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Physiological impact of hematocrit level during stress in broilers

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PHYSIOLOGICAL IMPACT OF HEMATOCRIT LEVEL DURING STRESS IN BROILERS

By

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Initial experiments evaluated the impact of hematocrit on a bird's ability to adapt to stress and what physiological mechanisms occurred to maintain oxygen carrying capacity (OCC). A final experiment was conducted to obtain proteomic evaluation of protein expression in monocytes of unstressed broilers. In initial experiments, ACTH treatment was applied to hematocrit separated broilers. Experiments evaluated effects of ACTH on broilers with low (19 to 22%, Experiment 1; 18-21%, Experiment 3), high (25 to 28%, Experiment 1; 24 to 27%, Experiment 3) or non-selected hematocrit levels (Experiment 2 and 3). After 4 d of ACTH, all treated birds had significantly increased ($P < 0.1$) pCO_2, HCO_3^-, and corticosterone levels, indicating as stress raises pCO_2, HCO_3^- must rise to maintain acid base balance. Birds not selected for hematocrit had significant drops in pO_2 when given ACTH. Broilers compensate for low OCC through release of red blood cells from storage sites, indicated by decreases in organ hemoglobin and increases in hematocrit and blood hemoglobin when birds are given ACTH. Accelerated red blood cell formation does not appear to occur, because erythropoietin decreases

following administration of ACTH to non-selected birds. ACTH induced stress, increased hemoglobin and hematocrit only in birds with low or non-selected hematocrit, suggesting high hematocrit birds prior to stress have an adaptive advantage during stress. Higher hematocrit prior to stress apparently provides ample OCC during stress. Unselected birds appear to require initiation of an inflammatory response to adapt to stress which can be noted by increases in total white blood cell count, monocytes, and heterophils and decreases in lymphocytes. High hematocrit birds appear less susceptible to stress effects by maintaining leukocytes at a constant level, while in non-selected birds lymphocyte percents drop. Proteomics was conducted on avian monocytes to reveal proteins related to immune functions, 3229 proteins were identified, with 46 involved in immune functions of professional antigen presenting cells. This protein data provides a means of comparing monocytes of stressed and unstressed animals in the future. In conclusion, evaluated hematocrit is advantageous in adaptation to stress through maintenance of high OCC, acid base balance and immune cells.

Key words: broiler, ACTH, stress, hematocrit, corticosterone, monocyte

DEDICATION

I would like to dedicate this research in memory of my major professor James Paul Thaxton. He was a wonderful mentor and friend.

I also want to dedicate this to my husband Marty McWilliams, precious daughter Marleigh Jo McWilliams, parents, Monte and Melissa Hale, my sister Scharidi Hale Barber and her family, Tom, Haeden, Kohlton and Jackson Barber.

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INTRODUCTION

Stress has been viewed in many ways by a variety of people. It was originally described through the process of fight or flight by Walter B. Cannon, 1915. One of the earlier definitions of stress, which is the most accepted, was depicted by Hans Selye in 1926 as a non-specific response to any demand on the body. For animals these demands or stressors may include environmental changes, crowding, feed restriction, and transportation. Selye proposed the general adaptation syndrome (GAS) to explain the physiological processes of stress. The purpose of the physiological processes of stress is to achieve a state of homeostasis and allow survival of the animal.

Stress is primarily viewed as undesirable, especially in animals, where performance may be negatively affected. However, not all stress is "bad" stress. Stress can be categorized into two groups: distress and eustress (Selye, 1973). Distress occurs when stressors appear to cause a negative outcome such as weight loss during cold temperatures. Eustress occurs when stressors have a positive outcome such as in childbirth. No matter the type of stress, the same overall processes occur to reach homeostasis.

Although many models have been developed in an attempt to understand the effects of stress on broiler physiology and well-being, a clear understanding of the effects

of stress is lacking. The first objective in this research was to evaluate the effect hematocrit level has on a bird's ability to adapt to stress. The second objective was to determine if a mechanism exists that provides additional red blood cells, either through production or from storage sites, to increase oxygen carrying capacity during stress. The final objective was to evaluate cells at the molecular level, by identifying protein profiles in monocytes, a leukocyte critical for innate immunity in the chicken. This provided initial information on protein expression in monocytes derived from normal animals. With this information future research can be conducted to observe the differences in protein expression when stress is induced.

CHAPTER II

LITERATURE REVIEW

Historical Perspective of Stress

From the perspective of most people, the term "stress" implies negative changes (Cordon, 2006) like pain, suffering, tension, and disease rather than positive changes, accepted by some segments of society, that lead to natural adaptation. The stress process plays an important role in maintaining a normal homeostatic state in the body of an animal. Although it often seems to have a detrimental effect on the well being and performance of an animal, these changes are necessary for survival and adaptation and are not always negative effects (Cordon, 2006).

Changes in the body due to stressors were first described by Walter B. Cannon, a Harvard Medical School Physiologist, in the early 1900's through the term "Fight or Flight". This is a process in which emergency adrenalin secretion occurs in the sympathico-adrenal system in response to acute emotions of fear, rage or pain (Cannon, 1929). Cannon and De La Paz demonstrated this process where an epinephrine-like compound was secreted in cat blood when the adrenal intact cat was exposed to a barking dog, but adrenalectomy prevented the appearance of the compound in experimental cats (Lightman, 1979). Characteristics of fight or flight were described as a specific response such as release of hormones like adrenaline and symptoms like dilation of the eyes, sweating, shivering and increasing heart rate and blood pressure depending on the

stressor. Cannon believed every stressor would elicit a different but specific response. These changes by the body allow the animal to adapt to the stressor presented. For instance, animals adapt to cold stress by shivering and constriction of blood vessels to conserve heat. During his study of stress, Cannon coined the term homeostasis, a set of acceptable ranges of values for internal variables (Goldstein and Kopin, 2007). He believed a threat to homeostasis induced activation of the sympathico-adrenal system and if an animal is unable to adapt, homeostasis cannot be maintained (Cannon, 1935).

In the 1930's, Hans Selye, a University of Montreal physiologist, (1973, 1976) described stress with a slightly different view, as a non-specific response to a demand or specific stimulus. Regardless of the stressor, adaptive non-specific responses will occur to alleviate physiological stress. Early on in his studies he proposed that all toxic substances produced the same signs: enlargement of the adrenals, shrinking of the thymicolymphatics, and gastrointestinal ulcers (Selye, 1973). These findings were later defined in Selye's concept termed the General Adaptation Syndrome (GAS), consisting of three descriptive stages: alarm, resistance and fatigue. In a brief overview of GAS, the alarm stage is characterized by initial release of epinephrine (EP) and norepinephrine (NE) ensuring bursts of energy needed to cope with the emergency (Cannon 1929) as well as stimulation of the hypothalamus, by a stressor, to release corticotrophin releasing factor. The latter leads to release of pro-opiomelanocortin (POMC) followed by adrenocorticotropin (ACTH) by the anterior pituitary. This in turn stimulates the adrenal cortex to release the glucocorticoid, corticosterone (CS) and lead into the stage of resistance. The resistance stage is defined by the adaptations of the body to resist the effects of the stressor. Corticosterone will signal changes in the intermediary metabolism

of carbohydrates, proteins and fats leading to an increase in cholesterol and glucose via gluconeogenesis. The effects from corticosteroids lead to many immunological changes and a long term effect of immunosuppression as well as a decrease in body weight due to tissue catabolism. During resistance, changes will be observed for blood gases, blood pH and acid base balance. If the animal is unable to resist the effects of the stressor, the result will often times be death from fatigue, the final stage of the general adaptation syndrome.

The definition introduced by Selye (1976), stating that stress is the nonspecific response of the body to any demand made upon it and that stressors are stress producing factors, is commonly used by physiologists. It appears though that the same stressors do not always elicit a stress response. Therefore, the meaning of stress is highly debated by scientists and researchers due to the complexity of responses that occur after a stressor is introduced.

