrease in the BCAA following an acute stressor in Sprague-Dawley rats on day 1 of the experiment. They reported an increase in BCAA leucine and valine during chronic stress lasting 44 days. In my study, valine was found in relatively low abundance in 14L and 17L and of high relative abundance in 20L and 23L by day 28 (Table 4.1). In chapter 3, the metabolic profile of treatment birds fed CORT

(30mg/kg diet) showed valine to be of high relative abundance around day 21 and 35. The 20L and 23L groups followed a similar pattern suggesting a stress process. Supplementation of valine in the diet prior to restraint stress reduces CORT secretion in the rat (Joseph & Kennett, 1983a). Valine competes with tryptophan an essential precursor of 5 hydroxytryptamine (Joseph & Kennett, 1983b) which is important in stimulating the secretion of corticotrophin hormone (CRH) from the hypothalamus (Pan & Gilbert, 1992). Valine in the 20L and 23L groups may be involved in reducing the secretion of CRH and in turn reduced CORT levels. Quantification and examination of the balance between the three BCAA during chronic stress conditions requires further investigation.

Threonine is an essential AA, found in abundance in blood plasma which is converted to acetyl CoA entering the citric acid cycle as a source for energy production (Murray et al., 2006). In this study, threonine was of high relative abundance in 20L and 23L groups on day 21 while it was of low relative abundance in 14L and 17L groups (Table 4.1). Low threonine was noted in birds fed CORT (30mg/kg diet, Chapter 3) on days 14, 21 and 35. Threonine stimulates the growth of the thymus (Murray et al., 2006; Wishart et al., 2009) and involves in the synthesis of intestinal mucin and maintaining intestinal integrity (Wu, 2009). Dietary sources of threonine have shown to increase antibody production and inhibit cell apoptosis (Li et al., 2007). Immune system impairment with constant lighting has been demonstrated in White Leghorn cockerels (Kirby and Froman, 1991) and Japanese quail (Moore & Siopes, 2000) increasing their susceptibility to disease. The presence of threonine in the 23L and 20L groups might be of a stimulatory effect on the immune system.

Lysine is converted to crotonyl CoA and further into acetyl CoA which enters the citric acid cycle for energy production or is converted to ketone bodies in the liver (Murray et al., 2006). Lysine was of relative low abundance in 20L and 23L groups on day 21 while 14L and 17L lighting groups had high relative abundance (Table 4.1). Similarly, the metabolic profile of birds fed CORT (30mg/kg diet) showed lysine in low relative abundance. Experiments using Sprague–Dawley rats subjected to restraint stress showed decreased levels of lysine (Teague et al., 2007). In contrast, requirements of lysine are reported to increase during stress in humans (Wishart et al., 2009). The

decrease in lysine might indicate a compromised immune response in 20L and 23L groups. Lysine deficiency reduces cytokine synthesis and proliferation of lymphocytes with an impaired immune response in chickens (Chen et al., 2003; Konashi et al., 2000; Li et al., 2007).

Histidine is an essential AA and is converted to  $\alpha$  ketoglutarate, which enters into the citric acid cycle for energy production (Murray et al., 2006). Histidine undergoes decarboxylation and yields histamine which is an important mediator of inflammatory reactions (Li et al., 2007). Histidine is also an important component of haemoglobin structure and in its function (Wu, 2009). Olanrewaju et al. (2006) reported an increase in erythropoiesis in stressed chickens. This provides evidence that elevated histidine aids in meeting increased oxygen demand during stress. In birds fed CORT (30mg/kg diet, Chapter 3) histidine was relatively high abundance on day 14 and low relative abundance on days 21 and 35. Histidine has also been reported to alleviate oxidative and inflammatory changes in the liver of rats chronically fed on ethanol (Liu et al., 2008) showcasing their anti oxidative and anti inflammatory properties. In this study, histidine was of high relative abundance in 14L and 20L lighting groups while it was in low relative abundance in 17L and 23L groups on day 21 (Table 4.1). The histidine found here might be involved in the regular process of histamine synthesis. Further quantification is warranted to understand the actual regulation.

Methionine a top metabolite was of high relative abundance in the 17L and 20L groups while 23L and 14L had a low relative abundance (Table 4.1). Low relative abundance of methionine was noted in treatment birds fed on CORT (30 mg/kg diet). Methionine enters the citric acid cycle for energy production as succinyl CoA (Murray et al., 2006) and is an indispensable dietary AA. Methionine is essential in normal growth of avian species (Murray et al., 2006; Wishart et al., 2009). Dietary supplementation of methionine improves weight gain and feed conversion ratio, (Garcia Neto et al., 2000) and improves production of antibodies and the immunoglobulin IgG in broilers (Tsiagbe et al., 1987). Increased levels of plasma methionine causes an increase in levels of its metabolite homocysteine (Murray et al., 2006). Methionine toxicity has been linked to depression, spleen and liver hemosiderosis (excess hemosiderin built up leading to organ damage) and reduced feed utilisation in chickens (Ekperigin & Vohra, 1981; Harter &

Baker, 1978). The varied regulation of methionine among the lighting groups suggests an altered methionine – homocysteine metabolism. Further study is warranted.

Choline is important in that it serves as a precursor of betaine and acetylcholine and is also a methyl donor (Murray et al., 2006; Wishart et al., 2009). Choline has also been reported to alleviate oxidative stress caused by asthma in rat models (Mehta et al., 2009). It was also found in relatively high abundance in the 20L and 23L groups on day 28 (Table 4.1). Similarly Teague et al. (2007) reported increased serum choline at day 44 in chronically stressed Sprague-Dawley rats subjected to restraint stress for 35 with a recovery period of 9 days. The presence of choline might be involved in reducing the oxidative stress in the 20L and 23L lighting groups. Environment factors are capable of causing oxidative stress (Altan et al., 2003; Hong et al., 2008; Hai Lin et al., 2006) leading to cellular dehydration and altered cell homeostasis (Haussinger et al., 1994). However, an assessment of plasma malondialdehyde, a biomarker of oxidative stress, is needed for meaningful conclusions (Nielsen et al., 1997).

Betaine is an organic osmolyte protecting cells and enzymes (Craig, 2004). It is found in animal tissues and is synthesized as an intermediate product from choline (Murray et al., 2006). Being a methyl donor (methyl groups are important for carnitine and creatine synthesis), betaine supplementation in poultry and pigs has been found to increase body weight gain and enhance intestinal cell activity (Eklund et al., 2005). In this study betaine was found in high relative abundance in 20L and 23L groups on day 28 while it was of low relative abundance in 14L and 17L lighting groups (Table 4.1). It was also found in low relative abundance in treatment birds fed CORT (30mg/kg diet, Chapter 3) on days 14, 21 and day 28. Environment factors can cause oxidative stress (Altan et al., 2003; Hong et al., 2008; Hai Lin et al., 2006) leading to cellular dehydration and altered cell homeostasis (Haussinger et al., 1994). Being an osmolyte (Kidd et al., 1997) it might be a valid reason for the presence of betaine among the top metabolites to enhance the osmoregulatory process. The difference in the pattern of regulation may be related to high exogenous CORT fed to the treatment birds (chapter 3) relative to birds experiencing changes in their lighting environments. Low levels in the treatment group (chapter 3) might suggest altered methionine–homocysteine metabolism and compromised response to oxidative stress.

In this study, proline was in high relative abundance in the 17L and 23L groups on day 28 while it was of low relative abundance in the 14L and 20L group (Table 4.1). Studies in Sprague-Dawley rats exposed to a various stressors like restraint, forced swimming and tail shock showed decreased levels of urinary proline in acute and chronic stress (Wang et al., 2009). Proline has known to be an osmoprotectant and been reported to be in increased abundance in *Drosophila melanogaster*, marine animals, bacteria and plants in times of osmotic stress (Burton, 1991; Hare et al., 1999; Misener et al., 2001). The high relative abundance of proline in the 17L and 23L groups may be related to its function to preserve the osmotic environment of the cell. Proline is synthesized from glutamic acid in the body and enters the citric acid cycle for energy production by getting converted to glutamate (Murray et al., 2006).

Creatine was found in high relative abundance in the 14L and 20L group but low relative abundance in the 23L and 17L groups on day 21 (Table 4.1). The increased abundance may infer increased protein accretion process going on in the body in the 20L and 14L lighting group. The fact that it was of low relative abundance in the 23L group may suggest birds in this group to be having poor muscle growth. It is interesting to note that 20L had increased body weight gain during the growing phase while 23L had poor body weight growth rate (Schwean Lardner & Classen, 2010). Creatine is primarily biosynthesized from guanidinoacetate that is formed from glycine and arginine in the kidney. Guanidinoacetate then enters the liver where it is methylated during the production of creatine (Wyss & Kaddurah-Daouk, 2000). Creatine is stored in skeletal muscle and other tissues as phosphocreatine, which serves as a ready source of ATP energy as a result of creatine kinase action. In energy requiring situations phosphocreatine under creatine kinase enzyme activity donates the phosphate group to ADP forming ATP (Murray et al., 2006; Pearlman & Fielding, 2006). Removal of a water molecule from creatine yields creatinine. Increased muscle growth have been associated with increased creatine kinase activity in broilers (Szabo & Milisits, 2007). Creatine supplement has shown to increase muscle mass in exercising human subjects (Volek et al., 1999).

## 4.5 Conclusion

In this experiment the changes seen in the H/L ratio are associated with age rather than lighting treatment. Reference values of 0.2, 0.5 and 0.8 of H/L ratio have been reported to indicate low, moderate and high stress levels in chickens (Gross, 1993). These reference values would infer that all lighting groups have experienced a level of moderate stress. This is a highly unlikely scenario when we consider the welfare data reported by Schwan Lardner and Classen (2010). Their report suggested the 14L and 17L lighting group to be more suitable for the birds on welfare terms with 23L having compromised welfare for the birds. The serum CORT levels for broilers in this experiment were well within the physiological levels for all lighting treatments and were affected by bird age. Comparison of metabolites from this study with exogenously CORT treated (30mg/kg feed, chapter 3) and other studies show that metabolites in 20L and 23L groups are most suggestive of stress. Welfare parameters determined by Schwan Lardner and Classen (2010) demonstrate that birds exposed to 23L have poor welfare scores followed by 20L while lighting groups 14L and 17L had best welfare scores. Metabolite profiles of birds in 14L and 17L groups have less metabolic perturbations. Comparison of metabolites from birds in this study with that birds fed CORT (30 mg/kg, chapter 3), identified 5 metabolites (valine, lysine, methionine, histidine and glucose) that had similar relative abundance and trends in lighting groups 20 L and 23 L. Metabolites such as lysine, and valine were highly suggestive of stress and behavioral changes while metabolites such as methionine, choline, betaine, histidine, proline were indicative of the methylation process, osmoprotection and oxidative stress response. Creatine was highly indicative of the ongoing protein accretion process. Metabolites threonine and histidine suggested an immune response while metabolites like glucose, fructose, glycerol and carnitine suggested the under going process of providing energy to the physiological needs of the body.

## CHAPTER 5

### COMPARING $^1\text{H}$ NMR METABOLOMICS AS A NOVEL TOOL OF ASSESSING STRESS IN COMPARISON WITH CORTICOSTERONE AND HETEROPHIL: LYMPHOCYTE RATIO IN BROILER CHICKENS (*GALLUS DOMESTICUS*) REARED UNDER 4 DIFFERENT LIGHTING INTENSITIES

#### 5.1 Introduction

Light is an important management technique in broiler production. Artificial lighting can be manipulated for broilers in three ways: photoperiod, wavelength, and light intensity. Lighting regimes have the potential to modulate various behavioral and physiological pathways, and therefore, may have significant effects on broiler production and welfare. Manipulation of light intensity (lux (**Lx**)) has also been used to reduce bird activity, thus maximize productivity, as well as to save fuel costs. However, low levels (less than 10 Lx) have been associated with reduced carcass yield, decreased early growth uniformity, increased incidence of leg disorders, ocular defects, and altered behavior including increased fearfulness in birds (Manser, 1996). Deep et al (2010) also found 1 Lx of light intensity resulted in increased eye weight and size, and increased the incidence of ulcerative footpad lesions in contrast to other intensities (10, 20 and 40 Lx). Blatchford et al (2008) showed altered behavior (increased resting) and heavier eyes of birds reared under an low light intensity of 5 Lx units with little effect on body weight and immunocompetence. Extended periods of dim lighting or darkness also caused a decrease in corneal thickness (Jenkins et al., 1979). Birds living in 5 Lx light intensity were lethargic and showed less change in activity between day and night than birds under 50 or 200 Lx lighting (Blatchford et al., 2009). Birds subjected to 200 Lx of light had increased activity with more hock and footpad bruising but less erosion of the hock joints than birds grown at 5 or 50 Lx (Blatchford et al., 2008). Similar results were reported by Prayitno et al (1997), where birds showed improved gait scores and less angular deformity of the legs when reared under high intensity red light early in the growth period. These studies indicate that light intensity can alter poultry welfare and may serve as a stressor.

Concern about stress in poultry production has long been an issue from both an animal welfare issue as well as a production concern. The “stress response”, is an

evolved trait that allows an animal to deal with changes or conditions within its environment. Proper response to stress can be beneficial in life threatening situations; however, chronic stress can have marked deleterious effects on reproduction, behavior, metabolism and immunocompetence (Siegel, 1980). Any situation that elicits a response (either psychological (i.e. fear, noise), pathological (i.e. disease) or physiological (i.e. overcrowding) can be considered a “stressor”. Also conditions within the environment including: climate (i.e. extreme heat, cold or humidity), environmental (i.e. poor ventilation, light regimes), nutritional (i.e. shortages of nutrients), physical (i.e. handling, transport), can act as stressors. Conditions which induce stress in an animal can reduce welfare and as well as performance (Scheele, 1997; Olanrewaju et al., 2006) thus measurement of stress can be used to evaluate and validate management techniques.

Identification of stress in the poultry industry has been done using various physiological endpoints but the two most accepted indicators of stress are elevated CORT and increased circulating heterophil/lymphocyte (H/L) ratios (Puvadolpirod and Thaxton, 2000). However, the length of time taken to obtain the blood sample can influence both CORT as early as 3 minutes (Voslarova et al., 2008) and H/L ratio within the first 15-20 minutes (Moneva et al., 2009). These errors may lead to problematic interpretation of the data. Lack of consistency between studies has resulted in skepticism when using them to quantify animal welfare in the poultry industry (Maxwell, 1993; Rushen, 1991).

I propose an alternative method of assessing lighting stress in broiler through the use of metabolomics. Serkova et al (2009), defines metabolomics as “an ‘omic’ science in systems biology, and as the global assessment and validation of endogenous small-molecule biochemicals (metabolites) within a biologic system”. Environmental metabolomics is an emerging approach for examining metabolic profiles in biological systems exposed to environmental stress (Lin et al., 2006). Utilization of 1-dimensional <sup>1</sup>H Nuclear Magnetic Resonance spectroscopy (NMR) can provide insightful mechanistic information on effects of stressors by simultaneously measuring a large number of low molecular weight endogenous metabolites (Viant et al., 2003). Metabolic profiles can be examined using pattern recognition algorithms to reveal subsets of metabolites, potentially identifying biomarkers (Lin et al., 2006; Viant et al., 2003). The advantage of



using NMR over more traditional methods is that it provides information about multiple metabolites, many that are not influenced by sampling handling time and may potentially identify biomarkers for stress. The objective of this study is to evaluate NMR as a method of stress assessment and compare the its use to more traditional methods of lighting stress assessment (CORT and H/L ratio) in broilers. In addition, I will compare the metabolite profile identified from this study with potential biomarkers of chronic stress identified from the experiment discussed in chapter 3 to provide additional criteria for selection of light intensity in broiler production.

## **5.2 Materials and methodology**

### **5.2.1 Animals**

A total of eight rooms were used with Genotype Ross 308 broilers sourced from Lilydale breeder flocks with a target stocking density being 32kg/m<sup>2</sup>. Eggs were hatched by Lilydale in Calgary; chicks were vaccinated with Coccivac® and then delivered to the University of Saskatchewan Poultry Centre. Feed was provided on an *ad libitum* basis with the major ingredients being corn and soybean meal and diet specifications and feeding amounts were according to Aviagen recommendations(Aviagen, 2007a). The feed was procured from a commercial feed mill (Federated Co-Operatives Ltd., Saskatoon, Saskatchewan, Canada). Feed did not contain a coccidiostat or a growth promotant. Straw of 7.5 to 10cm thick was used as litter material. Details of the birds housing are reported in the sister study (Deep et al., 2010). Chicks were placed into eight rooms without sex differentiation and lighting was set at 23 hours light (L): 1 hour dark (D) with a minimum of 40 Lx intensity for the first 7 days. On day 7, rooms were set at two replications of: (a) 1Lux (**1Lx**), (b) 10Lux (**10Lx**), (c) 20Lux (**20Lx**), and (d) 40Lux (**40Lx**) for the remainder of the study. Animals were treated in accordance with the Canadian Council on Animal Care guidelines and protocols were approved by the University of Saskatchewan's Animal Care Committee.