Stress Physiology

As previously noted, the stress cycle or GAS begins with an initial stimulus or stressor in the alarm phase. Stressors can range from environmental factors (temperature, overcrowding, feed deprivation), toxic substances, or disease to name a few. During the alarm phase of GAS, signals from potentially harmful stressors reach the central nervous system through various sensory receptors. Every receptor has a specific response profile for each particular stimulus. Therefore the body may receive a variety of messages when different receptors mediate more than one stressor. These messages lead to changes in the internal environment and release of neurotransmitters. The autonomic nervous

system, consisting of sympathetic and parasympathetic neural components, functions to stabilize the internal environment by controlling circulation, respiration, digestion, excretion and body temperature (Cannon, 1929; Monnier, 1968). The hypothalamus plays a central role in the autonomic nervous system and is recognized as the coordinator for the previously listed visceral and somatic functions. The hypothalamus is also responsible for endocrine functions by the body as well as emotional behavior (Monnier, 1968). Thus, the neurotransmitting responses that occur during GAS are transmitted via two divisions of the neuroendocrine system: sympathico-adrenal medullary (SAM) and the hypothalamic-pituitary adrenal (HPA) systems (Monnier, 1968; Siegel, 1980). Activation of SAM occurs to battle stressors, while activation of HPA occurs to create a physiological pathway to overcome or adapt to the stressor (Siegel, 1995). A great deal of research exists that indicate changes occur in the body affecting a variety of systems including immune, reproductive and overall growth in an attempt to reach homeostasis (Berne and Levy, 1996; Pulvadolipirod and Thaxton, 2000 a,b,c,d; Dhabhar, 2003; Dovio et al., 2003).

Sympathico-Adrenal Medullary System

The adrenal medulla and sympathetic nerves work together to compose what is known as the SAM system (Goldstein and McEwen, 2002). Catecholamines, EP and NE, are released into circulation within seconds of stimulation of the SAM system. The release of these catecholamines, allow the body to increase emergency response reactions during fight or flight (Cannon, 1929) or alarm phase of GAS (Selye, 1973).

Of the catecholamines, EP is the primary stimulator of metabolic activites. Early reports suggest that in carbohydrate metabolism, NE has a fraction of the hyperglycemic activity of EP (Bloom and Russell, 1955). The unique effects of EP and NE have are due to attractions to different membrane receptors. Effective binding of EP to β-adrenergic receptors coupled with a G_s protein (Enoksson et al., 2003; Storey, 2004) activate adenylate cyclase and adenyl kinase activity (Eckert et al., 1988). Epinephrine promotes breakdown of stored metabolic substrates into utilizable substrates via glycogenolysis, gluconeogenesis and or lipolysis (Berne and Levy, 1996). Through mobilization of these metabolic substrates catecholamines increase mobilization of glucose to vital organs during emergency responses (Berne and Levy, 1996). Some vital tissues in particular, the cardiovascular tissue, are able to utilize larges amounts of glucose, while suppressing uptake by skeletal muscle tissue (Enoksson et al., 2003; Guyton and Hall, 1996). Norepinephrine binds most effectively to α-adrenergic receptors (Sugano et al., 1980) coupled to G_i-mediated protein. This coupling causes a decrease in adenylate cyclase activity and/or elevation of intracellular Ca^{2+} concentration. Parallel to the effects of EP, NE increases basal metabolic rate by stimulating thermogenesis (Guyton and Hall, 1996) and providing energy for the skeletal muscles without limiting the action of EP to provide glucose to cardiovascular tissues (Berne and Levy, 1996). The net effects of this energy surge are increases in heart rate, respiration, blood pressure and nerve sensitivity (Guyton and Hall, 1996). The short lived actions of the sympathetic stress response during acute stress prepare the body to physiologically carry out amazing physical tasks (Guyton and Hall, 1996).

Hypothalamic-Pituitary Adrenal System

The HPA axis is a complex feedback and inhibitory system composed of the hypothalamus, pituitary, and the adrenals (Tsigos et al., 2006). During stress the SAM system is activated simultaneously with the HPA axis starting the resistance phase of GAS (Siegel, 1980). The hypothalamus is an important, but not the only source of corticotropin releasing factor (CRF). At first stimulation by a stressor, there is typically an increase in neurotransmitters and an excitatory neural drive of CRF neurons leading to secretion of CRF. The neurotransmitters involved in regulation include, acetylcholine (ACh), serotonin (5HT), noradrenalin (NA), dopamine (DA), EP and NE (Tilders et al., 1985; Widmaier et al., 1989; Song and Leonard, 2000).

After neural stimulation, CRF as well as arginine-vasopressin are released then carried to the anterior pituitary through the hypothalamic-hypophyseal portal system. Corticotropin releasing factor, after binding CRF receptors, stimulates production of the intracellular second messenger cAMP. Activation of this pathway leads to increased production of proopiomelanocortin (POMC) and increased release of adrenocorticotropic hormone (ACTH) and β-endorphin, proteolytic products of POMC (Seasholtz, 2000). While CRF is widely regarded as the major hypothalamic releasing factor for ACTH, other hypothalamic compounds such as vasopressin, oxytocin, and norepinephrine can also stimulate ACTH release (Plotsky, 1988; Seasholtz, 2000). Research in birds and humans indicate that the anterior pituitary is not the only source of ACTH or ACTH-like substance; leukocytes also play a key role in ACTH production (Siegel et al., 1985; Reder, 1992). This supports earlier findings that hypophysectomized mice and chickens are still capable of responding to stressors with elevated CS levels (Friedman, 1996) and

a pigeon's adrenals remain functional following hypophysectomy (Miller and Riddle, 1942).

Adrenocorticotropin primarily functions as a stimulator of the adrenals to produce the adrenocortical hormones (McEwen et al., 1997), cortisol or CS during acute stress and as a long term regulator of growth and maintenance for the adrenal cortex (Ramachandran et al., 1977). Adrenocorticotropin is secreted directly into circulation and transported to the adrenal cortex via blood. At the adrenal cortex, ACTH binds specific plasma membrane receptors of the cortical tissue. Research in mice shows a high affinity hybridization for the ACTH receptor mRNA in both the zona fasciculata and the zona glomerulosa with fewer cells hybridizing in the zona reticularis (Xia and Wikberg, 1996). Birds do not exhibit zones of the cortical layers, instead the adrenal cortex is intermingled with medullary tissue (Sturkie, 1976). Binding of the ACTH-receptor complex stimulates adenylate cyclase and formation of cyclic-AMP from ATP. Extracellular calcium is required for optimal ACTH binding and ACTH-induced steroid production (Litwack, 2005). Extracellular calcium enters via T-type calcium channels, independent of adenylyl cyclase activation. The cAMP activates protein kinase resulting in phosphorylation of specific proteins. The active protein kinase leads to increased hydrolysis of cholesteryl esters forming additional cholesterol, increased transport of free cholesterol into the mitochondria, and increased rates of synthesis of side chain cleavage enzymes responsible for catalyzing the rate-limiting step of cortisol synthesis. The high levels of cAMP may also stimulate Ca^{2+} channels leading to an increase in cytoplasmic Ca^{2+} which may stimulate corticosteroid release.

While the major glucocorticoid in humans is cortisol (Bao et al., 2008), in rats and birds it is CS (Puvadolpirod and Thaxton 2000b). Corticosterone is slightly different from cortisol, as cortisol has a hydroxyl at position 17 (Baxter and Rousseau, 1979). These corticosteroids act on two types of receptors, mineralcorticoid and glucocorticoid. Glucocorticoids play a major role as a negative feedback regulator of both CRF and ACTH during and after acute stress. It is believed that as cortisol levels rise in the extracellular space, negative feedback occurs on adrenocortical cells by increasing the rate of metabolism of intracellular cortisol to inactive forms and by enhancing the activity of cellular 5α-reductase (Norman and Litwack, 1987).

Corticosteroids may be found in both the protein-bound and unbound forms. Normally, 8 to 10% of corticosteroids in circulation are in the free, unbound form (Loriaux, 2006). These unbound corticosteroids are active components that are responsible for binding proper glucocorticoid receptors therefore initiating cellular mechanisms. The remainder of the corticosteroids are tightly bound to corticosteroid-binding globulin (CBG), around 70 to 77%, or loosely bound to albumin, 15 to 20% (Loriaux, 2006). While all corticosteroids will bind albumin, only natural corticosteroids bind CBG with the exceptions of dexamethasone in chickens (Gould and Siegel, 1978) and prednisolone (Ballard, 1979).

The glucocorticoid receptor (GR), a member of the nuclear receptor family (Robinson-Rechavi et al., 2001), is responsible for regulation of glucose homeostasis, lipid metabolism and inflammation (Reichardt et al, 2000). The GR is a transcription factor which is activated upon ligand binding with a glucocorticoid. At activation GR translocates from the cytoplasm into the nucleus where it serves as a transcriptional

regulator both directly or indirectly. Transrepression of genes is responsible for positive anti-inflammatory effects, while transactivation is the responsible mechanism for the negative effects of glucocorticoids (Adcock, 2000). Transactivation occurs via binding of GR to a glucocorticoid response element (GRE). This homodimer induces transcription of genes such as TAT (tyrosine aminotransferase) (Jantzen et al., 1987). Transrepression occurs through multiple mechanisms. Direct transrepression occurs with binding of activated GR to a negative GRE, inhibiting transcription. As a monomer activated GR can bind to pro-inflammatory transcription factors such as NF-κB leading to indirect transrepression (De Bosscher et al., 2000). This indirect mechanism, also called cross-talk, is thought to be the primary route by which glucocorticoids exert their anti-inflammatory effects. The anti-inflammatory effects of cortisol initially stabilize lysosomal membranes making it difficult for them to rupture and preventing release of the proteolytic enzymes that cause inflammation. Cortisol diminishes formation of prostaglandins and leukotrines therefore decreasing vasodilation, capillary permeability, migration of leukocytes to the damaged area and phagocytosis of the damaged cells. It suppresses lymphocyte, specifically T-lymphocyte, production; therefore further suppressing the inflammation process (Guyton and Hall, 1996).

Activated glucocorticoids have many roles as mentioned previously. Of these, the effects on metabolism are some of the most important. Glucocorticoids play a key role in stimulating glucose production from non-carbohydrate sources through gluconeogenesis by the liver. They increase all the enzymes required to convert amino acids into glucose in liver cells by activating DNA transcription in the liver nuclei and formation of mRNA (Guyton and Hall, 1996). The corticosteroids mobilize amino acids from muscle and

other extrahepatic tissues to be utilized in glucose production (Nussey and Whitehead, 2001). Cortisol causes moderate decreases in the rate of glucose utilization by cells throughout the body. This occurs possibly through a depression in the oxidation of, glycolysis required, nicotinamide-adenine dinucleotide to form NAD^+ (Guyton and Hall, 1996). Often, the increase in gluconeogenesis and reduction in glucose utilization leads to high concentrations of blood glucose causing hyperglycemia (Norris and Carr, 2006) or adrenal diabetes. The effects of the glucocorticoids can be counteracted with insulin.

In addition to the effects corticosteroids have on carbohydrate metabolism, effects can be observed on protein and lipid metabolism in the body. Cortisol causes a decrease in protein synthesis and increase in protein catabolism in cells of the body resulting in a reduction in protein stores throughout the body except the liver (Storey, 2004). Cortisol has also been known to decreases RNA formation leading to reductions in protein synthesis in muscle, lymphoid and other extrahepatic tissues. As proteins throughout the rest of the body decrease, liver proteins as well as plasma proteins produced by the liver, increase (Nussey and Whitehead, 2001). This enhancement of liver proteins occurs as amino acid transport to the liver cells and enzymes required for protein synthesis in the liver increase. The enhanced transport of amino acids from the muscles to the liver and increased utilization of these amino acids by the liver leads back to the enhancement of glucose metabolism via gluconeogenesis (Nussey and Whitehead, 2001). Much like mobilization of amino acids, cortisol and CS promote mobilization of fatty acids from adipose tissue. The mobilization increases free fatty acids in plasma and the utilization of these for energy. Lack of α-glycerophosphate, a derivative of glucose, may be the key to release of free fatty acids. Cortisol appears to enhance oxidation of fatty acids in the

cells. The increase in mobilization of fats in combination with increases in fatty acid oxidation in the cells shift the metabolic systems of cells to utilize fatty acids for energy rather than glucose during times of starvation or stress (Guyton and Hall, 1996).

Overall Effects of Stress

Metabolism

Stress, as indicated above, typically results in increases in catecholamines and glucocorticoids. During stress these neuroendocrine substances play important roles in intermediary metabolism of multiple tissues by promoting availability and mobilization of glucose (Felig and Frohman, 2001). Catecholamines and glucocorticoids work together to initiate normal glycogenolysis, gluconeogenesis, and lipolysis activities (Exton et al, 1972; Berne and Levy, 1996). These hormones are involved in carbohydrate, protein, fat and mineral metabolism.

Gluconeogenesis

In the presence of stress animals go through several processes of adaptation. One of the major events occurring during adaptation is when glucose and glycogen are synthesized from non-carbohydrate sources during gluconeogenesis. The sources include endogenous gluconeogenic substrates and specific natural amino acids (Exton, 1979). The amino acid precursors include glycine, serine, threonine, valine, histidine, cysteine, proline, alanine, methionine, glutamine, asparagine, glutamate, aspartate, arginine, isoleucine, phenylalanine, tyrosine, tryptophan, and endogenous gluconeogenic substrates

include lactate, pyruvate and glycerol (Exton, 1979; Nelson and Cox 2005). Studies have revealed that glucocorticoids play a key role in regulation of glucose homeostasis (Long et al., 1940; Haynes, 1964; Exton et al., 1972). One early study showed administration of adrenal cortex extracts to fasted, adrenalectomized rats lead to increases in blood glucose levels and liver glycogen deposition (Long et al., 1940). Although much of the effects of glucocorticoids have been attributed to glucogenic amino acid mobilization from peripheral tissues and a secondary rise in insulin levels, it has been demonstrated that direct addition of glucocorticoids to rat livers does indeed stimulate gluconeogenesis (Haynes, 1964). Studies comparing perfused livers in control and adrenalectomized rats, suggest glucocorticoids play a permissive role in hormonal regulation of gluconeogenesis (Friedmann et al., 1967; Exton et al., 1972). Exton (1979) found basal gluconeogenesis in the liver to not be impaired in adrenalectomized rats which were fed, however a decrease in response to glucagons and catecholamines was noted. Exton (1979) proposed that permissive effects of glucocorticoids involve maintenance of responsiveness of key enzymes to activation or inactivation by mechanisms involving cAMP or additional intracellular second messengers.

Glucocorticoids can have an overall effect on carbohydrate metabolism by increasing the rate of gluconeogenesis up to ten-fold (Guyton and Hall, 1996). Glucocorticoids directly activate at least 10 key hepatic gluconeogenic enzymes, such as glucose-6-phosphatase, fructose diphosphatase, aspartate aminotransferase, tyrosine aminotransferase, and phosphoenolpyruvate carboxykinase (PEPCK) (Baxter and Forsham, 1972; Fowden et al., 1993; Lin et al., 1998; Pech et al., 1997). Enzymes involved in gluconeogenesis and glycolysis are the same except for three enzymes which

act in three specific gluconeogenesis reactions. These include glucose-6-phosphatase, fructose-1,6-bisphosphatase and pyruvate carboxylase. The three enzymes are essential in the conversion of glucose-6-phosphate to glucose, fructose-1,6-bisphosphate to fructose-6-phosphate, and oxaloacetate to phosphoenolpyruvate (Nelson and Cox, 2005).

Protein Metabolism

Early research has revealed net catabolic effects of glucocorticoid hormones from endogenous or exogenous sources on skeletal muscle (Wool and Weinshelbaum, 1960). Net loss of muscle protein occurs due to breakdown rate exceeding synthesis rate. A decrease in muscle protein-synthesis rate after glucocorticoid administration has been established in early research (Wool and Weinshelbaum, 1960; Hanoune et al., 1972). Protein synthesis has been consistently inhibited in glucocorticoid treated animals (Odedra et al., 1983). Glucocorticoids have been shown to inhibit rate of collagen synthesis in dermal fibroblasts, skin, granulation tissue as well as hepatocytes (Kruse et al., 1978; McNelis and Cutroneo, 1978; Russell et al., 1981; Diegelmann et al., 1982). Studies suggest that collagen tissue declines nonselectively as a result of a generalized anti-anabolic effect which leads to decreases in cell growth, DNA synthesis, and protein synthesis (McCoy et al., 1980; Verbruggen and Salomon, 1980). Glucocorticoids inhibit DNA synthesis and reduce RNA formation in numerous extrahepatic tissues such as muscle tissue (Rannels and Jefferson, 1980), while RNA and protein synthesis in liver tend to be stimulated (Baxter and Rousseau, 1979). It has been shown that reductions in body weight gain in chicks consistently occurred after CS administration (Siegel and van

Kampen, 1984; Donker and Beuving, 1989). Glucocorticoids are generally considered to be growth inhibiting steroids because of catabolic effects on protein metabolism.

Although specific increases in rate of muscle protein breakdown has not been indisputably demonstrated, it is believed to occur due to increased proteolytic activity in the muscles of corticosteroid-treated animals (Mayer & Rosen, 1977). In one case an increase in the breakdown rate of muscle protein after treatment of rats with high doses of glucocorticoids was reported (Goldberg, 1975). Another study concluded that glucocorticoids do not influence the rate of muscle protein breakdown until plasma concentrations of the hormone rise to values observed in states of severe stress (Tomas et al., 1979). These contradicting results make it difficult to understand protein catabolism.