### **5.2.2 Sample collection**

Blood samples from birds were taken on days 7 (before lighting change), 14, 21 and 28. Birds were randomly selected from each room and a minimum time interval of 20 minutes was maintained between entering each room. Birds were blood sampled from the right jugular vein (n = 6 birds/room). Time of sample collection was recorded as was

the interval between when the room was entered and when the blood sample was obtained. Blood smears were made immediately and the remainder of the sample was transferred to sterile vacutainers and stored in ice water until centrifugation. After centrifugation at 3000 rpm for 15 minutes serum samples were harvested and frozen at -80° C for further sample analysis. Blood smears were fixed in 100% methanol and stained using May-Grunwald-Giemsa stain (Campo & Davila, 2002b; Gross & Siegel, 1983). A total of hundred cells including both granular (heterophils, eosinophils and basophils) and agranular (lymphocytes and monocytes) leukocytes were counted for each slide under oil immersion at 100 X. The H/L ratio was reported as the ratio of heterophils per hundred leukocytes to the number of lymphocytes per hundred leukocytes (Campo & Davila, 2002b; Jones et al., 1988). Serum CORT concentration was determined using a Double Antibody Rat Corticosterone Kit (ICN Pharmaceuticals Inc., Orangeburg, NY, (Sorenson et al., 1997).

### **5.2.3 Preparation of samples for NMR analysis**

Serum samples were prepared by diluting 150 uL of serum with sodium phosphate buffer at pH 7.0 made in Deuterium oxide (D<sub>2</sub>O, 99.9% Cambridge Isotope Inc., Andover, MA, U.S.A, 1:4 by volume) which included 0.5 mM TMSP (sodium-3-trimethylsilyl –[2, 2, 3, 3-d<sub>4</sub>] propionate, 98%, Cambridge Isotope Inc., Andover, M.A, U.S.A) added for referencing and locking (Viant, 2007). Ultrafiltration of samples was done using 10kda Nanosep centrifugal filters (Pall Life Sciences, Ontario, Canada). The filtration membranes were prepared by washing each filter 10 times with distilled water to remove glycerol (Tiziani et al., 2008). Diluted samples were filtered at 15,000 rpm for 15 minutes at 4° C to remove high molecular weight components. The filtrate was transferred into modified 3mm NMR tubes (New Era Enterprises Inc, Vineland, NJ, U.S.A.) and conventional NMR spectra obtained. The tubes were washed 5 times in distilled water and soaked in Extran (EMD chemicals Inc, Gibbstown, NJ, U.S.A) for 1 h to remove them of biological debris. The tubes were once again washed 5 times in distilled water to clear the Extran and then rinsed with acetone (BHA Inc, Toronto, Ontario, Canada) to remove any moisture adhering to the tubes before new samples were added.

#### 5.2.4 $^1\text{H}$ NMR spectroscopy

Conventional NMR spectra were recorded at a resonance frequency of 600.17 MHz on a Bruker Avance-600 spectrometer (Rheinstetten, Germany) equipped with an inverse triple resonance probe (TXI 5mm) at the Saskatchewan Structural Science Centre, University of Saskatchewan, SK, Canada. Water suppression using excitation sculpting with gradients was used to accomplish efficient suppression of the water resonance in the spectral data (Bollard et al., 2005). For each sample, 32 free induction decays (FID) were accumulated over a spectral width of 7183.91 Hz with an acquisition time of 2.28 seconds and relaxation delay of 5 seconds at a temperature of 298°K. The experimental time for each sample was 4.18 min. The resultant FID was Fourier transformed into one-dimensional  $^1\text{H}$ -NMR for further analysis. The resulting spectra were phased manually; baseline corrected, and calibrated (TMSP at 0.0 ppm) using XWIN-NMR (version 3.5, Bruker). Spectra were converted to an appropriate format for analysis using custom-written Prometab running within MATLAB (Version R2009a; The MathWorks, Natick, MA, USA). Spectra were segmented into equidistant 0.04-ppm bins between 0.2 and 10.0 ppm and the total spectra area within each bin was integrated, as described previously (Viant, 2003). Bins between 4.4 ppm and 6.0 ppm containing the suppressed water resonance were excluded (Aich et al., 2007).

#### 5.2.5 Statistical Analysis

A Shapiro Wilk normality test was done on H/L ratio and CORT to determine normality and data were log transformed where required. All data were log transformed and an analysis of variance (ANOVA) was done to determine statistical significance. Post hoc analysis was done using Fishers Least significant difference (LSD). Linear regression was used to examine the effect of sampling time on H/L ratio and CORT concentrations. The H/L ratio ( $R^2 = 0.048$ ,  $df = 48$ ,  $p = 0.005$ ) and serum CORT concentrations were correlated ( $R^2 = 0.03$ ,  $df = 48$ ,  $p = 0.002$ ) with the time interval between completion of blood sample and when the room was entered. Moneva et al (2009) has reported the H/L ratio levels to increase in as early as 15-20 minutes post handling for sample collection in normal chickens. Studies (Romero & Reed, 2005; Wingfield et al., 1982) have demonstrated that blood samples collected within 2-3 minutes of handling time have CORT concentrations which better represent an

unstressed state (i.e. baseline). To control for sampling time, H/L ratio and CORT were regressed against time, and the residuals were used in subsequent analyses (Wayland et al., 2003). Spectral resonance 2.22 ppm corresponding to acetone used to dry the NMR tubes was removed before further spectral analysis (Gottlieb et al., 1997). NMR data was imported into MetaboAnalyst (Xia et al., 2009) for multivariate analyses. Spectra were baseline corrected for noise, normalised and pareto-scaled. An unsupervised pattern recognition method (principal component analysis (PCA)) was used to examine intrinsic variation in the NMR data set and to identify and exclude outliers. A total of 11 birds (1Lx = 1, 10Lx = 4, 20Lx = 3, 40Lx = 3) from day 14, 6 birds (1Lx = 2, 10Lx = 3, 20Lx = 1) from day 21 and 6 birds (10Lx = 3, 20Lx = 3) from day 28 were identified as outliers and excluded from the model. A supervised partial least square-discriminant analysis (PLS-DA) was performed to identify the discriminating significant features between treatments. Permutation (Pr) by randomly assigning samples to different groups was done to assess the strength of the model (Bijlsma et al., 2005; Rubingh et al., 2006). Metabolites were assigned to peaks based on their resonance frequency using peer reviewed metabolite databases (Lindon et al., 1999; Lindon et al., 2000; D. S. Wishart et al., 2009). All metabolic data analysis and graph generation were done using MetaboAnalyst (Xia et al., 2009). Differences were considered significant when  $P \leq 0.05$  and noted when  $P \leq 0.10$ .

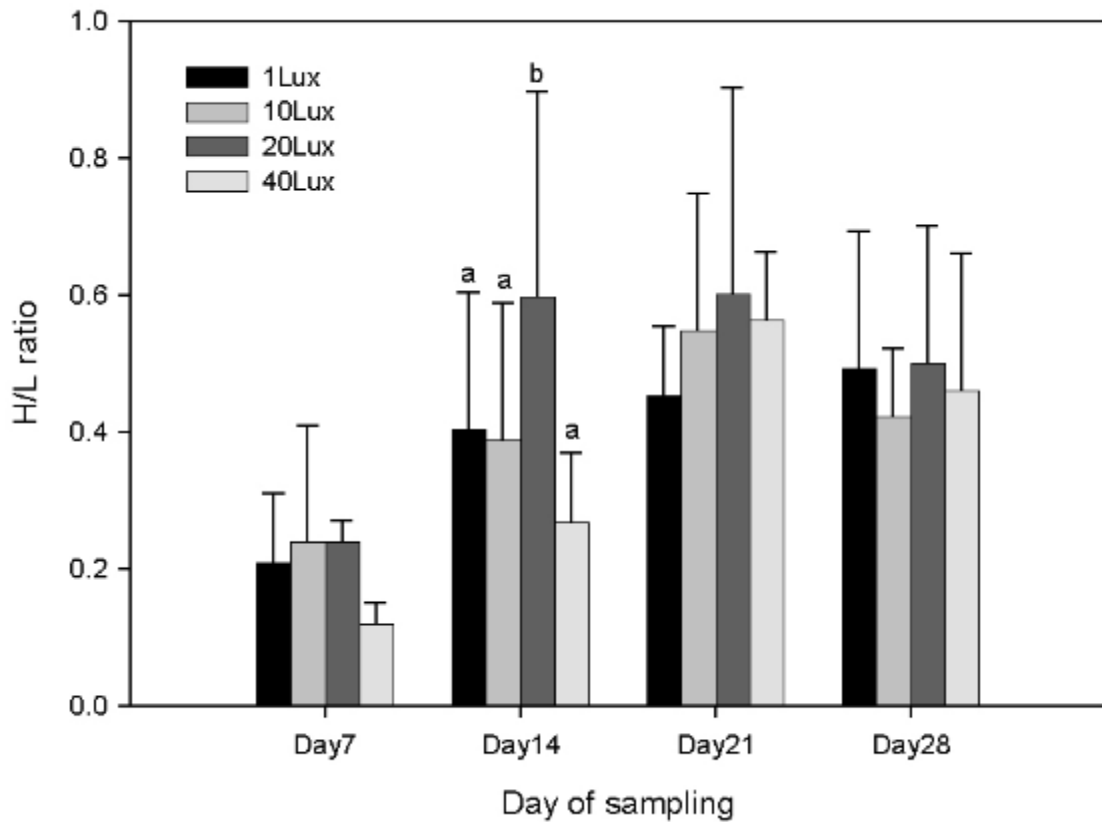
### 5.3 Result

The study was done concurrently with research undertaken to investigate the effect of light intensity on broiler production and welfare response criteria. Results for the concurrent study are reported elsewhere (Deep et al., 2010).

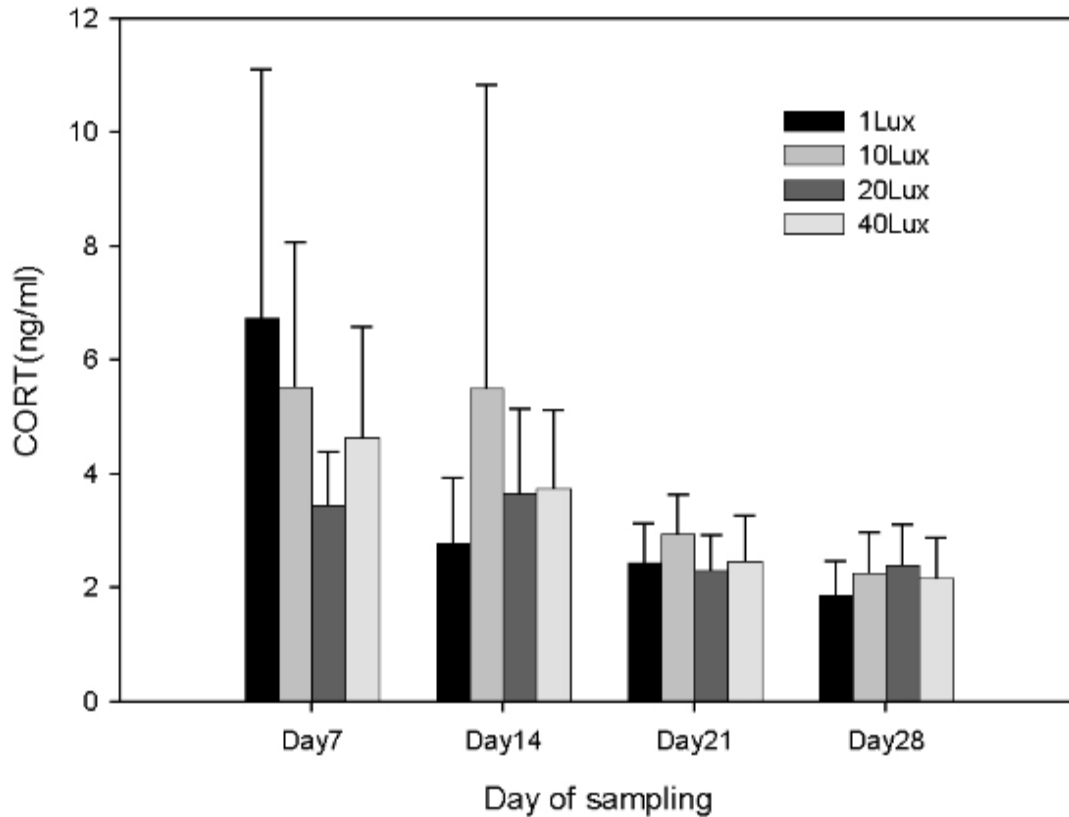
Mean sampling time was  $56 \pm 17$  (seconds) for all treatments across sampling days. There was a significant effect of sampling day ( $F_{3, 144} = 5.961$ ,  $P < 0.001$ ) and treatment ( $F_{3, 144} = 2.258$ ,  $P < 0.056$ ) on the H/L ratio (Figure 5.1). Post hoc analysis revealed H/L at 20 Lx was significantly different from treatments 1 Lx, 10 Lx and 40 Lx on day 14 (Figure 5.1). H/L ratio showed an increasing trend from day 14 to day 21 for the lighting groups 20 Lx and 40 Lx with the 20 Lx group having a maximum H/L ratio of 0.6 on days 14 and 21 and the 40 Lx having a reaching a maximum H/L ratio of 0.6 on day 21 (Figure 5.1). The 1 Lx group had a maximum of 0.50 H/L ratio on days 21 and 28

while the 10 Lx group had a maximum H/L ratio of 0.50 on day 21 followed by a decline to 0.4 H/L ratio by day 28(Figure 5.1). Serum CORT ( $F_{3, 144}=8.961$ ,  $P<0.001$ ) levels were lower on day 7 and 14 than days 21 and 28 (Figure 5.2). No treatment effect was detected ( $F_{3, 144}=1.795$ ,  $P<0.151$ ) for CORT.

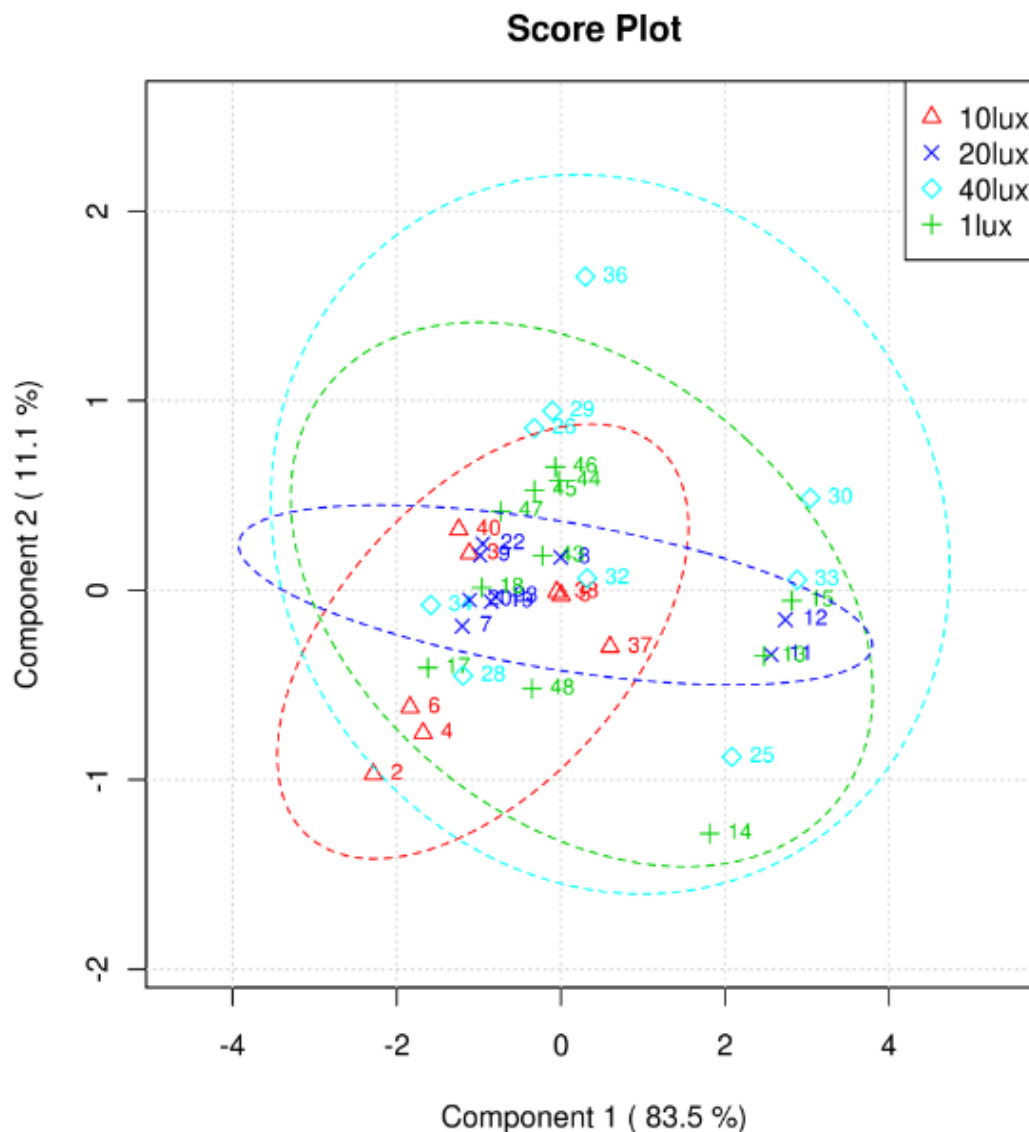
Metabolomic analysis showed the presence of amino acids isoleucine, threonine, histidine, methionine, tyrosine, serine and other metabolites including carnitine, choline, betaine, lactate, glucose and fructose. Two dimensional score plots of PLS-DA reveal that birds within lighting groups 10 Lx and 20 Lx had more homogeneity among their metabolites (Figure 5.5) while this was not the case in the 1 Lx and 40 Lx groups (Figure 5.5). Two dimensional (2D) score plots of PLS-DA analysis of the data showed separation of metabolites between the various lighting treatments on day 14 (Figure 5.3) with a permutation (Pr) value of  $Pr < 0.01$ . Similar 2D score plots of PLS-DA showed separation of metabolites between the lighting treatments on day 21 ( $Pr = 0.02$ , Figure 5.4), day 28 ( $Pr=0.02$ , Figure 5.5).