Lipid Metabolism

Stress causes lipolytic affects in adipose tissue via stimulation from catecholamines and glucocorticoids. The cleaved triglyceride becomes free fatty acids and a glycerol. There are at least three ß-adrenoceptor subtypes ($ß_1$-, $ß_2$-, and $ß_3$-) which coexist in fat cells of the white adipose tissue of rat (Hollenga and Zaasgma, 1989) or other mammalian species (Langin et al., 1991). The coexistence of these receptor subtypes, which are involved in the same biological effect, suggests a complex regulation of adipose tissue responses to endogenous catecholamines during physiological processes involving lipomobilization (Lafontan and Berlan, 1993). Adenylyl cyclase and ß-adrenoceptors are positively coupled, in order that an increase in cAMP levels causes stimulation of lipolysis after activation of cAMP-dependent protein kinase phosphorylates the hormone-sensitive lipase (Gilman, 1987; Langin et al., 1996). Elevated epinephrine

and norepinephrine levels lead to triglyceride breakdown in adipose tissue through the β_2-adrenergic stimulation of a hormone-sensitive lipase.

Two effects of elevated concentrations of glucocorticoids on lipid metabolism are redistribution of body fat and facilitation of actions of catecholamines and other lipolytic agents (Harvey, 1996). Research indicates enhanced hepatic rates of lipogenesis and plasma lipids are associated with increased levels of glucocorticoids in animals and humans (Amatruda et al., 1983). Glucocorticoids increase lipolysis and elevate plasma levels of free fatty acids via actions to inhibit glucose and glycerol production (Fain, 1979).

Mineral Metabolism

Catecholamines are involved in modulation of renal physiology by regulating renal blood flow, glomerular filtration, tubular transport and renin and erythropoietin secretion (Insel and Snavely, 1981). They directly influence sodium excretion through affects on the renal tubule, and, in animals, renal denervation or reflex suppression of the renal sympathetic activity acutely increase excretion (Grossman, 1998). Stimulation of calcitonin and parathyroid hormone by catecholamines results in changes in calcium, phosphate and magnesium metabolism.

Glucocorticoids are responsible for the most common type of secondary osteoporosis (Dovio et al., 2003). This osteoporosis appears to be a result of glucorticoid influence to decrease bone formation and bone reabsorption. Glucocorticoids act directly on intestinal absorption. In vivo research in the rat has indicated inhibition of calcium and phosphate absorption as a result of the stress hormones (Ferraro et al., 1976). It is

believed that glucocorticoids act on calcium absorption through two mechanisms: specific alteration of 1, 25-$(HO)_2$CC metabolism and non-specific biochemical alteration in function of intestinal epithelial cells.

Immune System

Stress is known to effect blood cell numbers in the body of animals and humans. Research suggests that overall immune responses are enhanced during acute stress but suppressed during chronic stress (Dhabhar, 2003). Adrenal hormones prepare the immune system for antigen invasion through the skin just as it prepares the musculoskeletal system and cardiovascular system for fight or flight. Without the adrenal glands it has been shown that delayed type hypersensitivity responses are not enhanced by stress (Dhabhar, 2003).

Effects on Cell and Tissue Types

Research has determined that avian lymphocytes are key sources of ACTH production (Elrom, 2000). The ACTH leads to increases in endogenous production of CS in cytoplasm and nuclei of sensitive lymphatic tissue (Elrom, 2000). Glucocorticoids generally suppress innate and cellular immune responses (Kapcala, 1995; Franchimont et al., 2000), however some research shows they may have immunoenhancing properties as well (Dhabar and McEwen, 1996; Dhabhar, 1998). In addition to glucocorticoid induced effects, CRF has been shown to increase lymphocyte production in cultures of human blood in both the absence and presence of T-cell mitogens (Singh, 1989). In the presence of stress and rising CS levels in treated chicken feed, it has been observed that

lymphocytes decrease (Elrom, 2000), while heterophils increase, indicating a rise in heterophil/lymphocyte (H/L) ratio in the presence of stress (Gross and Siegel, 1983; Puvadolpirod and Thaxton, 2000c). Glucocorticoid induced apoptosis is a well-recognized physiologic regulator of murine T-cell number and function (Brunetti et al., 1995). In one study, subjects experiencing physical stress had significantly increased granulocytes, monocytes and all lymphocyte, although B cells rose more than T cells and T (suppressor) cells more than T (helper) cells (Landmann et al., 1984).

One cell type particularly affected by stressors is monocytes, the precursor cells to both macrophages and dendritic cells. A complete understanding of the effects of glucocorticoids on monocytes are unknown, however, during both physical and psychological stress monocytes significantly increase (Landmann et al., 1984). Transcriptional effects of glucocorticoids on monocytes are believed to occur over a period of hours. The glucocorticoid receptor heterodimer binds to DNA at the glucocorticoid response elements, upstream of the promoter regions of glucocorticoid-responsive genes, inducing or repressing transcription, thus generating monocytes with broad anti-inflammatory and pro-resolution properties (Yona and Gordon, 2007). Glucocorticoids are known to suppress monocytic release of several cytokines, most noteably TNF-α (Joyce et al., 1997) therefore suppressing inflammation. Glucocorticoids are reported to induce high levels of CD163 expression on cultured human monocytes (Sulahian et al., 2000). This molecule is responsible for clearing free hemoglobin and therefore has anti-inflammatory properties.

In addition to stress induced changes in immune cell numbers, atrophy and decreases in weight of immune organs (thymus, spleen and bursa of Fabricius) (Guyton

and Hall, 1996) have been reported in broilers treated with ACTH (Puvadolpirod and Thaxton, 2000 a, c). The thymus, organ responsible for generation of the T cell repertoire, is subject to involution due to thymocytolytic effects of glucocorticoids. However, Rogerro and coworkers (2006) show partial reversal of thymic atrophy and decreased number of $CD4^{+}CD8^{+}$ thymocytes through blockage of glucocorticoid receptors with the glucocorticoid antagonist RU486.

Effects on Interleukins

Glucocorticoids are key regulators of production and action of immunoregulatory cytokines (Maes et al., 1998; Blotta et al., 1997). One cytokine in particular, interleukin-1 (IL-1) participates in an immunoregulatory feedback loop with these stress hormones. It appears the roles of IL-1 are to enhance release of dopamine, NE, serotonin, and secretion of CRF in response to stress (Shintani et al., 1995) as well as regulate the hypothalamic temperature control system (Guyton and Hall, 1996). Students under high levels of stress had significant stimulation of several pro-inflammatory cytokines, tumour necrosis factor α (TNF-α), interleukin 6 (IL-6), and interferon γ (IFN-γ), as well as, IL-1 receptor antagonist (IL-1Ra), and IL-10, an anti-inflammatory cytokine (Maes et al., 1999). Blotta and coworkers (1997) found corticosteroids significantly inhibited production of IL-12 in monocytes. This cytokine is responsible for potent enhancement of IFN-gamma and inhibition of IL-4 synthesis in T cells. Reduced production of IL-12 in corticosteroid-treated monocytes resulted in a decreased ability by monocytes to induce IFN-gamma and an increased ability to induce IL-4 in T cells (Blotta et al., 1997). Leukocytic cytokines produced by immune cells but inhibited by the stress hormones, are

prominent regulators of leukocyte maturation and recruitment, cellular and humoral immunity, inflammatory responses and overall disease resistance (Klasing, 1994).

Effects on Inflammation

Multiple events occur in the process of inflammation (Guyton and Hall, 1996). The first step to occur in inflammation is an increase in local blood flow due to vasodilatation of the local blood vessels, resulting in heat and redness. This is followed by increased permeability of the capillaries with leakage of large quantities of fluid into the interstitial spaces. There may be clotting of fluid in these spaces due to large amounts of fibrinogen and other proteins leaking through the capillary walls. Endothelial cells are activated to express adhesion molecules that promote leukocyte binding by slowing blood flow. Adhesion molecules allow the leukocytes to attach to the endothelium and migrate into the damaged tissue. These changes are controlled by cytokines from activated cells such as macrophages. These activated cells have the ability to produce chemokines to attract other cells like neutrophils and even more monocytes. Neutrophils are usually the first cells attracted after inflammation has started, followed my monocytes/macrophages, eosinophils and finally lymphocytes. Some of these cells such as macrophages become phagocytic and can consume the damaged cells.

Cortisol is well-known for two anti-inflammatory effects: preventing inflammation by blocking early stages or resolution of inflammation after it has began in combination with rapid healing. To prevent inflammation cortisol will stabilize lysosomal membranes of leukocytes (Persellin and Ku, 1974) decreasing rupture and release of proteolytic enzymes needed to induce inflammation. Another effect of cortisol

is decrease of capillary permeability thus a decrease in plasma leakage. Stress hormones are responsible for decreasing leukocyte migration to site of inflammation and phagocytosis of damaged cells by diminishing prostaglandin and leukotriene formation (Guyton and Hall, 1996). Prostaglandins and leukotrienes are key regulators of vasodilation, capillary permeability and leukocyte mobilization. As mentioned earlier, glucocorticoids are responsible for suppression of lymphocytes responsible for enhancing the process of inflammation. Due to the suppression of IL-1 by cortisol, fever and vasodilation are reduced therefore preventing inflammation. Cortisol is believed to resolve inflammation by blocking factors (cytokines) which promote it.

Erythropoiesis

Erythrocytes are the key cells responsible for hemoglobin, blood gas and carbonic anhydrase transport. These cells, as well as all blood cells, stem from pluripotential hematopoietic stem cells found in the bone marrow of adults. These cells can commit to become a colony forming unit or a lymphoid stem cell. A cell committed to eventually becoming an erythrocyte is referred to as a colony forming unit erythrocyte (Guyton and Hall, 1996). The process of erythrocyte production takes about 5 d. Exposure of the body to low levels of oxygen during long periods, such as stress or high altitude, results in growth induction, differentiation and production of greatly increased numbers of erythrocytes (Guyton and Hall, 1996). Erythropoietin (EPO) is the principal factor which stimulates erythrocyte production. In the absence of EPO, hypoxia has little effect in stimulating new red blood cell production. One case in rats has shown in the presence of ACTH, serum erythropoietin decreases as hematocrit and hemoglobin increase

suggesting stress causes a need for mature red blood cells but tends to suppress production and maturation of new immature cells (Zhang et al., 2004).

Evaluating Stress in Chickens

Models for Evaluating Stress in Chickens

Birds respond to stress much like mammals, however, the principal glucocorticoid in birds is CS (Vleck et al., 2000). Stress has been evaluated in chickens through various direct and indirect applications of stressors. Researchers have altered environmental conditions climatically (Thaxton et al., 1968; Thaxton and Siegel 1970, 1972, 1973; Bottje and Harrison, 1985; Wideman et al., 2003) and socially through overcrowding (Siegel and Gross, 1965; Pesti and Howarth, 1983; Turkylimaz, 2008,) have restricted feed (Bartov et al., 1988; Beuving et al., 1989; Latshaw, 1991; Webster, 2003), introduced toxic substances for ingestion (Thaxton et al., 1974, 1975), administered CS through feed, water, or by injection (Post et al., 2003; Lin et al., 2004a,b) and administered ACTH via injection or pump (Puvadolpirod and Thaxton 2000 a,b,c,d; Virden et al., 2005; Mumma et al., 2006) in search of a better understanding of the effects and adaptations that occur in commercial poultry to stressors . Commonly evaluated parameters examined in experimental birds include adrenal responses, blood chemistries and gases, changes in the immune system and commercial production (Gross and Siegel, 1981a;Puvadolpirod and Thaxton 2000 a, b, c, d, Virden et al., 2005, Mumma et al., 2006).

In recent years a model has been developed to observe physiological adaptive responses in broilers and layers under stressful conditions using a continuous infusion mini-osmotic ACTH pump (Puvadolpirod and Thaxton 2000 a, b, c, d). Responses observed in the model experiment included increased CS, glucose, cholesterol, triglycerides, lipoproteins, and heterophil/lymphocyte ratio, however blood gases and acid base balance were not evaluated. Using this model, research has been conducted to evaluate acid base balance and blood gas changes in broilers (Olanrewaju et al., 2006) and reproductive, digestive and immune parameters in layers (Mumma et al., 2006).

Avian Adrenal Glands

As in the mammal, the adrenal cortex is vital for an animal's adaptive abilities and survival during stress (Siegel 1995). Due to its ability to remain functional, producing CS, in hypophysectomized birds the avian adrenal is believed to be an independent organ (Siegel, 1995). Research has indicated additional sources of ACTH or ACTH-like substances beyond the adrenals (Hendricks and Mashaly, 1998). Stimulated leukocytes have the ability to increase CS through ACTH production and release into circulation of both mammals and avian species (Smith et al., 1983, 1986; Hendricks and Mashaly, 1998). Actions of stress hormones have direct effects on adrenal weight. Administration of ACTH has been shown to maximize adrenal weight (Freeman and Manning 1975; Thaxton et al., 1982).

Adrenal cholesterol, a steroid hormone precursor, is significantly reduced during stress exposure in normal (Challey, 1966; Freeman and Manning, 1975; Thaxton, et al., 1975) and bursectomized chicks (Sato and Glick, 1964). In vitro, a reduction in asorbic

acid concentration occurs in parallel with an increase in CS synthesis (Kitabchi and West, 1975). Reports of decreased adrenal asorbic acid levels have been noted in bursectomized chicks, although levels were not totally depleted after ACTH administration (Nir et al., 1975).

Avian Blood Chemistry, Gases, and Cells

As previously noted ACTH stimulates the adrenal cortex to produce and secrete CS into circulation. Therefore, circulating levels of CS in the blood are frequently measured to identify presence of stress. Changes in CS then lead to additional changes in levels of chemical components of the blood (glucose, cholesterol) as well as blood gases. As mentioned in earlier sections, glucocorticoids induce gluconeogenesis which plays a key role in glucose and energy metabolism and leads to the numerous changes observed in the blood during stress. Additionally, glucocorticoids have been shown to be required for sustained proliferation of erythroid progenitor cells (Bauer et al., 1999) and an inducer of erythropoiesis (Lugar et al., 2003).

A great deal of research exists indicating in the presence of various stressors, plasma CS levels rise in avian blood (Edens, 1978; Puvadolpirod and Thaxton 2000 a, b, c, d; Post et al., 2003). These stressors range from ACTH (Puvadolpirod and Thaxton 2000 a, b, c, d; Post et al., 2003; Olanrewaju et al., 2006) CS (Donker and Beuving, 1989; Post et al., 2003), temperature (Edens, 1978) and feed deprivation (Weber et al., 1990). Research conducted by Vandenborne and coworkers (2005) indicated within 30 min of corticosterone injection, circulating corticosterone concentration was significantly increased, compared with the control group. It is believed the highest level of

corticosterone, caused by the injection together with the stress of handling, occurs prior to the 30 min time limit. The single injection of corticosterone caused a significant decrease in plasma corticosterone levels after 2 h. Research suggests the low concentration is the result of a cascade of feedback effects beginning in the hypothalamus, passing through the pituitary and ending via inhibition of corticosterone production by the adrenal cortex (Vandenborne et al., 2005). Dose, route of administration and time lapse after stress induction appear to influence plasma CS levels (Davis and Siopes, 1987).

Stress induced through continuous delivery of ACTH via a mini-osmotic pump over 7 d has been shown to cause increases in not only plasma CS, but also increases in glucose, cholesterol, triglycerides, high-density lipoprotein and total protein (Puvadolpirod and Thaxton, 2000a, b; Olanrewaju et al., 2006). In one study, these results appeared to peak by d 4 and return to normal levels by d 12 post infusion (Puvadolpirod and Thaxton, 2000a, b). Results of plasma levels of CS, glucose, cholesterol, and total protein agree with metabolic changes associated with stress in chickens (Siegel, 1995).

The pattern of change of acids and bases depends upon the effects of stressors on the condition and rate of metabolism, respiration, and the mechanism of H+ equivalent exchange (Olanrewaju et al., 2006). Studies show that during heat stress an increase in respiratory rate, results in decreased pCO2 leading to decreased carbonic acid concentration (H_2CO_3) and hydrogen (H^+) (Borges et al., 2007; Teeter et al., 1985). Kidneys must respond by increasing HCO_3 excretion and reducing H^+ excretion in an attempt to maintain acid-base balance of the bird. These changes and overall effect on pH or acid-base balance may lead to respiratory alkalosis. In one study ACTH induced

broilers had increased effects on not only HCO_3^-, but also on anion gap, while reducing Na^+, K^+, and Cl^-. In this study, pH was not affected by ACTH, indicating a balance of acid and base (Olanrewaju et al., 2006).

Erythropoietin production by kidney cells and its stimulatory influence on bone marrow production of erythroblasts has been well studied in chickens (Pain et al., 1991; Wickeramasinghe et al., 1994). Shortage of oxygen in the body or hypoxia up-regulates erythropoietin gene transcription. As stress induces metabolic production of glucose by gluconeogenesis, one mol of O_2 is utilized for every mole of ATP required for each enzymatic step, indicating how costly gluconeogenesis is from the O_2 and energy standpoints. In broilers erythropoiesis is thought to increase in order to produce O_2 needed to support energy production by gluconeogenesis during stress. This hypothesis is supported by research indicating ACTH induced broilers have reduced partial pressure of O_2 (pO_2) but increased hematocrit, mean corpuscular hemoglobin concentration, and blood hemoglobin levels, as well as increased partial pressure of CO_2 (pCO_2) and HCO_3^- (Olanrewaju et al., 2006).