**Figure 5.1 Heterophil/Lymphocyte (H/L) ratio in broiler chickens day 7 (prior to lighting treatment, n = 16), and days 14, 21, 28 (following lighting treatments 1 Lux, 10 Lux, 20 Lux and 40 Lux from day 7, n = 12 for each group). Significant sampling day ( $F_{3, 144} = 5.961$ ,  $P < 0.001$ ) and treatment ( $F_{3, 144} = 2.258$ ,  $P < 0.056$ ) effect. Superscripts 'a' and 'b' represent means within a day with different superscripts differ significantly  $P \leq 0.05$ . Error bars represent  $\pm$  SD.**

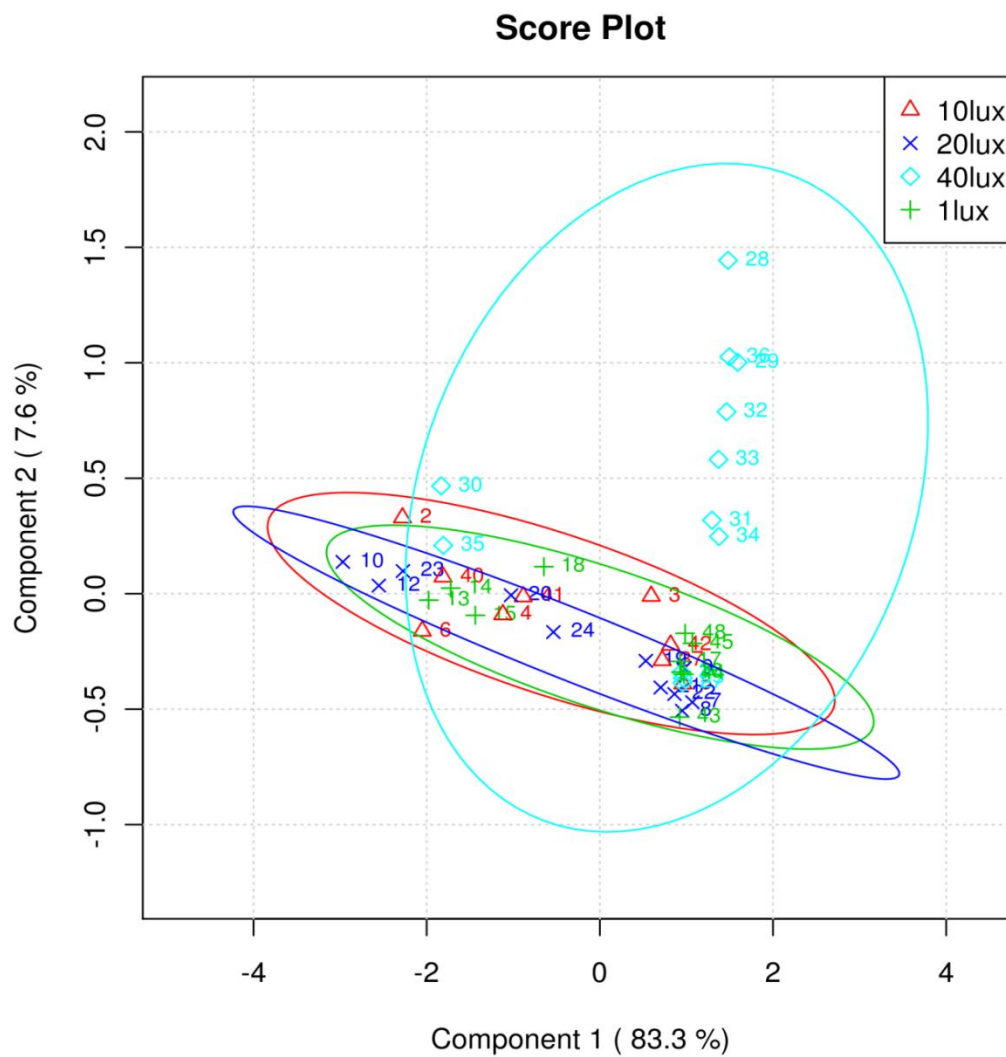


**Figure 5.2 Serum corticosterone (CORT) ng/ml in broiler chickens sampled on day 7 (prior to lighting treatment, n = 16), and days 14, 21, 28 (following start of lighting treatments 1 Lux, 10 Lux, 20 Lux and 40 Lux from day 7, n = 12 for each group). Showed there was a significant effect of day of sampling ( $F_{3,144} = 8.961$ ,  $P < 0.001$ ). Error bars represent  $\pm$  SD.**

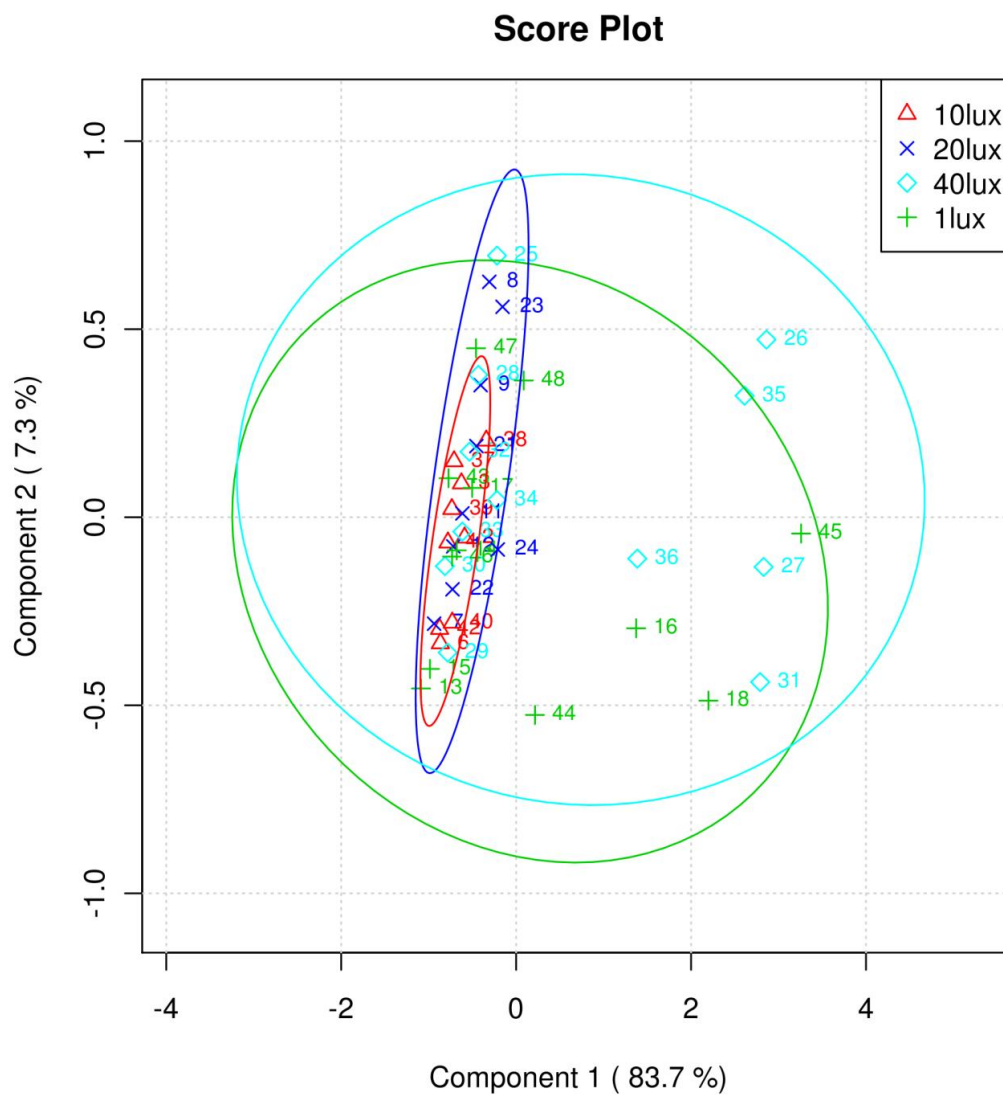


**Figure 5.3** 2D score plot of PLS-DA of broiler chickens aged 14 days following start of lighting treatments at day 7 of 1 Lux (n = 11), 10 Lux (n = 8), 20 Lux (n = 9) and 40 Lux (n = 9) shows a separation of metabolites between the various lighting intensity treatments. Component 1 accounted for 83.5% of the separation between the groups with  $P < 0.01$ . Generated using MetaboAnalyst (Xia et al., 2009).





**Figure 5.4** 2D score plot of PLS-DA of broiler chickens aged 21 days following start of lighting treatments at day 7 of 1 Lux (n = 10), 10 Lux (n = 9), 20 Lux (n = 11) and 40 Lux (n=12) shows a separation of metabolites between the various lighting intensity treatments. Component 1 accounted for 83.3% of the separation between the groups with  $P < 0.02$ . Generated using MetaboAnalyst (Xia et al., 2009).



**Figure 5.5** 2D score plot of PLS-DA of broiler chickens aged 28 days following start of lighting treatments at day 7 of 1Lux (n = 12), 10Lux (n = 9), 20Lux (n = 9) and 40Lux (n=12) shows separation of metabolites between the various lighting intensity treatments. Component 1 accounted for 83.7% of the separation between the groups with  $P < 0.024$ . Generated using MetaboAnalyst (Xia et al., 2009)

**Table 5.1 Relative abundance of the significant metabolites identified based on the variable importance in projection (VIP) scores of PLS-DA on respective sampling days. Arrows ↑ and ↓ respectively indicate an increased or decreased relative abundance while ↔<sup>++</sup> and ↔<sup>+</sup> represent moderately high and moderately low relative abundance respectively of metabolites in the treatment groups 1 lux, 10 lux, 20 lux and 40 lux.**

Spectral ppm	Metabolites	Relative abundance			
		1Lx	10Lx	20Lx	40Lx
0.86	2 hydroxybutyric acid	Day14↔ <sup>+</sup>	Day14↑	Day14↔ <sup>++</sup>	Day14↓
1.26	Isoleucine	Day14↔ <sup>+</sup> Day21↔ <sup>+</sup> Day28↔ <sup>++</sup>	Day14↑ Day21↑ Day28↓	Day14↔ <sup>++</sup> Day21↔ <sup>++</sup> Day28↔ <sup>+</sup>	Day14↓ Day21↓ Day28↑
1.3	Lactate	Day14↓ Day21↔ <sup>+</sup>	Day14↑ Day21↑	Day14↔ <sup>++</sup> Day21↔ <sup>++</sup>	Day14↔ <sup>+</sup> Day21↓
1.34	Threonine	Day14↔ <sup>++</sup> Day28↔ <sup>++</sup>	Day14↓ Day28↓	Day14↔ <sup>+</sup> Day28↔ <sup>+</sup>	Day14↑ Day28↑
3.26	Histidine	Day21↔ <sup>++</sup> Day28↔ <sup>++</sup>	Day21↔ <sup>+</sup> Day28↓	Day21↑ Day28↔ <sup>+</sup>	Day21↓ Day28↑
3.42	Carnitine	Day14↔ <sup>++</sup> Day21↔ <sup>++</sup> Day28↓	Day14↓ Day21↓ Day28↔ <sup>++</sup>	Day14↔ <sup>+</sup> Day21↔ <sup>+</sup> Day28↑	Day14↑ Day21↑ Day28↔ <sup>+</sup>
3.46	β-Glucose	Day14↔ <sup>+</sup> Day21↔ <sup>++</sup> Day28↔ <sup>+</sup>	Day14↓ Day21↓ Day28↑	Day14↔ <sup>+</sup> Day21↔ <sup>+</sup> Day28↔ <sup>++</sup>	Day14↑ Day21↑ Day28↓
3.5	Choline	Day14↔ <sup>++</sup> Day21↔ <sup>++</sup>	Day14↓ Day21↓	Day14↔ <sup>+</sup> Day21↔ <sup>+</sup>	Day14↑ Day21↑
3.82	Serine	Day28↑	Day28↔ <sup>++</sup>	Day28↔ <sup>+</sup>	Day28↓
3.86	Methionine	Day28↔ <sup>++</sup>	Day28↓	Day28↔ <sup>+</sup>	Day28↑
3.9	Betaine	Day28↔ <sup>+</sup>	Day28↔ <sup>++</sup>	Day28↔ <sup>++</sup>	Day28↓
3.94	Tyrosine	Day21↔ <sup>+</sup>	Day21↓	Day21↔ <sup>++</sup>	Day21↑
4.1	Fructose	Day14↔ <sup>++</sup>	Day14↓	Day14↔ <sup>+</sup>	Day14↑
4.14	β hydroxybutyrate	Day21↓	Day21↔ <sup>++</sup>	Day21↔ <sup>+</sup>	Day21↑

## 5.4 Discussion

In this study, neither H/L ratio nor CORT were affected by lighting treatment and therefore did not indicate differential stress among the four lighting regimes (1 Lx, 10 Lx, 20 Lx, and 40 Lx). Both H/L ratio and CORT were affected by age. Metabolic profiles (metabolomics) were able to differentiate treatments and indicate that birds reared under 1 Lx and 40 Lx are physiologically different from birds raised in 10 Lx and 20 Lx. The metabolic profiles suggest a stress component related to 1 Lx and 40 Lx treatments. My results support the sister study (Deep et al., 2010) birds reared under 1 Lx had enlarged heavier eyes and foot pad lesions suggesting compromised welfare, although these results contradict the findings at 40 Lx. Body weight, feed conversion ratio and mortality were unaffected at 1 Lx, 10 Lx, 20 Lx and 40 Lx (Deep et al., 2010) indicating that changes were subtle.

Evaluation of the H/L ratio has been used as reliable tool in assessing stress in chickens fed CORT (Gross and Siegel, 1983). An increase in circulating heterophils and a decrease in lymphocytes (high H/L) suggests stress (Gross & Siegel, 1983; Maxwell & Robertson, 1998; Puvadolpirod & Thaxton, 2000a; Shaniko Shini et al., 2008). In chickens 0.2, 0.5 and 0.8 of H/L ratios have been associated with low, moderate and high stress levels, respectively (Gross, 1993). Elevated CORT, generally increases H/L ratio (Post et al., 2003) through recruitment of heterophils from bone marrow and decrease exiting into the surrounding tissues or alternatively there is increased exit of lymphocytes from the circulation into lymphoid tissue (Athens et al., 1968; Ottaway and Husband, 1994; Dhabhar, 2006). In this study, the H/L ratio in the 1 Lx group (Figure 5.1) would be classified as unstressed. However the sister study found birds reared under 1 Lx had enlarged heavier eyes and foot pad lesions suggesting compromised welfare. Body weight, feed conversion ratio and mortality were unaffected at 1 Lx, 10 Lx, 20 Lx and 40 Lx (Deep et al., 2010). Birds fed CORT (30mg/kg, chapter 3) had an increased H/L ratio indicative of physiological stress (Figure 3.3A, Chapter 3). Lighting management studies have used H/L ratio to assess welfare of birds. Lien et al (2007) investigating the effect of lighting intensities 1 Lx and 10 Lx using 23L:1D and 18L:6D in broilers reported 0.45 H/L ratio that was not indicative of physiological stress. Other factors such as age, sex, breed, social ranking, rearing methods, etc. can alter H/L ratio (Campo & Davila,

2002a, 2002b; Gross & Siegel, 1983; Maxwell, 1993; Maxwell & Robertson, 1998; Moneva et al., 2009). Thus the H/L ratio changes seen in this study were mostly age related. Our findings add to the discrepancy of use of H/L ratio as an indicator of stress or welfare. Thus, H/L ratio should not be used as the sole indicator of stress (Gross & Siegel, 1983; Maxwell, 1993; Maxwell & Robertson, 1998; Moneva et al., 2009).

Corticosterone is often used to assess and is considered to be a sensitive indicator of stress (Post et al., 2003; Puvadolpirod & Thaxton, 2000a, 2000b, 2000c). The most immediate noticeable change following exposure to a stressor is an increase in CORT concentration. The half-life of CORT in chickens is 8 minutes (Carsia et al., 1988) following which levels are actively reduced through down regulation of synthesis. Studies conducted by (Romero & Reed, 2005; Wingfield et al., 1982) have demonstrated that blood samples collected within 2-3 minutes of handling time have CORT concentrations which better represent an unstressed state (i.e. baseline). In this study, the average time of sample collection across sampling days was  $56 \pm 17$  seconds which was well within the 2-3 minutes. In chapter 3, birds fed CORT (30mg/kg diet from day 7) showed a profound increase in serum CORT levels at day 14 (first blood sample following treatment) with a gradual decline by day 35. In this study, elevated CORT was not evident at day 14 or subsequent days (Figure 5.2). During chronic stress, birds can experience decreased basal CORT related to habituation to the stressor, adrenal exhaustion or down regulation in the synthesis and release of CORT to prevent its deleterious effects (Rich & Romero, 2005). Studies in broilers have also shown that there is an age related decrease in circulating CORT levels after 2 weeks of age (Thaxton et al., 2005) which was also seen here (Figure 5.2). Because CORT was measured at one week intervals, it is possible that a stress response was missed and that CORT measured here is more representative of adrenal exhaustion or down regulation.

In this study, CORT concentration among treatments was well within the unstressed level despite reduced welfare at 1 Lx. Other factors can influence CORT concentrations such as receptor availability, sensitivity and binding protein availability (Landys et al., 2006). These factors can differ between species and prior stressful events, therefore, it is difficult to compare or use CORT as a sole indicator of stress (Chloupek et al., 2009; Romero, 2004). Data compiled by (Rushen, 1991) demonstrated inconsistent

plasma CORT concentrations between experiments with similar conditions. For example, plasma CORT can be increased (Gibson et al., 1986), decreased (Koelkebeck et al., 1987) or show no change (Craig et al., 1986) in laying hens reared in battery cages compared with those reared in pens. To overcome the inconsistency, some researchers have resorted to using a combination of blood H/L ratio and CORT in the assessment of stress and welfare (Moneva et al., 2009). Low H/L ratios and CORT concentrations offered no additional advantage in this study. Once weekly sampling may not have been sufficient to identify peaks in CORT or H/L ratio which may have been present at some time points or shortly after lighting regimes were changed in the chicks.

Out of the top common metabolites identified between this study and that of the birds fed CORT (30 mg/kg diet, chapter 3), 5 metabolites (isoleucine, histidine, carnitine and glucose, betaine) in lighting groups 1 Lx and 40 Lx showed similar relative abundance and trends, while threonine in 10 Lx and 20 Lx,  $\beta$  hydroxybutyrate in 10 Lx and 40 Lx, lactate in 10 Lx (day 14) and 40 Lx (day 21) followed similar trends to the stressed birds. In response to a stressor, the main function of CORT is to provide the necessary gluconeogenic substrates and regulate the glucogenic pathways in the liver (Exton et al., 1972; Scanes, 2009). However concomitant changes in other catabolic hormones like glucagon and catecholamines and anabolic hormones such as insulin, growth hormone and insulin-like growth factor 1 during stress also play substantial role in the provision of energy (Carrasco & Van de Kar, 2003; Umpleby & Russell-Jones, 1996). In this study, AAs are highly unlikely to be a resultant of muscle protein breakdown but might be a result of the redox homeostasis occurring at the cellular level. Studies (Häussinger et al., 1993; Häussinger et al., 1994; Häussinger & Schliess, 1995) demonstrate that cellular hydration leads to protein synthesis while cellular dehydration leads to proteolysis. Hormones like insulin promote cell hydration and thereby protein synthesis while hormones like glucagon and catecholamines promote cell shrinkage and proteolysis. Cellular shrinkage leads to protein phosphorylation resulting in protein and glycogen breakdown along with an increase in expression of glucogenic enzymes (PEPCK) (Häussinger et al., 1993; Häussinger et al., 1994). Other factors that contribute to cellular shrinkage include stress, starvation, uraemia and oxidative stress (Häussinger et al., 1993). Oxidative stress can induce insulin resistance influencing glucose transport

and glycogen synthesis (Dokken et al., 2008). Environment factors can cause oxidative stress (Altan et al., 2003; Hong et al., 2008; Hai Lin et al., 2006) leading to cellular dehydration and altered cell homeostasis (Haussinger et al., 1994). Heat stress in chickens (Altan et al., 2003; Hai Lin et al., 2006) has been shown to contribute to oxidative stress altering the redox homeostasis at the cell level. With CORT being low and adequate dietary AAs, environmental factors (i.e. light intensity) may have produced oxidative stress resulting in cellular dehydration and proteolysis. However further research is warranted.

When exposed to a stressor, energy is required to fuel bodily activities that will deal with the stressor and restore homeostasis (Sapolsky et al., 2000; Selye, 1951). The stress response involves increased gluconeogenesis (synthesis of glucose from non carbohydrate sources) ensuring availability of glucose and insulin resistance preventing storage of glucose (Tsigos, 2002; Dupont et al., 1999b; Sapolsky et al., 2000). The brain and erythrocytes require a readily available source of glucose. The brain derives about 20 % of its energy from ketone bodies and the remainder 80% from glucose, whereas, erythrocytes lacking mitochondria are solely dependent on glucose as the primary source of energy (Murray et al., 2006). Because available carbohydrate sources of glucose are being used up by the brain and erythrocytes, others tissues must meet their energy requirements via gluconeogenesis. This is achieved through a variety of routes including glycolysis using lactate from glycogen, amino acids (AAs) from protein degradation of muscle reserves, ketone bodies synthesized from acetyl coA in liver and glycerol from adipose tissue.

Of the three branched chain amino acids (BCAA) (i.e, isoleucine, leucine and valine), isoleucine was found in the top metabolites (Table 5.1). Despite the absence of leucine and valine from the top metabolites (Table 5.1) it is worth mentioning them as the three BCAA are integral to each other's physiological function (Harper et al., 1984). Branched chain amino acids, isoleucine, leucine and valine have been found to be involved in stress, energy and muscle metabolism (Wishart et al., 2009). They are primarily metabolized in the skeletal muscles with tissue aminotransferases reversibly converting all the BCAAs to  $\alpha$  keto acids and thereby maintaining a balance between them. Stress, trauma, starvation and fever increase requirements of these BCAA (Li et

al., 2007). With enhanced gluconeogenesis during stress (Dupont et al., 1999; Sapolsky et al., 2000), isoleucine and leucine are converted to acetyl CoA to enter the TCA primarily involved in the production of energy (ATP) while valine gets converted to succinyl CoA to enter the TCA cycle. Acetyl CoA is also used in the synthesis of ketone bodies in the liver, which in turn serve as energy fuel during prolonged fasting and chronic stress (Murray et al., 2006). Branched chain amino acids are important sources of non carbohydrate energy (Wu, 2009).

Teague et al(2007) reported a decrease in BCAAs isoleucine, leucine and valine following acute restraint stress in Sprague-Dawley rats on day 1 of the experiment and an increase in BCAAs leucine and valine during chronic stress lasting 44 days. In this study, isoleucine followed a similar trend with low relative abundance on day 14 and 21 in 1 Lx and 40 Lx groups with subsequent high relative abundance by day 28 in 1 Lx and 40 Lx lighting groups (Table 5.1). In chapter 3, the metabolic profile of treatment birds fed CORT (30mg/kg diet) showed isoleucine to be of high relative abundance on day 14, 28 and 35 with low relative abundance on day 21. This difference in regulation between CORT fed (30 mg/kg, chapter 3) birds and this study might be due to the difference in the degrees of stress experienced. Studies have shown that with stress induced hyperglycaemia and ketoacidosis, an increase isoleucine is expected (McCowen et al., 2001; Soriano et al., 1967). While this was true for birds fed CORT(30mg/kg diet, chapter) where stress was extreme, birds in the 1 Lx and 40 Lx groups of this study had increased isoleucine by day28, following a similar trend to birds fed CORT (30 mg/kg diet, Chapter 3), potentially signifying involvement of a stress component in them. Excess leucine and/or isoleucine or valine by themselves do not lead to protein synthesis (Harper et al., 1984). Excessive leucine can increase depletion of isoleucine and valine leading to accelerated protein degradation (Antalikova et al., 1999; Harper et al., 1984).Quantification and examination of the balance between the three BCAA under stress conditions requires further investigation.

Threonine is an essential AA, found in abundance in blood plasma which is converted to acetyl CoA entering the TCA cycle as a source for energy production (Murray et al., 2006). Threonine was in high relatively abundance in 1 Lx and 40 Lx lighting groups on day 14 and 28, while low relative abundance was noted in the 10 Lx



and 20 Lx on days 14 and 28 (Table 5.1). Low relative abundance of threonine was also seen in CORT fed birds (30mg/kg diet, chapter 3) on days 14, 21 and 35. Threonine deficiency has been reported to cause neurologic dysfunction in kittens (Titchenal et al., 1980). Adverse effects of threonine deficiency in the lighting groups 10 Lx and 20 Lx can be safely ruled out as the physiological data provided for the same experiment (Deep et al., 2010) showed them to have acceptable growth rate, mortality rate, gait scores and eye health. The other possible reason could be due to a serine–threonine imbalance. Increased levels of serine have been reported to cause a reduction in plasma threonine levels in chickens (Davis & Austic, 1982). 10 Lx had high relative abundance of serine (Table 5.1) Threonine stimulates the growth of the thymus (Murray et al., 2006; Wishart et al., 2009) and is an important AA in the synthesis of intestinal mucin and maintenance of intestinal integrity (Wu, 2009). Dietary supplementation of threonine has been reported to increase growth rate, antibody production and inhibit cell apoptosis in pigs (Defa et al., 1999). Further quantification studies of threonine are warranted.

Tyrosine is a nonessential AA that is important in synthesizing catecholamines and dopamine during periods of stress. Chronic administration of corticotrophin releasing hormone (CRH) in rats increases tyrosine hydroxylase activity producing catecholamines from tyrosine. (Carrasco & Van de Kar, 2003; Melia & Duman, 1991). Likewise, dietary supplement of tyrosine prevented the depletion of norepinephrine in stressed rats (Lehnert et al., 1984; Reinstein et al., 1985). In this study, tyrosine was among the top metabolites in high relative abundance in the 40 Lx lighting group on day 21 while it was of low relative abundance in the 1 Lx and 10 Lx groups (Table 5.1). The increased levels seen may be due to the necessity to replenish norepinephrine levels that are being depleted as a result of stress. Varying housing conditions in mice have shown to activate the sympathetic neurotransmission mechanism (D'Arbe et al., 2002). Dietary supplementation of tyrosine has been shown to suppress the secretion of CORT in stressed rats (Reinstein et al., 1985). On the other hand, a tyrosine deficient diet resulted in symptoms of reduced growth, anorexia, lethargy and muscle weakness in young children having type 1 hereditary tyrosinemia (inability of the liver and kidney to metabolize tyrosine) which were promptly alleviated by a tyrosine rich diet (Cohn et al., 1977). Tyrosine deficiency has been associated with reduced growth, degeneration of eye

and central nervous system in chick embryos perfused with a tyrosine and phenylalanine (precursor of tyrosine) deficient solution (Austic & Grau, 1971). Low levels of tyrosine in the 1 Lx and 10 Lx groups might predispose them to the conditions like reduced growth and degeneration of the eye. It would be interesting to know if dietary supplementation of tyrosine would help in alleviating stress in chickens.

Methionine is essential in normal growth of avian species (Murray et al., 2006; Wishart et al., 2009). Dietary supplementation of methionine improves weight gain and feed conversion ratio (Garcia Neto et al., 2000) and also improves antibody and the immunoglobulin G production in broilers (Tsiagbe et al., 1987). Methionine is an indispensable dietary AA (supplemented through diet). It enters the citric acid cycle for energy production as succinyl CoA (Murray et al., 2006). On day 28, methionine was of high relative abundance in 1 Lx and 40 Lx groups while 10 Lx and 20 Lx were low (Table 5.1). Low relative abundance of methionine was also noted in treatment birds (CORT 30 mg/kg diet, chapter 3) but only on day 35. Deficient methionine levels has been associated with fatty liver (steatohepatitis) degeneration in rats (Oz et al., 2008). Autistic children had low levels of plasma methionine with increased vulnerability to oxidative stress (James et al., 2004a). Methionine toxicity has been linked to depression, spleen and liver hemosiderosis (excess iron built up leading to organ damage) and reduced feed utilisation in chickens (Ekperigin & Vohra, 1981; Harter & Baker, 1978). Increased methionine causes and an increase in levels of its metabolite homocysteine (Murray et al., 2006). Hyperhomocysteneimia has been associated with tibial dyschondroplasia in chickens (Orth et al., 1992). The high relative abundance of methionine in 1 Lx and 40 Lx birds is highly suggestive of an altered methionine-homocysteine metabolism and might predispose them to the above said conditions. Since all the lighting treatments had no effect on production parameters with only 1 Lx and 40 Lx groups having behavioral changes, there is a high likelihood of altered methionine-homocysteine metabolism in these groups. However, a thorough study in alteration of methionine – homocysteine metabolism in relevance to various lighting treatments in warranted.

Choline is important in that it serves as a precursor of betaine and acetylcholine and is also a methyl donor (methyl groups are important for carnitine, creatine,

polyamines and spermidine synthesis) (Murray et al., 2006; Wishart et al., 2009). It was found in relatively high abundance in the 1 Lx and 40 Lx lighting groups on day 14 and 21 (Table 5.1). Similarly, Teague et al., (2007) reported an increase in plasma levels of choline at day 44 in chronically stressed Sprague-Dawley rats subjected to restraint stress for 35 days. Increased choline has been shown to reduce oxidative stress in asthmatic rats (Mehta et al., 2009). The increased choline in 1 LX and 40 Lx may suggest oxidative stress caused by environment (Altan et al., 2003; Hong et al., 2008; Hai Lin et al., 2006).

Serine is a nonessential AA biosynthesized in the body vice versa from glycine (Murray et al., 2006). The carbon skeleton of serine is catabolised to form pyruvate which in turn is converted to acetyl CoA thereby entering the citric acid cycle for ATP production. Serine was found in increased relative abundance in the 1 Lx and 10 Lx lighting groups on day 28 while being in low relative abundance in 20Lx and 40Lx groups on day 28 (Table 5.1). Serine plays an important role in maintaining the methionine-homocysteine metabolism in that it helps in maintaining the homocysteine within physiological limits (Dudman et al., 1987; Verhoef et al., 2004).

Hyperhomocysteinemia has been associated with tibial dyschondroplasia (excess mass of cartilage at the proximal metaphysis of tibiotarsal bones) in chickens (Orth et al., 1992). It should be noted that physiological data collected on skeletal health and mobility for the same experiment as assessed by gait scoring (Garner et al., 2002) did not show any difference between lighting treatments (Deep et al., 2010). However Garner et al. (2002) reported that the presence and/or severity of tibial dyschondroplasia did not affect gait scoring. Further research in terms of altered methionine–homocysteine metabolism due to various lighting treatments and homocysteine interference in mineralization of bone matrix is warranted. Increased plasma serine levels have also been reported in depression associated with schizophrenia in humans (Maes et al., 1995). Birds reared under low light intensity 1 Lx (Deep et al., 2010) and 5 Lx (Blatchford et al., 2008) showed increased periods of resting indicative of behavioral change. Low relative abundance of serine levels seen in 20 Lx and 40 Lx groups in this study might be regulating the methionine - homocysteine metabolism. While high relative abundance levels seen in 1 Lx might be related to behavioral changes.

In this study, betaine was found in low relative abundance in 1 Lx and 40 Lx groups on day 28 (Table 5.1). It was also found in low relative abundance in CORT (30 mg/kg diet, Chapter 3) fed birds on day 28. Being a methyl donor (methyl groups are important for carnitine and creatine synthesis), betaine supplementation in poultry and pigs has been found to increase body weight gain and enhance intestinal cell activity (Eklund et al., 2005). Betaine is also important in the methionine – homocysteine metabolism as it aids in the remethylation of homocysteine to methionine. It is essential to have adequate levels of methionine as it gets converted to S Adenosyl methionine an important metabolite involved in the maintenance of DNA structure, synthesis of epinephrine and choline (Murray et al., 2006). Betaine supplementation has been reported to alleviate the compromised methylation process and poor oxidative stress response in autistic children.(James et al., 2004b). Being an osmolyte (Kidd et al., 1997), betaine can enhance the osmoregulatory process resulting from oxidative stress (Altan et al., 2003; Hong et al., 2008; Hai Lin et al., 2006)

Histidine is converted to  $\alpha$  ketoglutarate and then enters into the TCA cycle for energy production (Murray et al., 2006). It was found in relatively high abundance in birds fed on CORT (30 mg/kg diet, chapter 3) on days 14, and 35 and low relative abundance on day 21. Low relative abundance of histidine was seen in 10 Lx and 40 Lx lighting groups on day 21 while high relative abundance was seen in 1 Lx and 20 Lx groups on day 21 in this study. By day 28 1 Lx and 40 Lx treatments had high relative abundance of histidine (Table 5.1). Histidine undergoes decarboxylation and yields histamine which is an important mediator of inflammatory reactions (Li et al., 2007). Histidine has also been reported to alleviate oxidative and inflammatory changes in the liver of rats chronically fed on ethanol (Liu et al., 2008) showcasing their antioxidative and anti-inflammatory properties. Increased incidence of ulcerative foot pad lesions were seen in birds in the 1 Lx group in the sister study (Deep et al., 2010). Blatchford et al (2008) also reported increased incidence of hock and footpad bruising with birds reared under 200 Lx. Even though hock pad bruising was not been reported in the concurrent study with 40 Lx treated birds, mild superficial lesions of the footpad increased linearly with increasing intensity (Deep et al., 2010). Olanrewaju et al (2006) reported an increase in erythropoiesis in stressed chickens. This provides evidence that more erythrocytes

with increased hemoglobin levels are needed to meet increased oxygen demand during stress. Histidine is also an important component of hemoglobin structure and in its function (Wu, 2009). The histidine found in this study might be involved in the anti oxidative, anti inflammatory and erythropoietin process.

In a stressed scenario, insulin resistance due to increased CORT accompanied by increased glucagon, catecholamines (Exton et al., 1972), stimulates the release of hormone sensitive lipase thereby providing abundant plasma free fatty acids. These free fatty acids serve as source of acetyl CoA which is an important substrate for energy synthesis via the citric acid cycle. Apart from entering the citric acid cycle for energy production the acetyl CoA formed from the fatty acid oxidation are converted into ketone bodies, namely acetone, acetoacetate and  $\beta$ -hydroxybutyrate in the liver and serve as substrates for the gluconeogenic process (Murray et al., 2006). Since CORT was low in this experiment it is unlikely that a ketogenic process occurred. However the role of glucagon and catecholamines, which are not involved in ketone body synthesis from fatty acids (Alberti et al., 1978), cannot be ruled out as their levels were not determined. Varying housing conditions in mice have shown to activate the sympathetic neurotransmission mechanism profoundly (D'Arbe et al., 2002). In this study,  $\beta$ -hydroxybutyrate was found in relatively high abundance in the 40 Lx on day 21 (Table 5.1). This might indicate an increased energy demand in these lighting groups that is being compensated via gluconeogenesis. Birds reared under 40 Lx were reported to be more active than other treatment groups (Deep et al., 2010). A similar high relative abundance of ketone bodies were noted in the treatment birds fed CORT (30 mg/kg diet, chapter 3). Ketone bodies are known to be acidic and when accumulated in large amounts for long periods of time lead to ketoacidosis (Murray et al., 2006).