Avian Immune System

Stress is believed to be a major factor leading to immunosuppression and pathology in the bird (Isohe and Lillehoj, 1992; Maxwell, 1993). Corticosterone and neurotransmitters of the sympathetic nervous system have a significant impact on lymphoid tissue and their functionality. Corticosteroids and other stress hormones in poultry have been shown to cause atrophy of the spleen, thymus and bursa of Fabricius (Gross and Siegel, 1981a; Puvadolpirod and Thaxton, 2000 a, c), decreased antibody

responses (Gross et al., 1980), decreased production of lymphokines, interferon, decreased numbers of both T-lymphocytes and natural killer cells (Isohe and Lillehoj, 1992), and enhanced susceptibility to infections.

Changes in immune cells are some of the most reliable and least variable indicators of avian stress. Environmental stressors such as road transportation and heat stress in birds produced significantly reduced lymphocytes, raised heterophils and H/L ratios (Borges et al., 2004) and basophilias (Maxwell, 1993). Bedánová and coworkers (2003) showed chronic heat stress caused significantly decreased lymphocyte counts and increased H/L ratio in male and female broilers. Acute heat stress, 30± 1°C for 24 h, led to a decrease in heterophils and H/L ratio in males only; however males and females both had significantly increased basophil counts. Although heterophil numbers typically increase in circulation, their phagocytic and bactericidal activities have been shown to decrease (Borges et al., 2004). Exposure of broilers to acute heat stress also results in decreased monocytes and lymphocyte proportions with no effect on eosinophil counts (Altan et al., 2000). Stress induced via ACTH infusion in broilers (Puvadolpirod and Thaxton, 2000b) and laying hens (Mumma et al., 2006) and short term administration (10 d) of the aflatoxin, ochratoxin (Janaczyk et al., 2006) have been shown to have similar effects on leukocyte levels increasing the ratio of heterophils to lymphocytes. However, long term (20 d) administration of ochratoxin, appears to have an opposite effect decreasing heterophils and increasing lymphocyte levels (Janaczyk et al., 2006). During severe feed restriction the H/L ratio appears to be an inadequate indicator of stress, because in this situation birds respond with heteropenia, lymphocytosis and significant basophilia (Maxwell, 1993).

Pronounced reductive effects of stress induced by hormones, nutritional and environmental factors on the thymus, spleen and bursa of Fabricius are apparent in poultry. Corticosterone (Donker and Beuving, 1989; Post et al., 2003), ACTH (Thaxton et al., 1982; Puvadolpirod and Thaxton, 2000a, c) administration and feed deprivation (Griffiths et al., 1985) significantly reduce growth of spleen, thymus and bursa of Fabricius. Hale and coworkers (2004) showed that amino acid deficiency will also lead to reductions in immune organs, specifically the thymus and CD 8^+ T-cells produced by the thymus. Graczyk and coworkers (2003) conducted a study to evaluate the effect of ACTH on the morphology and reactivity of the lymphatic system of chickens immunized with sheep erythrocytes (SRBC). Administration of ACTH reduced lymphocytes produced in the bursa and thymus after response to SRBC. Morphometric analysis of the spleen revealed an increase in type II, immature, germinal centers after administration of SRBC and ACTH (Graczyk et al., 2003).

Thymus derived T-cells characterize cell mediated immunity. In recent years, cell mediated immune responses have been typically measured via cutaneous baspohil hypersensitivity or delayed type hypersensitivity to a mitogen (El-Lethey, 2003), however graft *vs.* host reactions are reliable measurements. Stress promotes sensitization of cell mediated immune responses but tends to inhibit effectiveness of the response (Gross and Siegel, 1988). Corticosterone administration has been shown to decrease T cell-mediated delayed type hypersensitivity responses to PHA-P (El-Lethey, 2003) in chickens. In an experiment observing natural stressors in nestling barn swallows, Saino and coworkers (2003) found as CS levels rose, T-cell mediated immune responses were depressed. Mumma and coworkers (2006) observed reduced response to PHA-P in

evaluated ACTH treated laying hens. Gross and Siegel (1988) noted cell mediated defenses against Marek's disease tumors and coccidiosis are inhibited by high levels of social stress. Stress at time of challenge appeared to only inhibit cell mediated immunity to coccidiosis (Gross, 1976).

Bursal derived B-lymphocytes characterize humoral immunity in poultry. Studies have shown that humoral immune responses to SRBC and *Salmonella pollurum* have been suppressed by stressors. Thaxton and coworkers (1968), observed reduced antibody titers in poultry injected with ACTH 6 and 24 h prior to antigen injection. Chickens introduced to environmental stressors (overheating, chilling, or water deprivation) within the 1st week of life may have lasting effects on the immune system for up to 18 wk (Gross and Siegel, 1988). Antibody responses appear to differ in the presence of various stressors as indicated in a study where serum gamma globulin was reduced in animals feed deoxycorticosterone while an increase was noted in birds feed corticosterone (Gross and Siegel, 1981b).

Several nutritional and chemical additives have been found to alleviate the effects of stress in poultry (Gross and Chickering, 1987; Gore and Qureshi, 1997. Enhanced cell-mediated and humoral immune responses have been reported in broiler chicks receiving 10 IU of vitamin E *in ovo* on d 18 of incubation (Gore and Qureshi, 1997). In a study conducted to observe the effects of various levels (0, 10, 50, 100, 200-ppm) of ascorbic acid on immune responsiveness, lymphocyte subpopulations measured higher CD4 and T-cell receptor-II cells with 100-ppm supplementation of ascorbic acid. The 200-ppm supplemented group had significantly higher titers to infectious bursal disease. Metyropene inhibits the effects on environmental stress by inhibition of CS synthesis

(Gross and Chickering, 1987). This inhibition of stress results in rapid remission of Marek's tumors and the deleterious effects on antibody responsiveness (Gross and Siegel, 1988). These nutritional and chemical supplementations prove to be beneficial to improving immunity and performance.

Avian Growth and Reproductive Performance

Temperature (Hester et al., 1981; Suk and Washburn, 1995; Baziz et al., 1996), hormones (Thaxton et al., 1982; Siegel and van Kampen, 1984; Donker and Beuving, 1989; Puvadolpirod and Thaxton 2000c; Mumma et al., 2006; Virden et al., 2004, 2007), overcrowding (Patterson and Siegel, 1998), and toxicity (Thaxton et al., 1975; Edrington et al., 1997; Shi et al., 2006) are key causes of reduced performance.

Glucocorticoid hormones are essential in facilitating the catabolism of protein and fat to produce glucose for energy during stress. When excessive levels of blood CS are maintained, detrimental effects on growth can occur due to extreme turnover of body tissue for gluconeogenesis (Virden et al., 2004). One study found that administration of CS did not affect rate of protein degradation but resulted in significantly decreased rates of protein synthesis (Klasing et al., 1987). An immunological mediator of stress, IL-1, on the other hand resulted in significant increases in protein degradation (Klasing et al., 1987). Stimulation of CS in combination with IL-1 appears to be partially responsible for decreases in feed efficiency and body weight (BW). Decreases in digestion of dry matter, proteins, gross energy, and carbohydrates as a result of ACTH induced stress is another possible cause of poor growth and BW gain in broilers (Puvadolpirod and Thaxton 2000d).

Virden and coworkers conducted experiments in which dietary CS caused significantly decreased body weight, gain, feed intake, and livability from d 1 to 7 (2007) and 0 to 21 (2004). From 0 to 21, 0 to 34, and 0 to 42 d dietary CS decreased BW gain and increased feed conversion (2004, 2007). Injection of ACTH resulted in significantly depressed performance responses, however not as highly significant as CS treatments (Virden et al., 2007). Puvadolpirod and Thaxton (2000, c) showed ACTH infusion not only depresses body weight, but also immune organ weights. Birds grown in higher temperature environments have been reported to have depressed growth rate and decreased feed consumption (Suk and Washburn, 1995). Suk and Washburn, (1995) also observed decreased efficiency of feed utilization with increased environmental temperatures. Often birds will consume more feed during stress, however poor digestion mentioned earlier causes a decrease in efficiency and therefore a decrease in BW gain.

Temperature, stress hormones, overcrowding, and poor management often negatively impact reproductive performance in males and females; decreasing egg production, egg quality, fertility, and causing organ atrophy (Mashaly et al., 2004; Mumma et al., 2006). The negative effects of the stressors are well known, but the mechanisms involved are not completely understood.