Glucose was higher in relative abundance in the 1 Lx and 40 Lx groups lower in the 10 Lx and 20 Lx groups (Table 5.1). Similar trends in glucose regulation were seen in the CORT (30 mg/kg diet, chapter 3) fed birds. Fructose, an isomer of glucose, was also found in high relative abundance in the 1 Lx and 40 Lx lighting groups and has a similar regulatory trend as that of glucose. Stress leads to gluconeogenesis and thereby elevated plasma glucose which provides energy to tissues (Sapolsky et al., 2000; Selye, 1951).

Lactate, synthesised anaerobically during glycolysis in skeletal muscles and erythrocytes, is used as a glucogenic substrate by the liver in the production of glucose via the lactic acid cycle (Murray et al., 2006). Increased levels of glucagon and catecholamines aids in the hepatic gluconeogenic process utilising pyruvates and lactate (Garrison & Haynes, 1975; Kraus-Friedmann, 1984). Gluconeogenesis is a highly oxygen dependant process with consumption of one oxygen molecule for every ATP formed (Olanrewaju et al., 2006). This leads to an imbalance in the oxygen leading to reversible adaptative process of anaerobic glycolysis producing lactate rather than the normal aerobic glycolysis of pyruvate production by the skeletal muscles (Murray et al., 2006; Wishart et al., 2009). Olanrewaju et al (2006) reported an increase in erythropoiesis in stressed chickens demonstrating more erythrocytes and increased haemoglobin levels are required to meet increased oxygen demand during stress. Increased lactate may be attributed to the provision of gluconeogenic substrates for energy production during stress and to compensate for the excessive oxygen demand created by gluconeogenesis. Lactate was found in high relative abundance in 10 Lx and 20 Lx lighting groups and not in 1 Lx and 40 Lx lighting groups on day 14 and 21 (Table 5.1). Lactate was also found in high abundance in day 14 followed by low relative abundance in day 21 and subsequently high relative abundance in days 28 and 35 in birds fed CORT (30mg/kg diet, chapter 3). Similarly, Teague et al (2007) demonstrated a decrease in lactate during acute stress at 9 days and increase when chronically stressed at 35 days in Sprague-Dawley rats. By days 14 and 21 birds in 1 Lx and 40 Lx had low relative abundance of lactate similar to the study of Teague et al (2007), which may suggest acute stress. It should be noted that the stressed birds fed CORT (30 mg/kg diet, chapter 3) also had reduced lactate levels on day 21. This variation in regulation may be attributed to differences in the level of stressed experienced by birds in this study and that of birds fed CORT (30 mg/kg, chapter 3).

In the stress scenario, where hyperglycaemia is the norm with an enhanced gluconeogenesis (Dupont et al., 1999, Sapolsky et al., 2000), increased carnitine aids in production of acetyl CoA and ketone bodies from fatty acids for the provision of energy. Fat metabolism, in this study, was altered as indicated by an increased relative abundance of carnitine seen with 1 Lx and 40 Lx on day 14 and 21 (Table 5.1). Likewise, birds fed

CORT (30 mg/kg diet, chapter 3) had a high relative abundance of carnitine on day 14 with low relative abundance by day 21. Similarly birds reared in 1 Lx and 40 Lx had low relative abundance of carnitine by day 28 (Table 5.1). The difference in the days taken for down regulation to low relative abundance between the two experiments might be due to different degrees of stress being experienced. Carnitine is synthesised endogenously by the body in muscles, kidney and liver (Murray et al., 2006; Wishart et al., 2009). Broilers supplemented with dietary carnitine had reduced abdominal fat accumulation (Rabie & Szilagyi, 1998; Xu et al., 2003). Carnitine has been reported to enhance the fatty acid oxidation process and decrease adiposity (Janssens et al., 1998; Murray et al., 2006; Xu et al., 2003). Conversely, reduced plasma carnitine has shown to decrease the  $\beta$  oxidation process of fatty acids leading to hypoglycaemia and increased adiposity (Murray et al., 2006).

## **5.5 Conclusion**

Neither H/L ratio nor CORT were indicative of stress or welfare (buphthalmia and increased food pad lesions) reported in the sister study (Deep et al., 2010). The H/L ratio increased with age, while CORT concentrations declined with age and were within physiological limits reported in unstressed chickens (Thaxton et al., (2005). Of the top metabolites differentiating 1 Lx and 40 Lx from 10 Lx and 20 Lx, five were also seen in the birds fed CORT (30 mg/kg, chapter 2). While birds in the 1 Lx lighting group had physical changes, 40 Lx did not appear to produce any physical effects on the birds. Weight gain, feed intake, feed gain ratio, mortality, skeletal health and bird mobility were not different between any of the lighting treatments of this experiment (Deep et al., 2010). I am unable to determine why the metabolite profiles of 40 Lx birds are similar to the 1 Lx birds; it is possible that the increased mobilization of energy sources is a result of increased activity. Increased light intensity has no effect on performance (Newberry et al., 1988) but it does not rule out a stress component. Further studies are required.

## CHAPTER 6

### SUMMARY

The “stress response”, is an evolved primary defensive, adaptive mechanism that allows an animal to deal with changes or conditions within its environment and to restore homeostasis. Any situation that elicits a response (either behavioral or physiological) can be considered a “stressor” and any combination of circumstances in the bird’s environment may potentially act as stressors. These include: climatic (i.e. extreme heat, cold or humidity), environmental (i.e. poor ventilation, light regimes, housing), nutritional (i.e. shortages of nutrients), physical (i.e. handling, transport), social (i.e. overcrowding), physiological (i.e. rapid growth), psychological (i.e. fear, noise), and pathological (i.e. disease). The purpose of physiological changes following an exposure to a stressor aids in minimizing detrimental effects of the stressor. Proper response to a stressor can be beneficial in life threatening situations; however, chronic or repeated exposure can have marked deleterious effects on reproduction, behavior, metabolism and immunocompetence (Siegel, 1980). Conditions which induce stress in an animal can reduce welfare and as well as performance (Olanrewaju, 2006; Scheele, 1997).

Identification of stress in the poultry industry has been done using various physiological endpoints with the two most accepted indicators of stress being elevated CORT and increased circulating heterophil/lymphocyte (H/L) ratios (Puvadolpirod & Thaxton, 2000a). The lack of consistency between studies measuring CORT and H/L ratios results in some skepticism when using them to quantify animal welfare and stress in poultry science (Maxwell, 1993; Rushen, 1991). Research using H/L as an indicator of stress demonstrates differences between studies with H/L ratio being influenced by such factors as age, sex, breed, social ranking, rearing methods (Campo & Davila, 2002a, 2002b; Gross & Siegel, 1983; Maxwell, 1993; Maxwell & Robertson, 1998; Moneva et al., 2009). Data compiled by (Maxwell, 1993) on the avian blood leukocyte response to stress have shown that severe food restriction causes a heteropenia, whereas climate, environmental, transport and heat stress causes heterophilia. In life threatening situations heteropenia and basophilia are seen and H/L ratio cannot be used as measure of stress in



such conditions (Maxwell, 1993). Thus, H/L ratios may not indicate the magnitude of stress experienced (Moneva et al., 2009).

The most immediate noticeable change following exposure to a stressor is the spike in plasma CORT concentrations. The half life of CORT in chicken is 8 minutes (Carsia et al., 1988). Studies conducted by (Romero & Reed, 2005; Wingfield et al., 1982) have demonstrated that blood samples collected within 2-3 minutes of handling time have CORT concentrations which better represent an unstressed state (i.e. baseline). In chickens, peak CORT is reached as quickly as 3 minutes (Voslarova et al., 2008). CORT has been successfully used for assessment of stressful events (Post et al., 2003; Puvadolpirod & Thaxton, 2000a, 2000b, 2000c), but there has been controversy regarding its usage as a reliable indicator of stress (Rushen, 1991). Data compiled by (Rushen, 1991) demonstrated varying plasma CORT concentrations between experiments studied with similar conditions. Given that the effect of CORT depends on a number of other factors such as receptor availability, sensitivity and binding protein availability (Landys et al., 2006), and as these factors can differ between species and prior stressful events, it becomes more difficult to compare or use CORT as a sole indicator of stress (Chloupek et al., 2009; Romero, 2004).

Environmental metabolomics is an emerging approach for examining metabolic profiles in biological systems exposed to environmental stress (Lin et al., 2006). Utilization of 1-dimensional <sup>1</sup>H Nuclear Magnetic Resonance spectroscopy (NMR) can provide insightful mechanistic information on effects of stressors by simultaneously measuring a large number of low molecular weight endogenous metabolites (Viant et al., 2003). Metabolic profiles can be examined using pattern recognition algorithms to reveal subsets of metabolites, potentially identifying biomarkers (Lin et al., 2006; Viant et al., 2003). Metabolomics has been successfully used to identify stressed and control groups in cattle (Aich et al., 2007) and Sprague-dawley rats (Teague et al., 2006). The advantage of using NMR over more traditional methods is that it provides information about abundance of multiple metabolites that gives information about the physiological changes occurring within the organism. With chicken meat topping the highest per capita consumption in Canada at 31.34 kg and the farm cash receipts of revenues and payments

from poultry and egg being \$3billion CAD by December 2010 (Statistics Canada), identification and reduction of stressors in chickens becomes important to the poultry industry both from a welfare and production standpoint.

In this study, comparison of H/L ratio results between the three experiments of this thesis showed that it can be used as an indicator of stress under extreme stressful conditions as seen in the treatment birds (TRT) fed exogenous CORT 30 mg/kg diet. High H/L ratio in the TRT is similar to other research showing increased H/L ratio following a stressor (Gross & Siegel, 1983; Maxwell & Robertson, 1998; Puvadolpirod & Thaxton, 2000a; Shaniko Shini et al., 2008). Our findings also suggest that H/L ratio is less variable with time as it remained consistently at an increased level of over 1.5 H/L ratio for 4 weeks, a period largely indicative of long term stress. However H/L ratio in the lighting experiments (chapters 4 and 5) were unable to differentiate or identify stress due to treatment. Investigation of H/L ratio in birds exposed to four different photoperiods showed an H/L ratio of  $0.41 \pm 0.05$  in the 23L group which also had significantly compromised welfare scores (Schwean Lardner & Classen, 2010). Birds reared in this group had enlarged heavier eyes, increased resting time and poor gait scores in comparison to other photoperiods. Likewise, investigation of the H/L ratio in birds exposed to four light intensities showed an H/L ratio of  $0.45 \pm 0.12$  H/L ratio for 1 Lx group that also had a compromised welfare with increased eye size and foot pad lesions in the sister study done by Deep et al., (2010). Body weight, feed conversion ratio and mortality were unaffected by the various lighting intensities of 1 Lx, 10 Lx, 20 Lx and 40 Lx (Deep et al., 2010). Thus the H/L ratio in both the photoperiod and light intensity experiments were highly suggestive of age related changes. They were not indicative of stress or compromised welfare in spite of the birds in 23L and 1Lx having compromised welfare, evincing behavioral and adaptive changes to the environment.

Serum CORT was increased dramatically in birds fed CORT (30 mg/kg feed, chapter 2) measured at day 14 (7 days after treatment, Figure 3.3B) While the concentration remained elevated in comparison to the control (CON) group, there was a gradual decline in CORT to day 35. This decline may be suggestive of a down regulation process to bring the serum CORT to physiological levels and thereby preventing deleterious effects. During chronic stress, birds can experience decreased basal CORT

related to habituation to the stressor, adrenal exhaustion or down regulation in the synthesis and release of CORT to prevent its deleterious effects (Rich & Romero, 2005). In comparison CORT in the CON group decreased between days 7 to day 14 and then remained fairly constant and less than the TRT group for the remainder of the study. Broilers between 2 to 7 weeks of age can have decreasing levels of CORT during acclimation to their environment (Thaxton et al., 2005). In this study, mortality was higher and body weight was significantly lighter in the TRT group compared with the CON group. Despite CORT at 30 mg/kg diet being a standard dose used in most poultry studies, the degree of stress was extreme and likely not representative of stress that most birds experience even under what would be considered stressful housing conditions.

In both lighting studies (photoperiod, chapter 4 and light intensity, chapter 5) CORT was a poor indicator of welfare or stress. CORT patterns were consistent with age related changes but were not affected by lighting treatment. In the photoperiod study (chapter 4), welfare scores in birds exposed to 23L followed by 20L suggested a compromised welfare (Schwean Lardner & Classen, 2010). Likewise, Deep et al. (2010) reported welfare issues related to heavier eyes and a higher incidence of footpad lesions for ) for birds receiving 1 Lx light intensity. Although concurrent examination of H/L ratio and CORT together can sometimes offer additional sensitivity for interpretation of results it offered no additional benefits here. These studies demonstrate that when stress is extreme as in chapter 3 with exogenously administered CORT (30 mg/kg feed) measuring H/L ratio and CORT can indicate stress. However, when stress is more subtle CORT and H/L ratio offer no advantages to measuring welfare parameters. This demonstrates that environmental impacts do not necessarily result in traditional measurable stress response variables.

The use of metabolomics differentiated the TRT birds from the CON birds in chapter 3, identifying amino acids isoleucine, valine, threonine, lysine, glutamine, histidine, methionine, and tryptophan, ketone body  $\beta$  hydroxybutyrate and other metabolites like carnitine, betaine, lactate, and sugars like glucose as being important. Of the top metabolites identified, metabolites including isoleucine, lysine, valine and tryptophan are important in stress induced anxiety, depression, behavioral changes and ketoacidosis while metabolites methionine, betaine, histidine suggest a compromised

methylation process and oxidative stress response involving osmoregulation. Metabolites threonine, glutamine and histidine also suggested altered immune response while metabolites including lactate, glucose and  $\beta$  hydroxybutyrate suggest an undergoing process of providing energy to the physiological needs of the body.

Comparison of metabolites from the photoperiod study (chapter 4) with fed CORT (30 mg/kg, chapter 3), identified 5 metabolites (valine, lysine, methionine, histidine and glucose) that had similar relative abundance and trends in lighting groups 20 L and 23 L. Welfare parameters determined by Schwan-Lardner and Classen, (2010) demonstrate that birds exposed to 23L have poor welfare scores followed by 20L while 14L and 17L groups had the best welfare scores. Metabolite profiles of birds in 14L and 17L groups also had less metabolic perturbations. Metabolites such as lysine and valine are important in stress and behavioral changes while metabolites methionine, choline, betaine, histidine, proline were indicative of the methylation process, osmoprotection and oxidative stress response. Creatine is highly indicative of the ongoing protein accretion process. Metabolites threonine and histidine suggested an immune response while metabolites like glucose, fructose, glycerol and carnitine are important for providing ongoing energy for the physiological needs of the body. Additional metabolites like choline, creatine and proline that were not among the top metabolites seen in the CORT (30 mg/kg diet, chapter 3) fed birds were among the top metabolites differentiating photoperiod treatments.

Comparison of metabolites from the light intensity study (chapter 5) with birds fed CORT (30 mg/kg, chapter 3), had 5 metabolites (isoleucine, histidine, carnitine and glucose, betaine) in lighting groups 1 Lx and 40 Lx that showed similar relative abundance. Additional metabolites like choline, serine and tyrosine that were not among the top metabolites seen in the CORT (30 mg/kg diet, chapter 3) fed birds were among the top metabolites differentiating lighting intensity treatments. Metabolomics was helpful in identifying metabolites like isoleucine, serine and tyrosine that might potentially signify behavioral changes. Increased mobilization of energy sources like glucose and ketone bodies in lighting group 40 Lx might also suggest an energy demanding than energy conserving state of homeostasis mostly a resultant of increased activity. The high relative abundance of energy metabolites in the 40Lx group might also

suggest an increased growth rate in them however further studies is warranted. By day 28, 1 Lx and 40 Lx groups had more metabolic perturbations within group members while 10 Lx and 20 Lx had more homogeneity of metabolites within group members (Figure 5.5). While birds in the 1 Lx lighting groups had physical changes, 40 Lx did not appear to produce any visible adverse effect on the birds. Weight gain, feed intake, feed gain ratio, mortality, skeletal health and bird mobility were not different between any of the lighting treatments (Deep et al., 2010). I am unable to determine why the metabolite profiles of 40 Lx birds are similar to the 1Lx birds; but it does not rule out a stress component as these groups had five metabolites following the pattern and trends seen in stressed birds fed CORT 30 mg/kg feed. As the difference between the lighting intensities was small (1 Lx to 40 Lx) compared to other studies which have used up to 200 Lx(Blatchford et al., 2008),future behavioral and metabolomics research in lighting range of 10 Lx to 20 Lx in comparison with higher lighting intensities of 40Lx and above is warranted.

While H/L ratio and CORT values were able to successfully differentiate the stressed birds from the control birds under extreme stressful conditions as seen in chapter 3, they failed to distinguish or identify any sort of stress in the lighting experiments in spite of concurrent sister studies showing compromised welfare in some of the lighting treatments. Metabolomics was also able to successfully differentiate the stressed birds from the control birds in chapter 3 with valuable insights about the physiological process involved in the stress response. In the lighting experiments metabolomics was able to identify metabolites suggestive of the behavioral changes, energy homeostasis, immune response, oxidative stress and osmoregulation. Metabolomics supported evidence of compromised welfare photoperiod ( 20L and 23L, chapter 4) and lighting intensity(1 Lx, chapter 5) by Schwean-Lardner and Classen (2010) and Deep et al. (2010), respectively. I am unable to determine why the metabolite profiles of 40 Lx birds are similar to the 1 Lx birds; it is possible that the increased mobilization of energy sources is a result of increased activity. Increased light intensity has no effect on performance(Newberry et al., 1988) but it does not rule out a stress component. Further studies are required.

Future metabolomic experiments should be directed at producing a more physiologically relevant stress and examining age related homeostatic response to stress.

Four groups of birds subjected to stress at different ages (7-14 days, 14-21 days, 21-28 days and 28-35 days) followed by recovery period with subsequent metabolite quantification would help to understand age related stress response and metabolism in broilers. This would potentially help us in identifying necessary dietary interventions and other environmental manipulations during that age group to alleviate stress. The role of other catabolic hormones like catecholamines and glucagon and anabolic hormone like insulin in maintaining homeostatic regulation could not be underestimated. Future experiments should be directed at measuring these hormones for a holistic understanding of the metabolic regulation. Metabolomics suggested that oxidative stress was more important than a glucocorticoid response, therefore to better understand biochemical pathways and the role of these metabolites in energy homeostasis and behavior, more studies are warranted. Examination of metabolites such as methionine, serine, betaine, and proline highly related to methylation process, osmoprotection and oxidative stress response need further attention. It would be prudent to measure for malondialdehyde levels to have an understanding of the magnitude of oxidative stress in relation to altered environmental conditions.

## References

- Adam, K. (1980) Sleep as a Restorative Process and a Theory to Explain Why. *Vol. 53* (pp. 289-305).
- Adam, K., & Oswald, I. (1977). Sleep is for tissue restoration. *Journal of the Royal College of Physicians of London*, 11(4), 376-388.
- Aich, P., Jalal, S., Czuba, C., Schatte, G., Herzog, K., Olson, D. J., Ross, A. R., Potter, A. A., Babiuk, L. A., & Griebel, P. (2007). Comparative approaches to the investigation of responses to stress and viral infection in cattle. *OMICS*, 11(4), 413-434.
- Alberti, K. G., Johnston, D. G., Gill, A., Barnes, A. J., & Orskov, H. (1978). Hormonal regulation of ketone-body metabolism in man. *Biochem Soc Symp*(43), 163-182.
- Altan, Ö., Pabuçcuoğlu, A., Altan, A., Konyalıoğlu, S., & Bayraktar, H. (2003). Effect of heat stress on oxidative stress, lipid peroxidation and some stress parameters in broilers. *British Poultry Science*, 44(4), 545-550.
- Antalikova, J., Jankela, J., & Baranovska, M. (1999). The effect of branched chain amino acids on protein synthesis in two skeletal muscles of Japanese quail. *Physiol Res*, 48(1), 59-63.
- Archer, J. (1979). Behavioural aspects of fear. In: W. Sluckin, Editor, *Fear in Animals and Man*.
- Austic, R. E., & Grau, C. R. (1971). Degeneration of the eyes and central nervous system of chick embryos subjected to phenylalanine or tyrosine deficiency. *Developmental Biology*, 25(1), 159-175.
- Aviagen. (2007a). *Ross 308 Broiler Nutrition Specifications*. Huntsville,Alabama 35805, USA: Aviagen.
- Aviagen. (2007b). *Ross 308 Broiler Performance Objectives*. Huntsville,Alabama 35805, USA: Aviagen.
- Bellinger, D. L., Lorton, D., Romano, T. D., Olschowka, J. A., Felten, S. Y., & Felten, D. L. (1990). Neuropeptide Innervation of Lymphoid Organs. *Annals of the New York Academy of Sciences*, 594(1), 17-33.
- Besedovsky. (1985). immune -neuroendocrine interaction
- Besedovsky, H., del Rey, A., Sorkin, E., & Dinarello, C. (1986). Immunoregulatory feedback between interleukin-1 and glucocorticoid hormones. *Science*, 233(4764), 652-654.
- Besedovsky, H., Sorkin, E., Felix, D., & Haas, H. (1977). Hypothalamic changes during the immune response. *European Journal of Immunology*, 7(5), 323-325.
- Besedovsky, H. O., & Rey, A. d. (1996). Immune-Neuro-Endocrine Interactions: Facts and Hypotheses. *Endocr Rev*, 17(1), 64-102.
- Bessei, W. (2006). Welfare of broilers: a review. *World's Poultry Science Journal*, 62(03), 455-466.

- Bijlsma, S., Bobeldijk, I., Verheij, E. R., Ramaker, R., Kochhar, S., Macdonald, I. A., van Ommen, B., & Smilde, A. K. (2005). Large-Scale Human Metabolomics Studies: A Strategy for Data (Pre-) Processing and Validation. *Analytical Chemistry*, 78(2), 567-574.
- Bishop, C. R., Athens, J. W., Boggs, D. R., Warner, H. R., Cartwright, G. E., & Wintrobe, M. M. (1968). Leukokinetic studies :XIII. A non-steady-state kinetic evaluation of the mechanism of cortisone-induced granulocytosis. *The Journal of Clinical Investigation*, 47(2), 249-260.
- Black, H. E. (1988). The Effects of Steroids Upon the Gastrointestinal Tract. *Toxicologic Pathology*, 16(2), 213-222.
- Blair, R., Newberry, R. C., & Gardiner, E. E. (1993). Effects of Lighting Pattern and Dietary Tryptophan Supplementation on Growth and Mortality in Broilers. *Poult Sci*, 72(3), 495-502.
- Blatchford, R. A., Klasing, K. C., Shivaprasad, H. L., Wakenell, P. S., Archer, G. S., & Mench, J. A. (2008). The effect of light intensity on the behavior, eye and leg health, and immune function of broiler chickens. *Poultry Science*, 88(1), 20-28.
- Bollard, M. E., Stanley, E. G., Lindon, J. C., Nicholson, J. K., & Holmes, E. (2005). NMR-based metabonomic approaches for evaluating physiological influences on biofluid composition. *NMR in Biomedicine*, 18(3), 143-162.
- Boushey, C. J., Beresford, S. A. A., Omenn, G. S., & Motulsky, A. G. (1995). A Quantitative Assessment of Plasma Homocysteine as a Risk Factor for Vascular Disease. *JAMA: The Journal of the American Medical Association*, 274(13), 1049-1057.
- Breuner, C., & Orchinik, M. (2002). Plasma binding proteins as mediators of corticosteroid action in vertebrates. *J Endocrinol*, 175(1), 99-112.
- Brosnan, J. T. (2003). Interorgan Amino Acid Transport and its Regulation. *The Journal of Nutrition*, 133(6), 2068S-2072S.
- Bryan Jones, R., & Mills, A. D. (1983). Estimation of fear in two lines of the domestic chick: Correlations between various methods. *Behavioural Processes*, 8(3), 243-253.
- Buckley, T. M. (2005). On the Interactions of the Hypothalamic-Pituitary-Adrenal (HPA) Axis and Sleep: Normal HPA Axis Activity and Circadian Rhythm, Exemplary Sleep Disorders. *Journal of Clinical Endocrinology & Metabolism*, 90(5), 3106-3114.
- Bundy, J., Davey, M., & Viant, M. (2009). Environmental metabolomics: a critical review and future perspectives. *Metabolomics*, 5(1), 3-21.
- Bundy, J. G., Keun, H. C., Sidhu, J. K., Spurgeon, D. J., Svendsen, C., Kille, P., & Morgan, A. J. (2007). Metabolic Profile Biomarkers of Metal Contamination in a Sentinel Terrestrial Species Are Applicable Across Multiple Sites. *Environmental Science & Technology*, 41(12), 4458-4464.
- Burton, R. S. (1991). Regulation of proline synthesis during osmotic stress in the copepod *Tigriopus californicus*. *Journal of Experimental Zoology*, 259(2), 166-173.
- Campo, J., & Davila, S. (2002a). Effect of photoperiod on heterophil to lymphocyte ratio and tonic immobility duration of chickens. *Poult Sci*, 81(11), 1637-1639.



- Campo, J., & Davila, S. (2002b). Estimation of heritability for heterophil:lymphocyte ratio in chickens by restricted maximum likelihood. Effects of age, sex, and crossing. *Poult Sci*, 81(10), 1448-1453.
- Campo, J. L., Gil, M. G., Davila, S. G., & Munoz, I. (2007). Effect of Lighting Stress on Fluctuating Asymmetry, Heterophil-to-Lymphocyte Ratio, and Tonic Immobility Duration in Eleven Breeds of Chickens. *Poult Sci*, 86(1), 37-45.
- Carew, L., McMurtry, J., & Alster, F. (2003). Effects of methionine deficiencies on plasma levels of thyroid hormones, insulin-like growth factors-I and -II, liver and body weights, and feed intake in growing chickens. *Poultry Science*, 82(12), 1932-1938.
- Carrasco, G. A., & Van de Kar, L. D. (2003). Neuroendocrine pharmacology of stress. *European Journal of Pharmacology*, 463(1-3), 235-272.
- Carsia, R. V., Weber, H., & Lauterio, T. J. (1988). Protein Malnutrition in the Domestic Fowl Induces Alterations in Adrenocortical Function. *Endocrinology*, 122(2), 673-680.
- Chen, C., Sander, J. E., & Dale, N. M. (2003). The Effect of Dietary Lysine Deficiency on the Immune Response to Newcastle Disease Vaccination in Chickens. *Avian Diseases*, 47(4), 1346-1351.
- Cheng, L. L., Chang, I.-W., Louis, D. N., & Gonzalez, R. G. (1998). Correlation of High-Resolution Magic Angle Spinning Proton Magnetic Resonance Spectroscopy with Histopathology of Intact Human Brain Tumor Specimens. *Cancer Research*, 58(9), 1825-1832.
- Chloupek, Voslářová, E., Suchý, P., Bedáňová, I., Pištěková, V., Vitula, F., Chloupek, J., & Večerek, V. (2009). Influence of Pre-Sampling Handling Duration on Selected Biochemical Indices in the Common Pheasant (*Phasianus colchicus*). *Acta Veterinaria Brno*, 78(1), 23-28.
- Classen, H. L., & Riddell, C. (1989). Photoperiodic effects on performance and leg abnormalities in broiler-chickens. [Article]. *Poultry Science*, 68(7), 873-879.
- Cohn, R., Yudkoff, M., Yost, B., & Segal, S. (1977). Phenylalanine-tyrosine deficiency syndrome as a complication of the management of hereditary tyrosinemia. *The American Journal of Clinical Nutrition*, 30(2), 209-214.
- Craig, J. V., Craig, J. A., & Vargas Vargas, J. (1986). Corticosteroids and other indicators of hens' well-being in four laying-house environments. *Poultry Science*, 65(5), 856-863.
- Craig, S. A. (2004). Betaine in human nutrition. *The American Journal of Clinical Nutrition*, 80(3), 539-549.
- D'Arbe, M., Einstein, R., & Lavidis, N. A. (2002). Stressful animal housing conditions and their potential effect on sympathetic neurotransmission in mice. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, 282(5), R1422-R1428.
- Dantzer, R., & Kelley, K. W. (1989). Stress and immunity: An integrated view of relationships between the brain and the immune system. *Life Sciences*, 44(26), 1995-2008.
- Davis, A. K., Maney, D. L., & Maerz, J. C. (2008). The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Functional Ecology*, 22(5), 760-772.

- Davis, A. T., & Austic, R. E. (1982). Threonine Metabolism of Chicks Fed Threonine-Imbalanced Diets. *The Journal of Nutrition*, 112(11), 2177-2186.
- Davis, G., Anderson, K., & Carroll, A. (2000). The effects of long-term caging and molt of Single Comb White Leghorn hens on heterophil to lymphocyte ratios, corticosterone and thyroid hormones. *Poultry Science*, 79(4), 514-518.
- Dawkins, M., Donnelly, C. A., & Jones, T. A. (2004). Chicken welfare is influenced more by housing conditions than by stocking density. [10.1038/nature02226]. *Nature*, 427(6972), 342-344.
- de Graaf, R. A., & Behar, K. L. (2003). Quantitative <sup>1</sup>H NMR Spectroscopy of Blood Plasma Metabolites. *Analytical Chemistry*, 75(9), 2100-2104.
- Deep, A., Schwan-Lardner, K., Crowe, T. G., Fancher, B. I., & Classen, H. L. (2010). Effect of light intensity on broiler production, processing characteristics, and welfare. *Poult Sci*, 89(11), 2326-2333.
- Defa, L., Changting, X., Shiyang, Q., Jinhui, Z., Johnson, E. W., & Thacker, P. A. (1999). Effects of dietary threonine on performance, plasma parameters and immune function of growing pigs. *Animal Feed Science and Technology*, 78(3-4), 179-188.
- deRoos, R. (1961). The corticoids of the avian adrenal gland. *General and Comparative Endocrinology*, 1(5-6), 494-512.
- Dettmer, K., & Hammock, B. D. (2004). Metabolomics--a new exciting field within the "omics" sciences. *Environ Health Perspect*, 112(7), A396-397.
- Dhabhar, F. S. (2006). Stress-induced Changes in Immune Cell Distribution and Trafficking: Implications for Immunoprotection versus Immunopathology. In C. J. Welsh, M. W. Meagher & E. M. Sternberg (Eds.), *Neural and Neuroendocrine Mechanisms in Host Defense and Autoimmunity* (pp. 7-25): Springer US.
- Dokken, B. B., Saengsirisuwan, V., Kim, J. S., Teachey, M. K., & Henriksen, E. J. (2008). Oxidative stress-induced insulin resistance in rat skeletal muscle: role of glycogen synthase kinase-3. *American Journal of Physiology - Endocrinology And Metabolism*, 294(3), E615-E621.
- Drucker-Colín, R., & Spanis, C. W. (1976). Is there a sleep transmitter? *Progress in Neurobiology*, 6(Part 1), 1-22.
- Dudman, N. P. B., Tyrrell, P. A., & Wilcken, D. E. L. (1987). Homocysteinemia: Depressed plasma serine levels. *Metabolism*, 36(2), 198-201.
- Dupont, J., Derouet, M., Simon, J., & Taouis, M. (1999). Corticosterone alters insulin signaling in chicken muscle and liver at different steps. *Journal of Endocrinology*, 162(1), 67-76.
- Eklund, M., Bauer, E., Wamatu, J., & Mosenthin, R. (2005). Potential nutritional and physiological functions of betaine in livestock. *Nutrition research reviews*, 18(1), 31-48.
- Ekperigin, H. E., & Vohra, P. (1981). Histopathological and biochemical effects of feeding excess dietary methionine to broiler chicks. *Avian Dis*, 25(1), 82-95.
- Estevez, I. (2007). Density Allowances for Broilers: Where to Set the Limits? *Poult Sci*, 86(6), 1265-1272.
- Exton, J. H., Friedmann, N., Wong, E. H.-A., Brineaux, J. P., Corbin, J. D., & Park, C. R. (1972). Interaction of Glucocorticoids with Glucagon and Epinephrine in the

- Control of Gluconeogenesis and Glycogenolysis in Liver and of Lipolysis in Adipose Tissue. *Journal of Biological Chemistry*, 247(11), 3579-3588.
- Felten, D. L., Felten, S. Y., Bellinger, D. L., Carlson, S. L., Ackerman, K. D., Madden, K. S., Olschowki, J. A., & Livnat, S. (1987). Noradrenergic Sympathetic Neural Interactions with the Immune System: Structure and Function. *Immunological Reviews*, 100(1), 225-260.
- Fleischer, B. (1981). The avian immune system. *Immunology Today*, 2(10), 195-200.
- Fraser, D. (1993). Assessing Animal Well-Being: Common Sense, Uncommon Science.
- Fulmer, G. R., Miller, A. J. M., Sherden, N. H., Gottlieb, H. E., Nudelman, A., Stoltz, B. M., Bercaw, J. E., & Goldberg, K. I. (2010). NMR Chemical Shifts of Trace Impurities: Common Laboratory Solvents, Organics, and Gases in Deuterated Solvents Relevant to the Organometallic Chemist. *Organometallics*, 29(9), 2176-2179.
- Garcia Neto, M., Pesti, G., & Bakalli, R. (2000). Influence of dietary protein level on the broiler chicken's response to methionine and betaine supplements. *Poult Sci*, 79(10), 1478-1484.
- Garner, J. P., Falcone, C., Wakenell, P., Martin, M., & Mench, J. A. (2002). Reliability and validity of a modified gait scoring system and its use in assessing tibial dyschondroplasia in broilers. *British Poultry Science*, 43(3), 355-363.
- Garrison, J. C., & Haynes, R. C. (1975). The hormonal control of gluconeogenesis by regulation of mitochondrial pyruvate carboxylation in isolated rat liver cells. *Journal of Biological Chemistry*, 250(8), 2769-2777.
- Gibson, S. W., Hughes, B. O., Harvey, S., & Dun, P. (1986). Plasma concentrations of corticosterone and thyroid hormones in laying fowls from different housing systems. *British Poultry Science*, 27(4), 621-628.
- Glaser, R., & Kiecolt-Glaser, J. K. (2005). Stress-induced immune dysfunction: implications for health. [10.1038/nri1571]. *Nat Rev Immunol*, 5(3), 243-251.
- Glick, B. (1979). The Avian Immune System. *Avian Diseases*, 23(2), 282-289.
- Gottlieb, H. E., Kotlyar, V., & Nudelman, A. (1997). NMR Chemical Shifts of Common Laboratory Solvents as Trace Impurities. *The Journal of Organic Chemistry*, 62(21), 7512-7515.
- Greef, J. v. d., Stroobant, P., & Heijden, R. v. d. (2004). The role of analytical sciences in medical systems biology. *Current Opinion in Chemical Biology*, 8(5), 559-565.
- Gross, W. B., & P. B. Siegel. (Ed.). (1993). *General principles of stress and welfare*. Wallingford, UK.: CAB International.
- Gross, W. B., & Siegel, H. S. (1983). Evaluation of the Heterophil/Lymphocyte Ratio as a Measure of Stress in Chickens. *Avian Diseases*, 27(4), 972-979.
- Hare, P. D., Cress, W. A., & van Staden, J. (1999). Proline synthesis and degradation: a model system for elucidating stress-related signal transduction. *Journal of Experimental Botany*, 50(333), 413-434.
- Harmon, B. (1998). Avian heterophils in inflammation and disease resistance. *Poult Sci*, 77(7), 972-977.
- Harper, A. E., Miller, R. H., & Block, K. P. (1984). Branched-Chain Amino Acid Metabolism. *Annual Review of Nutrition*, 4(1), 409-454.
- Harter, J. M., & Baker, D. H. (1978). Factors Affecting Methionine Toxicity and Its Alleviation in the Chick. *The Journal of Nutrition*, 108(7), 1061-1070.