Short and long term heat exposure has been shown to cause reductions of egg production, egg weight, ovarian weight, and the number of large follicles. Additionally a significant reduction in key reproductive hormones, plasma progesterone and testosterone, has been detected within 2 d of heat exposure, and a reduction in plasma 17ß-estradiol within 14 d of heat exposure (Rozenboim et al., 2007). Rozenboim and coworkers observed the effects of stress at the molecular level and observed that short

term exposure to heat stress caused significant reduction in mRNA expression of cytochrome P450 17-α hydroxylase. Exposing birds to long-term heat stress lead to a significant reduction in expression of mRNA of steroidogenic enzymes. Reproduction in hens has been shown to be negatively affected by direct infusion of ACTH, resulting in cessation of laying, atretic follicles, and decreased oviduct weight (Mumma et al., 2006). Egg production, egg weight, shell weight, shell thickness, and specific gravity were significantly inhibited among heat stressed hens (Mashaly et al., 2004). Reduced feed consumption in these hens may be contributing factors in the egg quality, as nutrients need to support egg production are likely deficient.

Very little research addresses the effects of stress on male reproduction. However, some research exists suggesting that heat stress negatively effects male reproduction by decreasing fertility, sperm viability, and sperm quality index (McDaniel et al., 2004). Karaca and coworkers (2002) conducted an experiment observing the effects of heat stress on rooster fertility, specifically the roles of seminal plasma and sperm. They found seminal plasma from heat stressed males caused lower sperm quality index values and diminished fertility of control sperm. Sperm from heat stressed birds always had a lower fertilization rate than control sperm and seminal plasma from semen samples with heat stressed sperm contained lower ion concentrations. They concluded that high temperatures decrease seminal plasma and intracellular ion concentrations leading to male infertility (Karaca et al., 2002).

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CHAPTER III

EVALUATION OF THE EFFECTS ON BLOOD GASES OF HIGH AND LOW HEMATOCRIT LEVELS ON BROILERS DURING STRESS

Abstract

An experiment was conducted to evaluate the effects of ACTH on broilers with high and low hematocrit levels. An initial evaluation separated the forty broilers into two groups low (19 to 22%) and high (25 to 28%) based on hematocrit level. Treatment was delivered via mini-osmotic pumps, which infused ACTH at 8 IU/kg BW/d for 7 days at a rate of 1uL/h. A 2 (hematocrit level) x 2 (ACTH treatment) factorial arrangement in a completely randomized design with ten replications per treatment was performed; measured effects were ACTH treatment and hematocrit level. Blood was evaluated for pH, partial pressure of CO_2 (pCO_2) partial pressure of O_2 (pO_2), hematocrit, hemoglobin, electrolytes (Na^+, K^+, Ca^+, Cl^- and HCO_3^-), and corticosterone (CS) levels. Immediately prior to the start of ACTH treatment on d 43, birds differed significantly ($P < 0.1$) with high hematocrit birds having increased hematocrit, hemoglobin, HCO_3^-, and decreased pO_2 levels. After 4 d of continuous infusion of ACTH, blood was taken and evaluated for the same characteristics as those evaluated on d 43. Adrenocorticotropin hormone treated birds had significantly increased ($P < 0.1$) hematocrit, hemoglobin, pCO_2, HCO_3^-, and corticosterone levels. High hematocrit grouped broilers had significantly higher hematocrit, hemoglobin, pCO_2, and HCO_3^- levels and decreased Cl^-, Na^+, anion gap levels

compared to the low hematocrit group. Corticosterone levels were not significantly different between low and high hematocrit birds. An ACTH x hematocrit response interaction was observed for hematocrit and hemoglobin. The results indicate birds are capable of maintaining pH and acid base balance through constant shifts in blood gas and electrolyte levels. High hematocrit birds exhibit a minimal increase in hematocrit and hemoglobin in an attempt to improve oxygen carrying capacity, making it easier to adapt to stress than low hematocrit birds.

Introduction

Stress is most recently defined as a non-specific response to a demand or specific stimulus (Selye, 1976). These specific stimuli, which often reduce poultry performance, consist of environmental conditions (excess heat or cold, poor ventilation), disease, nutritional deficiencies or excesses, rapid growth, overcrowding, noise, and harsh catching to describe a few (Feltenstein et. al, 2003; Freeman, 1987). Following presentation with a stressor, the animal will respond via the general adaptation syndrome (Selye, 1976) classified by three stages: alarm, resistance, and fatigue.

The alarm stage, a neurogenic stage (Olanrewaju et al., 2006), is characterized by initial stimulation of the hypothalamus to release corticotrophin releasing factor. This leads to release of pro-opiomelanocortin (POMC) followed by adrenocorticotropin (ACTH) by the anterior pituitary. This in turn stimulates the adrenal cortex to release the glucorticoid, corticosterone (CS). Corticosterone will then signal metabolic changes such as an increase in gluconeogenesis, as well as, immunological changes to try to maintain a state of homeostasis. This increase in gluconeogenesis leads into the stage of resistance.

The resistance stage is defined by the changes in the body to resist the effects of the stressor. During this time changes in blood gases, blood pH and acid base balance are observed. If the bird is unable to resist the effects of the stressor, the result will often be death from fatigue, the final stage of the general adaptation syndrome.

Blood gas and acid base balance are influenced by many environmental factors in various species. In broilers an environmental stressor leading to changes in blood gases or electrolyte balance often observed is heat stress (Altan et al., 2000; Borges et al., 2004: Deyhim et al., 1990; Sandercock et al., 2001). Changes many times are hard to interpret during stress because blood gases and electrolytes are constantly changing simultaneously in the same or opposite direction. Every stressor has the ability to have a unique effect on the rate of metabolism, respiration, and mechanism of H^+ equivalent exchange, therefore causing its own pattern of change (Olanrewaju et al., 2006).

A model was recently developed to observe physiological adaptive responses in broilers and layers under stressful conditions using a continuous infusion mini-osmotic ACTH pump (Puvadolpirod and Thaxton 2000 a,b,c,d). Responses observed in the model experiment included increased CS, glucose, cholesterol, triglycerides, lipoproteins, and heterophil/lymphocyte ratio, however blood gases and acid base balance were not evaluated. Using this model research has been conducted to evaluate acid base balance and blood gas changes in broilers. Through this research it has been observed that ACTH induced broilers have higher hemtocrit, partial pressure of CO_2 (pCO_2), anion gap, CS, mean corpuscular hemoglobin concentration, blood hemoglobin, and HCO_3^-, but lower partial pressure of O_2 (pO_2), Na^+, K^+, and Cl^-. The pH was shown to not be affected by ACTH, indicating a balance of acid and base (Olanrewaju et al., 2006). Erythropoiesis is

also thought to increase, in order to supply O_2 needed to support energy production through gluconeogenesis.

As suggested by previous research, there is a major need for an increase in oxygen carrying capacity in stressed broilers. High packed red blood cell volumes (hematocrit) should allow for higher oxygen carrying capacity. Research evaluating the effects of stress on broilers with various hematocrit levels is sparse. Therefore, the purpose of this research is to evaluate whether a higher hematocrit can improve a bird's ability to adapt to stress

Materials and Methods

Treatment

Mini-osmotic pumps were loaded with porcine pituitary ACTH dissolved in 0.85% saline solution, and subcutaneously implanted on the backs of broilers, above the scapula bone. The interscapular tract skin was sterilized with betadine, anesthetized locally by infusing with Lidocain HCl, and a small incision (10mm) was made. A mini-pump was inserted between the skin and the muscle, and the incision site was closed with one to two surgical staples. The delivery rate of the pump was 1µL/h for 7 consecutive d; dosage was adjusted to 8 IU/kg of BW/d.

Blood Sample Preparation

Blood samples were collected via a wing vein. Blood CS level increases if broilers are restrained for periods exceeding 45 s (Beuving and Vonder, 1978); therefore,

sampling time never surpassed 45 s. Samples of 3 to 5ml of blood were collected into a syringe with lithium-heparin or EDTA. Whole blood samples were centrifuged at 4,000 x g for 15 min and plasma samples were collected. Plasma samples were stored at -20 C for later analysis of CS.

Analysis of Blood by ABL 77

A 70µl sample of whole blood was analyzed using the ABL 77 blood gas analyzer[1]. Parameters evaluated included pH, pCO_2, pO_2, hematocrit, hemoglobin, and electrolytes (Na^+, K^+, Ca^+, Cl^-). Blood gases and pH were corrected for temperature according to the ABL 77 formulations. Bicarbonate and anion gap were also calculated.

Enzyme Linked Immunoassay and Immunosorbant Assay Kit Analysis

Plasma CS concentrations were determined by an enzyme linked immunoassay kit (ELISA) as described by Odhiambo (2004). To assay, 25ul of each plasma sample was transferred into an Eppendorf tube and 25ul of steroid displacement buffer (20ul steroid displacement reagent + 980 ul Tris buffer) was added and then vortexed. All samples were brought to a 1:10 dilution using Tris buffer solution with sodium azide before application on the 96 well plate. Standard curves and sample concentrations were calculated using KC Junior data analysis software.