- Häussinger, D., Gerok, W., Roth, E., & Lang, F. (1993). Cellular hydration state: an important determinant of protein catabolism in health and disease. *The Lancet*, 341(8856), 1330-1332.
- Haussinger, D., Lang, F., & Gerok, W. (1994). Regulation of cell function by the cellular hydration state. *American Journal of Physiology - Endocrinology And Metabolism*, 267(3), E343-E355.
- Häussinger, D., & Schliess, F. (1995). Cell volume and hepatocellular function. *Journal of Hepatology*, 22(1), 94-100.
- Holmes, W. N., and J. G. Phillips. (1976). The adrenal cortex of birds. In Academic Press (Ed.), *General, Comparative and Clinical Endocrinology of the Adrenal Cortex*. (pp. 292–420 ). New York.
- Hong, Y., Cho, S., Kim, H., Kim, Y., Cheong, H., Sohn, J., Kwon, M., Park, S., Cho, M., & Im, H. (2008). Oxidative Stress from Environmental Exposure to PAHs, VOCs and Formaldehyde in the Elderly. *Epidemiology*, 19(6), S283  
210.1097/1001.ede.0000340324.0000342190.d0000340322.
- Iwakabe, K., Shimada, M., Ohta, A., Yahata, T., Ohmi, Y., Habu, S., & Nishimura, T. (1998). The restraint stress drives a shift in Th1/Th2 balance toward Th2-dominant immunity in mice. *Immunology Letters*, 62(1), 39-43.
- James, S. J., Cutler, P., Melnyk, S., Jernigan, S., Janak, L., Gaylor, D. W., & Neubrandner, J. A. (2004a). Metabolic biomarkers of increased oxidative stress and impaired methylation capacity in children with autism. *The American Journal of Clinical Nutrition*, 80(6), 1611-1617.
- James, S. J., Cutler, P., Melnyk, S., Jernigan, S., Janak, L., Gaylor, D. W., & Neubrandner, J. A. (2004b). Metabolic biomarkers of increased oxidative stress and impaired methylation capacity in children with autism. *The American Journal of Clinical Nutrition*, 80(6), 1611-1617.
- Janssens, G., Buyse, J., Seynaeve, M., Decuypere, E., & De Wilde, R. (1998). The reduction of heat production in exercising pigeons after L-carnitine supplementation. *Poult Sci*, 77(4), 578-584.
- Johnson, E. O., Kamilaris, T. C., Chrousos, G. P., & Gold, P. W. (1992). Mechanisms of stress: A dynamic overview of hormonal and behavioral homeostasis. *Neuroscience & Biobehavioral Reviews*, 16(2), 115-130.
- Jones, R. B. (1986). Tonic immobility in the domestic fowl: A descriptive profile. *Applied Animal Behaviour Science*, 15(2), 182-182.
- Jones, R. B., Beuving, G., & Blokhuis, H. J. (1988). Tonic immobility and heterophil/lymphocyte responses of the domestic fowl to corticosterone infusion. *Physiology & Behavior*, 42(3), 249-253.
- Jones, R. B., & Faure, J. M. (1981). Sex and strain comparisons of tonic immobility ("Righting time") in the domestic fowl and the effects of various methods of induction. *Behavioural Processes*, 6(1), 47-55.
- Joseph, M. H., & Kennett, G. A. (1983a). Corticosteroid response to stress depends upon increased tryptophan availability. *Psychopharmacology*, 79(1), 79-81.
- Joseph, M. H., & Kennett, G. A. (1983b). Stress-induced release of 5-HT in the hippocampus and its dependence on increased tryptophan availability: an in vivo electrochemical study. *Brain Research*, 270(2), 251-257.

- Kaiser, P., Wu, Z., Rothwell, L., Fife, M., Gibson, M., Poh, T.-Y., Shini, A., Bryden, W., & Shini, S. (2009). Prospects for understanding immune-endocrine interactions in the chicken. *General and Comparative Endocrinology*, 163(1-2), 83-91.
- Khansari, D. N., Murgo, A. J., & Faith, R. E. (1990). Effects of stress on the immune system. *Immunology Today*, 11, 170-175.
- Kidd, M. T., Ferket, P. R., & Garlich, J. D. (1997). Nutritional and osmoregulatory functions of betaine. *World's Poultry Science Journal*, 53(02), 125-139.
- Kim, Y. S., Maruvada, P., & Milner, J. A. (2008). Metabolomics in biomarker discovery: future uses for cancer prevention. *Future Oncology*, 4(1), 93-102.
- Koelkebeck, K. W., Amoss Jr, M. S., & Cain, J. R. (1987). Production, physiological, and behavioral responses of laying hens in different management environments. *Poultry Science*, 66(3), 397-407.
- Konashi, S., Takahashi, K., & Akiba, Y. (2000). Effects of dietary essential amino acid deficiencies on immunological variables in broiler chickens. *The British journal of nutrition*, 83(4), 449-456.
- Kraus-Friedmann, N. (1984). Hormonal regulation of hepatic gluconeogenesis. *Physiological Reviews*, 64(1), 170-259.
- Kristensen, H. H., & Wathes, C. M. (2000). Ammonia and poultry welfare: a review. *World's Poultry Science Journal*, 56(03), 235-245.
- Kvetnansky, R., Pacak, K., Fukuhara, K., Viskupič, E., Hiremagalur, B., Nankova, B., Goldstein, D. S., Sabban, E. L., & Kopin, I. J. (1995). Sympathoadrenal System in Stress. *Annals of the New York Academy of Sciences*, 771(1), 131-158.
- Lacey, J. M., & Wilmore, D. W. (1990). Is Glutamine a Conditionally Essential Amino Acid? *Nutrition Reviews*, 48(8), 297-309.
- Landys, M. M., Ramenofsky, M., & Wingfield, J. C. (2006). Actions of glucocorticoids at a seasonal baseline as compared to stress-related levels in the regulation of periodic life processes. *General and Comparative Endocrinology*, 148(2), 132-149.
- Leeuwenberg, J. F., Smeets, E. F., Neefjes, J. J., Shaffer, M. A., Cinek, T., Jeunhomme, T. M., Ahern, T. J., & Buurman, W. A. (1992). E-selectin and intercellular adhesion molecule-1 are released by activated human endothelial cells in vitro. *Immunology*, 77(4), 543-549.
- Lehnert, H., Reinstein, D. K., Strowbridge, B. W., & Wurtman, R. J. (1984). Neurochemical and behavioral consequences of acute, uncontrollable stress: Effects of dietary tyrosine. *Brain Research*, 303(2), 215-223.
- Leveille, G. A., O'Hea, E. K., & Chakrabarty, K. (1968). In Vivo Lipogenesis in the Domestic Chicken. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)*, 128(2), 398-401.
- Lewis, P. D., & Morris, T. R. (2000). Poultry and coloured light. *World's Poultry Science Journal*, 56(03), 189-207.
- Li, P., Yin, Y. L., Li, D., Kim, S. W., & Wu, G. (2007). Amino acids and immune function. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S. Review]. *The British journal of nutrition*, 98(2), 237-252.

- Li, R., Kerr, W. L., Toledo, R. T., & Carpenter, J. A. (2000).  $^1\text{H}$  NMR Studies of Water in Chicken Breast Marinated with Different Phosphates. *Journal of Food Science*, 65(4), 575-580.
- Lien, R. J., Hess, J. B., McKee, S. R., Bilgili, S. F., & Townsend, J. C. (2007). Effect of Light Intensity and Photoperiod on Live Performance, Heterophil-to-Lymphocyte Ratio, and Processing Yields of Broilers. *Poult Sci*, 86(7), 1287-1293.
- Lin, C. Y., Viant, M. R., & Tjeerdema, R. S. (2006). Metabolomics: Methodologies and applications in the environmental sciences. *Journal of Pesticide Science*, 31(3), 245-251.
- Lin, H., Decuypere, E., & Buyse, J. (2006). Acute heat stress induces oxidative stress in broiler chickens. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology*, 144(1), 11-17.
- Lin, H., Sui, S. J., Jiao, H. C., Buyse, J., & Decuypere, E. (2006). Impaired development of broiler chickens by stress mimicked by corticosterone exposure. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology*, 143(3), 400-405.
- Lindon, J. C., Holmes, E., & Nicholson, J. K. (2006). Metabonomics techniques and applications to pharmaceutical research & development. [Review]. *Pharmaceutical research*, 23(6), 1075-1088.
- Lindon, J. C., Nicholson, J. K., & Everett, J. R. (1999). NMR Spectroscopy of Biofluids. In G. A. Webb (Ed.), *Annual Reports on NMR Spectroscopy* (Vol. Volume 38, pp. 1-88): Academic Press.
- Lindon, J. C., Nicholson, J. K., Holmes, E., & Everett, J. R. (2000). Metabonomics: Metabolic processes studied by NMR spectroscopy of biofluids. *Concepts in Magnetic Resonance*, 12(5), 289-320.
- Liu, W. H., Liu, T. C., & Yin, M. C. (2008). Beneficial effects of histidine and carnosine on ethanol-induced chronic liver injury. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, 46(5), 1503-1509.
- Lynn, S. E., Prince, L. E., & Phillips, M. M. (2010). A single exposure to an acute stressor has lasting consequences for the hypothalamo-pituitary-adrenal response to stress in free-living birds. *General and Comparative Endocrinology*, 165(2), 337-344.
- Maes, M., De Backer, G., Suy, E., & Minner, B. (1995). Increased plasma serine concentrations in depression. *Neuropsychobiology*, 31(1), 10-15.
- Malleau, A., Duncan, I., Widowski, T., & Atkinson, J. (2007). The importance of rest in young domestic fowl. *Applied Animal Behaviour Science*, 106(1-3), 52-69.
- Malmendal, A., Overgaard, J., Bundy, J. G., Sørensen, J. G., Nielsen, N. C., Loeschcke, V., & Holmstrup, M. (2006). Metabolomic profiling of heat stress: hardening and recovery of homeostasis in *Drosophila*. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, 291(1), R205-R212.
- Martí, O., García, A., Vellès, A., Harbuz, M. S., & Armario, A. (2001). Evidence that a single exposure to aversive stimuli triggers long-lasting effects in the hypothalamus-pituitary-adrenal axis that consolidate with time. *European Journal of Neuroscience*, 13(1), 129-136.

- Maser, J. D., & Gallup, G. G., JR. (1974). Tonic Immobility in the Chicken: Catalepsy Potentiation by Uncontrollable Shock and Alleviation by Imipramine. *Psychosom Med*, 36(3), 199-205.
- Maxwell, M. H. (1993). Avian blood leucocyte responses to stress. *World's Poultry Science Journal*, 49(01), 34-43.
- Maxwell, M. H., & Robertson, G. W. (1998). The avian heterophil leucocyte: a review. *World's Poultry Science Journal*, 54(02), 155-178.
- McCowen, K. C., Malhotra, A., & Bistrian, B. R. (2001). Stress-Induced Hyperglycemia. *Critical Care Clinics*, 17(1), 107-124.
- McEwen, B. S. (1997). The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions
- McEwen, B. S., Biron, C. A., Brunson, K. W., Bulloch, K., Chambers, W. H., Dhabhar, F. S., Goldfarb, R. H., Kitson, R. P., Miller, A. H., Spencer, R. L., & Weiss, J. M. (1997). The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions. *Brain Research Reviews*, 23(1-2), 79-133.
- Meerlo, P., Koehl, M., Van Der Borght, K., & Turek, F. W. (2002). Sleep Restriction Alters the Hypothalamic-Pituitary-Adrenal Response to Stress. *Journal of Neuroendocrinology*, 14(5), 397-402.
- Mehta, A. K., Arora, N., Gaur, S. N., & Singh, B. P. (2009). Choline supplementation reduces oxidative stress in mouse model of allergic airway disease. *European Journal of Clinical Investigation*, 39(10), 934-941.
- Melia, K. R., & Duman, R. S. (1991). Involvement of corticotropin-releasing factor in chronic stress regulation of the brain noradrenergic system. *Proceedings of the National Academy of Sciences*, 88(19), 8382-8386.
- Menaker, M., & Underwood, H. (1976). Extraretinal photoreception in birds. *Photochemistry and Photobiology*, 23(4), 299-306.
- Misener, S. R., Chen, C.-P., & Walker, V. K. (2001). Cold tolerance and proline metabolic gene expression in *Drosophila melanogaster*. *Journal of Insect Physiology*, 47(4-5), 393-400.
- Moneva, P., Popova-Ralcheva, S., Abadjieva, D., Gudev, D., & Sredkova, V. (2009). Poultry welfare assessment; is it possible to avoid handling-induced mental stress interference? *Biotechnology in Animal Husbandry*, 25(5/6), 1055-1062.
- Moore, C. B., & Siopes, T. D. (2000). Effects of Lighting Conditions and Melatonin Supplementation on the Cellular and Humoral Immune Responses in Japanese Quail *Coturnix coturnix japonica*. *General and Comparative Endocrinology*, 119(1), 95-104.
- Murray, R., Granner, D., Mayes, P., & Rodwell, V. (2006). *Harper's Illustrated Biochemistry (Harper's Biochemistry)*: McGraw-Hill Medical.
- Nash, R. F., Gallup, G. G., Jr., & Czech, D. A. (1976). Psychophysiological correlates of tonic immobility in the domestic chicken (*Gallus gallus*). *Physiol Behav*, 17(3), 413-418.
- Newberry, R. C., Hunt, J. R., & Gardiner, E. E. (1988). Influence of Light Intensity on Behavior and Performance of Broiler Chickens. *Poult Sci*, 67(7), 1020-1025.

- Nielsen, F., Mikkelsen, B. B., Nielsen, J. B., Andersen, H. R., & Grandjean, P. (1997). Plasma malondialdehyde as biomarker for oxidative stress: reference interval and effects of life-style factors. *Clin Chem*, 43(7), 1209-1214.
- Nijdam, E., Delezie, E., Lambooij, E., Nabuurs, M., Decuypere, E., & Stegeman, J. (2005). Comparison of bruises and mortality, stress parameters, and meat quality in manually and mechanically caught broilers. *Poult Sci*, 84(3), 467-474.
- Nimet, İ.-U., A˘aç, A., & Deri, N. (2001). Effect of carnitine on stress-induced lipid peroxidation in rat gastric mucosa. *Journal of Gastroenterology*, 36(4), 231-236.
- Odedra, B. R., Bates, P. C., & Millward, D. J. (1983). Time course of the effect of catabolic doses of corticosterone on protein turnover in rat skeletal muscle and liver. *Biochem J*, 214(2), 617-627.
- Olanrewaju, H., Wongpichet, S., Thaxton, J., Dozier, W., 3rd, & Branton, S. (2006). Stress and acid-base balance in chickens. *Poult Sci*, 85(7), 1266-1274.
- Olanrewaju, H. A. (2006). A Review of Lighting Programs for Broiler Production. *International Journal of Poultry Science*, 5 (4), 301-308.
- Ononiwu, J. C., Thomson, R. G., Carlson, H. C., & Julian, R. J. (1979). Studies on Effect of Lighting on Sudden-Death syndrome in Broiler-Chickens. [Article]. *Canadian Veterinary Journal-Revue Veterinaire Canadienne*, 20(3), 74-77.
- Orth, M. W., Bai, Y., Zeytun, I. H., & Cook, M. E. (1992). Excess Levels of Cysteine and Homocysteine Induce Tibial Dyschondroplasia in Broiler Chicks. *The Journal of Nutrition*, 122(3), 482-487.
- Ottaway, C. A., & Husband, A. J. (1994). The influence of neuroendocrine pathways on lymphocyte migration. *Immunology Today*, 15(11), 511-517.
- Oz, H., Chen, T., & Neuman, M. (2008). Methionine Deficiency and Hepatic Injury in a Dietary Steatohepatitis Model. *Digestive Diseases and Sciences*, 53(3), 767-776.
- Pacak, K., Palkovits, M., Kopin, I. J., & Goldstein, D. S. (1995). Stress-Induced Norepinephrine Release in the Hypothalamic Paraventricular Nucleus and Pituitary-Adrenocortical and Sympathoadrenal Activity: In Vivo Microdialysis Studies. *Frontiers in Neuroendocrinology*, 16(2), 89-150.
- Pan, L., & Gilbert, F. (1992). Activation of 5-HT1A receptor subtype in the paraventricular nuclei of the hypothalamus induces CRH and ACTH release in the rat. *Neuroendocrinology*, 56(6), 797-802.
- Pearlman, J. P., & Fielding, R. A. (2006). Creatine Monohydrate as a Therapeutic Aid in Muscular Dystrophy. *Nutrition Reviews*, 64(2), 80-88.
- Petros, A. M., Gyenes, M., Patthy, L., & Lliná, M. (1988). Analysis of the aromatic 1H NMR spectrum of chicken plasminogen kringle 4. *Archives of Biochemistry and Biophysics*, 264(1), 192-202.
- Post, J., Rebel, J., & ter Huurne, A. (2003). Physiological effects of elevated plasma corticosterone concentrations in broiler chickens. An alternative means by which to assess the physiological effects of stress. *Poult Sci*, 82(8), 1313-1318.
- Prayitno, D., Phillips, C., & Stokes, D. (1997). The effects of color and intensity of light on behavior and leg disorders in broiler chickens. *Poult Sci*, 76(12), 1674-1681.
- Prescott, N. B., & Wathes, C. M. (1999). Spectral sensitivity of the domestic fowl (*Gallus g. domesticus*). [Research Support, Non-U.S. Gov't]. *British Poultry Science*, 40(3), 332-339.



- Prescott, N. B., Wathes, C. M., & Jarvis, J. R. (2003). Light, vision and the welfare of poultry. *Animal Welfare*, 12(2), 269-288.
- Puvadolpirod, S., & Thaxton, J. (2000a). Model of physiological stress in chickens 1. Response parameters. *Poult Sci*, 79(3), 363-369.
- Puvadolpirod, S., & Thaxton, J. (2000b). Model of physiological stress in chickens 2. Dosimetry of adrenocorticotropin. *Poult Sci*, 79(3), 370-376.
- Puvadolpirod, S., & Thaxton, J. (2000c). Model of physiological stress in chickens 3. Temporal patterns of response. *Poult Sci*, 79(3), 377-382.
- Puvadolpirod, S., & Thaxton, J. (2000d). Model of physiological stress in chickens 4. Digestion and metabolism. *Poult Sci*, 79(3), 383-390.
- Quirk, P. G., King, G. F., Campbell, I. D., & Boyd, C. A. (1989). Quantitation of metabolites of isolated chicken enterocytes using NMR spectroscopy. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, 256(2), G423-G429.
- Raamsdonk, L. M., Teusink, B., Broadhurst, D., Zhang, N., Hayes, A., Walsh, M. C., Berden, J. A., Brindle, K. M., Kell, D. B., Rowland, J. J., Westerhoff, H. V., van Dam, K., & Oliver, S. G. (2001). A functional genomics strategy that uses metabolome data to reveal the phenotype of silent mutations. [10.1038/83496]. *Nat Biotech*, 19(1), 45-50.
- Rabie, M. H., & Szilagy, M. (1998). Effects of L-carnitine supplementation of diets differing in energy levels on performance, abdominal fat content, and yield and composition of edible meat of broilers. *The British journal of nutrition*, 80(4), 391-400.
- Rechtschaffen, A., Gilliland, M., Bergmann, B., & Winter, J. (1983). Physiological correlates of prolonged sleep deprivation in rats. *Science*, 221(4606), 182-184.
- Reinstein, D. K., Lehnert, H., & Wurtman, R. J. (1985). Dietary tyrosine suppresses the rise in plasma corticosterone following acute stress in rats. *Life Sciences*, 37(23), 2157-2163.
- Rich, E. L., & Romero, L. M. (2005). Exposure to chronic stress downregulates corticosterone responses to acute stressors. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, 288(6), R1628-R1636.
- Romero, L. M. (2004). Physiological stress in ecology: lessons from biomedical research. *Trends in Ecology & Evolution*, 19(5), 249-255.
- Romero, L. M., & Reed, J. M. (2005). Collecting baseline corticosterone samples in the field: is under 3 min good enough? *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology*, 140(1), 73-79.
- Rubingh, C. M., Bijlsma, S., Derks, E. P. P. A., Bobeldijk, I., Verheij, E. R., Kochhar, S., & Smilde, A. K. (2006). Assessing the performance of statistical validation tools for megavariate metabolomics data. *Metabolomics*, 2(2), 53-61.
- Rushen, J. (1991). Problems associated with the interpretation of physiological data in the assessment of animal welfare. *Applied Animal Behaviour Science*, 28(4), 381-386.
- Russo, S., Kema, I. P., Fokkema, M. R., Boon, J. C., Willemse, P. H. B., de Vries, E. G. E., den Boer, J. A., & Korf, J. (2003). Tryptophan as a Link between Psychopathology and Somatic States. *Psychosomatic Medicine*, 65(4), 665-671.

- Sapolsky, R. M., Romero, L. M., & Munck, A. U. (2000). How Do Glucocorticoids Influence Stress Responses? Integrating Permissive, Suppressive, Stimulatory, and Preparative Actions. *Endocr Rev*, 21(1), 55-89.
- Scahaw. (2000). *The welfare of chickens kept for meat production (broilers). Report of the Scientific Committee on Animal Health and Welfare Adopted 21 March 2000.*
- Scanes, C. G. (2009). Perspectives on the endocrinology of poultry growth and metabolism. *General and Comparative Endocrinology*, 163(1-2), 24-32.
- Schade, D. S., & Eaton, R. P. (1979). Pathogenesis of Diabetic Ketoacidosis: A Reappraisal. *Diabetes Care*, 2(3), 296-306.
- Scheele, C. (1997). Pathological changes in metabolism of poultry related to increasing production levels. *Veterinary Quarterly* 19, 127-130.
- Schmidt, C. W. (2004). Metabolomics: what's happening downstream of DNA. *Environ Health Perspect*, 112(7), A410-415.
- Schwean-Lardner, K., Fancher, B. I., & Classen, H. L. (2012a). Impact of daylength on behavioural output in commercial broilers. *Applied Animal Behaviour Science*, 137(1), 43-52.
- Schwean-Lardner, K., Fancher, B. I., & Classen, H. L. (2012b). Impact of daylength on the productivity of two commercial broiler strains. *British Poultry Science*, 53(1), 7-18.
- Schwean Lardner, K., & Classen, H. (2010). *Lighting for Broilers* Aviagen.
- Selye, H. (1951). The general-adaptation-syndrome. *Annu Rev Med*, 2, 327-342.
- Serkova, N. J., & Glunde, K. (2009). Metabolomics of Cancer. In M. A. Tainsky (Ed.), *Tumor Biomarker Discovery* (Vol. 520, pp. 273-295): Humana Press.
- Shanaiah, N., Zhang, S., Desilva, M. A., & Raftery, D. (2008). NMR-Based Metabolomics for Biomarker Discovery. In F. Wang (Ed.), *Biomarker Methods in Drug Discovery and Development* (pp. 341-368): Humana Press.
- Sharma, J. M. (1991). Overview of the avian immune system. *Veterinary Immunology and Immunopathology*, 30(1), 13-17.
- Shini, S., Kaiser, P., Shini, A., & Bryden, W. (2008). Biological response of chickens (*Gallus gallus domesticus*) induced by corticosterone and a bacterial endotoxin. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 149(2), 324-333.
- Shini, S., Kaiser, P., Shini, A., & Bryden, W. L. (2008). Differential alterations in ultrastructural morphology of chicken heterophils and lymphocytes induced by corticosterone and lipopolysaccharide. *Veterinary Immunology and Immunopathology*, 122(1-2), 83-93.
- Siegel, H. S. (1980). Physiological Stress in Birds. *BioScience*, 30(8), 529-534.
- Siegel, H. S., & Vankampen, M. (1984). Energy relationships in growing chickens given daily injections of corticosterone. [Article]. *British Poultry Science*, 25(4), 477-485.
- Silverstein, L. R., Francis X. Webster, David Kiemle. (2005). *Spectrometric Identification of Organic Compounds* (7th Edition ed.). United States of America: John Wiley & Sons, Inc.
- Sorenson, L. G., Nolan, P. M., Brown, A. M., Derrickson, S. R., & Monfort, S. L. (1997). Hormonal dynamics during mate choice in the northern pintail: a test of the 'challenge' hypothesis. *Animal Behaviour*, 54(5), 1117-1133.

- Soriano, J. R., Taitz, L. S., Finberg, L., & Edelman, C. M. (1967). Hyperglycinemia with ketoacidosis and leukopenia. *Pediatrics*, 39(6), 818-828.
- Sullivan, D. A., & Wira, C. R. (1979). Sex Hormone and Glucocorticoid Receptors in the Bursa of Fabricius of Immature Chicks. *The Journal of Immunology*, 122(6), 2617-2623.
- Szabo, A., Kenesei, E., Korner, A., Miltenyi, M., Szucs, L., & Nagy, I. (1991). Changes in plasma and urinary amino acid levels during diabetic ketoacidosis in children. *Diabetes Res Clin Pract*, 12(2), 91-97.
- Szabo, A., & Milisits, G. (2007). Clinicochemical follow-up of broiler rearing - A five-week study. [Article]. *Acta Veterinaria Hungarica*, 55(4), 451-462.
- Tankson, J., Vizzier-Thaxton, Y., Thaxton, J., May, J., & Cameron, J. (2001). Stress and nutritional quality of broilers. *Poult Sci*, 80(9), 1384-1389.
- Teague, C. R., Dhabhar, F. S., Barton, R. H., Beckwith-Hall, B., Powell, J., Cobain, M., Singer, B., McEwen, B. S., Lindon, J. C., Nicholson, J. K., & Holmes, E. (2007). Metabonomic Studies on the Physiological Effects of Acute and Chronic Psychological Stress in Sprague-Dawley Rats. *Journal of Proteome Research*, 6(6), 2080-2093.
- Ter Kuile, B. H., & Westerhoff, H. V. (2001). Transcriptome meets metabolome: hierarchical and metabolic regulation of the glycolytic pathway. *FEBS Letters*, 500(3), 169-171.
- Thaxton, J., & Puvadolpirod, S. (2000e). Model of physiological stress in chickens 5. Quantitative evaluation. *Poult Sci*, 79(3), 391-395.
- Thaxton, J. P., Stayer, P., Ewing, M., & Rice, J. (2005). Corticosterone in Commercial Broilers. *J APPL POULT RES*, 14(4), 745-749.
- Titchenal, C. A., Rogers, Q. R., Indrieri, R. J., & Morris, J. G. (1980). Threonine imbalance, deficiency and neurologic dysfunction in the kitten. *J Nutr*, 110(12), 2444-2459.
- Tiziani, S., Emwas, A. H., Lodi, A., Ludwig, C., Bunce, C. M., Viant, M. R., & Gunther, U. L. (2008). Optimized metabolite extraction from blood serum for 1H nuclear magnetic resonance spectroscopy. [Research Support, Non-U.S. Gov't]. *Analytical biochemistry*, 377(1), 16-23.
- Tobler, I., Murison, R., Ursin, R., Ursin, H., & Borbély, A. A. (1983). The effect of sleep deprivation and recovery sleep on plasma corticosterone in the rat. *Neuroscience Letters*, 35(3), 297-300.
- Tsiagbe, V. K., Cook, M. E., Harper, A. E., & Sunde, M. L. (1987). Enhanced Immune Responses in Broiler Chicks Fed Methionine-Supplemented Diets. *Poult Sci*, 66(7), 1147-1154.
- Umpleby, A. M., & Russell-Jones, D. L. (1996). The hormonal control of protein metabolism. *Baillière's Clinical Endocrinology and Metabolism*, 10(4), 551-570.
- Van Horne, P. L. M., & Achterbosch, T. J. (2008). Animal welfare in poultry production systems: impact of EU standards on world trade. [Review]. *Worlds Poultry Science Journal*, 64(1), 40-51.
- Verhoef, P., Steenge, G. R., Boelsma, E., van Vliet, T., Olthof, M. R., & Katan, M. B. (2004). Dietary serine and cystine attenuate the homocysteine-raising effect of dietary methionine: a randomized crossover trial in humans. *The American Journal of Clinical Nutrition*, 80(3), 674-679.

- Viant, M. R. (2007). Revealing the metabolome of animal tissues using  $^1\text{H}$  nuclear magnetic resonance spectroscopy. *Methods Mol Biol*, 358, 229-246.
- Viant, M. R. (2008). Recent developments in environmental metabolomics. *Molecular BioSystems*, 4(10), 980-986.
- Viant, M. R., Rosenblum, E. S., & Tjeerdema, R. S. (2003). NMR-Based Metabolomics: A Powerful Approach for Characterizing the Effects of Environmental Stressors on Organism Health. *Environmental Science & Technology*, 37(21), 4982-4989.
- Virden, W. S., Thaxton, J. P., Corzo, A., Dozier, W. A., & Kidd, M. T. (2007). Evaluation of Models Using Corticosterone and Adrenocorticotropin to Induce Conditions Mimicking Physiological Stress in Commercial Broilers. *Poultry Science*, 86(12), 2485-2491.
- Volek, J. S., Duncan, N. D., Mazzetti, S. A., Staron, R. S., Putukian, M., Gomez, A. L., Pearson, D. R., Fink, W. J., & Kraemer, W. J. (1999). Performance and muscle fiber adaptations to creatine supplementation and heavy resistance training. *Med Sci Sports Exerc*, 31(8), 1147-1156.
- Voslarova, E., Chloupek, P., Bedanova, I., Suchy, Pistekova, V., & Vecerek. (2008). *The effect of pre-sampling handling time on blood plasma biochemical parameters of broilers* (Vol. 115). Alfeld, Allemagne: Schaper.
- Wang, X., Zhao, T., Qiu, Y., Su, M., Jiang, T., Zhou, M., Zhao, A., & Jia, W. (2009). Metabonomics Approach to Understanding Acute and Chronic Stress in Rat Models†. *Journal of Proteome Research*, 8(5), 2511-2518.
- Wayland, M., Smits, J. J. E. G., Grant Gilchrist, H., Marchant, T., & Keating, J. (2003). Biomarker Responses in Nesting, Common Eiders in the Canadian Arctic in Relation to Tissue Cadmium, Mercury and Selenium Concentrations. *Ecotoxicology*, 12(1), 225-237.
- Wilmore, D. W. (1991). Catabolic illness. Strategies for enhancing recovery. *The New England journal of medicine*, 325(10), 695-702.
- Wingfield, J. C., Smith, J. P., & Farner, D. S. (1982). Endocrine Responses of White-Crowned Sparrows to Environmental Stress. *The Condor*, 84(4), 399-409.
- Wishart, Knox, C., Guo, A. C., Eisner, R., Young, N., Gautam, B., Hau, D. D., Psychogios, N., Dong, E., Bouatra, S., Mandal, R., Sinelnikov, I., Xia, J., Jia, L., Cruz, J. A., Lim, E., Sobsey, C. A., Shrivastava, S., Huang, P., Liu, P., Fang, L., Peng, J., Fradette, R., Cheng, D., Tzur, D., Clements, M., Lewis, A., De Souza, A., Zuniga, A., Dawe, M., Xiong, Y., Clive, D., Greiner, R., Nazyrova, A., Shaykhutdinov, R., Li, L., Vogel, H. J., & Forsythe, I. (2009). HMDB: a knowledgebase for the human metabolome. *Nucleic Acids Res*, 37(Database issue), D603-610.
- Wishart, D. S., Knox, C., Guo, A. C., Eisner, R., Young, N., Gautam, B., Hau, D. D., Psychogios, N., Dong, E., Bouatra, S., Mandal, R., Sinelnikov, I., Xia, J., Jia, L., Cruz, J. A., Lim, E., Sobsey, C. A., Shrivastava, S., Huang, P., Liu, P., Fang, L., Peng, J., Fradette, R., Cheng, D., Tzur, D., Clements, M., Lewis, A., De Souza, A., Zuniga, A., Dawe, M., Xiong, Y., Clive, D., Greiner, R., Nazyrova, A., Shaykhutdinov, R., Li, L., Vogel, H. J., & Forsythe, I. (2009). HMDB: a knowledgebase for the human metabolome. *Nucleic Acids Research*, 37(suppl 1), D603-D610.

- Wu, G. (2009). Amino acids: metabolism, functions, and nutrition. *Amino Acids*, 37(1), 1-17.
- Wyss, M., & Kaddurah-Daouk, R. (2000). Creatine and Creatinine Metabolism. *Physiological Reviews*, 80(3), 1107-1213.
- Xia, J., Psychogios, N., Young, N., & Wishart, D. S. (2009). MetaboAnalyst: a web server for metabolomic data analysis and interpretation. *Nucleic Acids Research*, 37(suppl 2), W652-W660.
- Xu, Z., Wang, M., Mao, H., Zhan, X., & Hu, C. (2003). Effects of L-carnitine on growth performance, carcass composition, and metabolism of lipids in male broilers. *Poult Sci*, 82(3), 408-413.
- Yang, E. V., & Glaser, R. (2002). Stress-induced immunomodulation and the implications for health. *International Immunopharmacology*, 2(2-3), 315-324.
- Yogman, M. W., & Zeisel, S. H. (1983). Diet and Sleep Patterns in Newborn Infants. *New England Journal of Medicine*, 309(19), 1147-1149.
- Zawilska, J. B., Berezinska, M., Lorenc, A., Skene, D. J., & Nowak, J. Z. (2004). Retinal illumination phase shifts the circadian rhythm of serotonin N-acetyltransferase activity in the chicken pineal gland. *Neuroscience Letters*, 360(3), 153-156.