[1] Radiometer America, Inc., Westlake, OH 44145

Experimental Facility

The experiment was conducted in a facility containing floor pens on a concrete pad with side wall curtains. All floor pens measured 1.5 x 2.9m. Each pen contained one tube feeder, one bell type drinker, and used litter supplemented with new shavings. The facility temperature was maintained at an average low of 69°F and a high of 80°F using curtain ventilation. The lighting program consisted of 22 h of light throughout the trial period.

Experimental Design, Bird Husbandry, Treatment and Diets

One strain of commercial broilers was obtained from a local hatchery after being set and hatched in a common incubator. The vaccination program consisted of Marek's vaccination *in ovo*. Forty broiler chicks were distributed into four pens in the experimental facility, each containing ten birds.

All birds were fed a common diet throughout the trial period. On d 43 all birds were statistically sorted by hematocrit level into two groups of high (25 to 28%) and low (19 to 22%) hematocrit. Pumps were inserted into two groups of birds, one high hematocrit and one low hematocrit. The two remaining groups, one high and low hematocrit, were used as controls creating a 2(ACTH treatment) x 2(hematocrit level) factorial arrangement in a completely randomized design with ten replications per treatment.

Hematology and Hormone Measurements.

Blood from all broilers were evaluated for hematocrit, hemoglobin, electrolyte (K^+, Na^+, Cl^-, Ca^+, HCO_3^-), pCO_2, pO_2, pH, and CS as a baseline for d 43. After 4 d of continuous infusion of ACTH, d 47, blood was taken and evaluated for hematocrit, hemoglobin, electrolyte (K^+, Na^+, Cl^-, Ca^+, HCO_3^-), pCO_2, pO_2, pH, and CS. Anion gap was calculated from anions and cations. The pH, pCO_2 and pO_2 were corrected for average (41.5 C) bird temperature. Bicarbonate was calculated from the pH and pCO_2.

Statistical Analysis

A 2x2 factorial arrangement of treatments in a completely randomized design was employed in the experiment. Data was analyzed by a general ANOVA (Statistix, 2003). Means were compared for significant ($P \leq 0.1$) differences by using the means and all-pair wise comparison options of Statistix (Statistix, 2003). Statements of significance are based on $P \leq 0.1$ unless otherwise noted.

Results and Discussion

The experiment presented herein tested whether a higher hematocrit prior to stress would give a bird a greater advantage in its ability to adapt to stress. Because stress can have such a large effect on broiler performance, research evaluating ways to reduce negative stress is important in maintaining the well-being of the bird.

The present study as well as past studies (Puvadolpirod and Thaxton 2000 a,b,c,d; Olanrewaju et al., 2006) indicate physiological stress will be induced by stimulation of CS in broiler chickens via continuous delivery of ACTH at 8 IU/kg of BW/d for 7 d at a

rate of 1uL/h by a mini osmotic pump. Previous research has demonstrated that ACTH induced stress can increase hematocrit, pCO_2, anion gap, CS (Olanrewaju et al., 2006; Puvadolpirod and Thaxton, 2000a,b,c,d), mean corpuscular hemoglobin concentration, blood hemoglobin, HCO_3^- (Olanrewaju et al., 2006), glucose, cholesterol, triglycerides, lipoprotein, and heterophil:lymphocyte ratio (Puvadolpirod and Thaxton, 2000a,b,c,d). It may reduce pO_2, plasma K^+, Na^+, and Cl^- with no effect on pH or plasma Ca^+. The balance in pH suggests birds are capable of maintaining acid base balance even in the presence of stress. Present research indicates similar effects from ACTH with significant increases ($P < 0.1$) in pCO_2 and HCO_3^- noted in Table 3.1 and increases in hematocrit, hemoglobin, and corticosterone levels noted in Table 3.2. No significant effects due to ACTH were noted for pO_2 (Table 3.1), electrolytes (Table 3.3), anion gap or pH (Table 3.4). Stress from heat has been shown to have similar effects on electrolytes (HCO_3^-, K^+, Na^+, and Cl^-) but an opposite effect on pCO_2 (Borges et al., 2004). This indicates that type and level of stress can cause various physiological changes and that blood gases are constantly changing to adapt.

Research has suggested that the changes due to stress in glucose, cholesterol, and triglycerides may alter the chicken's metabolism. As circulating glucocorticoid levels increase, the result is an increase in gluconeogenesis leading to a rise in glucose and cholesterol (Olanrewaju et al., 2006; Siegel, 1971). When stressors are present the body prepares for a "fight or flight" response (Cannon, 1915). Hormone levels, catecholamine, epinephrine and glucocorticoids, increase resulting in stored energy from fat and glucose, be released for use by the body. Glucocorticoids stimulate gluconeogenesis, leading to new glucose production. This occurs at the expense of

available oxygen for the body or cells, as suggested by reductions in pO_2 levels in the blood. Therefore, birds must increase erythropoiesis to increase oxygen carrying capacity and fulfill oxygen needs. Increased hematocrit and blood hemoglobin levels in present and previous research (Olanrewaju et al., 2006; Muldoon et al., 1995) support the previous statement.

Little research has evaluated the differences in the physiological parameters tested herein in animals with various hematocrit levels. Data shows high hematocrit grouped broilers had significantly higher hematocrit and hemoglobin (Table 3.2), as expected, higher pCO_2 and HCO_3^- levels (Table 3.1) and decreased Cl^-, Na^+ (Table 3.3),and anion gap (Table 3.4) levels compared to the low hematocrit group. There was no significant effect on pH (Table 3.4) or pO_2 (Table 3.1) indicating all birds, no matter what the level of hematocrit, are capable of maintaining blood oxygen levels and acid base balance in the absence of stress. Corticosterone levels (Table 3.2) were not significantly different between low and high hematocrit birds.

An ACTH x hematocrit selection interaction occurred for hematocrit and hemoglobin (Table 3.2). Following stress induction, broilers selected for low hematocrit revealed approximately a 30% increase in hematocrit and hemoglobin compared to only a 12% increase in hematocrit and hemoglobin for birds selected for high hematocrit. This minimal increase in hematocrit and hemoglobin when high hematocrit birds are stressed gives high hematocrit birds an adaptive advantage to stress over low hematocrit birds. These results explain how the high hematocrit bird may have the ability to maintain pO_2 when presented with stress via ACTH pump due to the advantage of ample hemoglobin.

It appears from past and present research that birds are capable of maintaining pH and acid base balance through constant changes in electrolyte levels. The constant changes in blood gases, hematocrit, and hemoglobin levels, may occur via increased erythropoiesis or release of cells from possible storage, allowing birds to maintain proper oxygen needed to support gluconeogenesis and physiological changes in the body. Future studies evaluating these parameters are being conducted.

TABLE 3.1. Impact of Adrenocorticotropin (ACTH) Treatment and Hematocrit (Hct) Level on Broiler Blood Gases

ACTH	Hct Level	pO_2	pCO_2	HCO_3[1]
		----------(mm/Hg)----------		
Control		69	56[b]	25.8[b]
ACTH		62	66[a]	29.0[a]
SEM		3.2	3.1	0.84
	Low	67	53[y]	23.7[y]
	High	63	70[x]	31.0[x]
SEM		3.2	3.1	0.83
CV		21.69	22.56	13.54
Source of variation				
ACTH		0.118	0.035	0.011
Hct Level		0.369	0.001	0.001
ACTH x Hct Level		0.216	0.919	0.197

[1]HCO_3 is calculated from pH and pCO_2.

[a-b, x-y] Means within a column for a main effect response that do not share common superscripts differ ($P \leq 0.1$).

TABLE 3.2. Impact of Adrenocorticotropin (ACTH) Treatment and Hematocrit (Hct) Level on Broiler Blood Parameters

ACTH	Hct Level	Hemoglobin	Hct	Corticosterone
		g/dL	%	pg/mL
Control		7.1[b]	22.1[b]	1413[b]
ACTH		8.5[a]	26.3[a]	28324[a]
SEM		0.16	0.47	6972.0
	Low	7.0[n]	21.8[n]	19158
	High	8.6[m]	26.6[m]	10579
SEM		0.16	0.47	6972.0
Control x Low		6.1[z]	19.1[z]	1233
Control x High		8.1[y]	25.1[y]	10260
ACTH x Low		7.9[y]	24.6[y]	9733
ACTH x High		9.1[x]	28.1[x]	9733
SEM		0.22	0.66	9865.0
CV		8.52	8.85	207.01
Source of Variation				
ACTH		0.001	0.001	0.010
Hct Level		0.001	0.001	0.391
ACTH x Hct Level		0.068	0.072	0.371

[a-b, m-n, x-z,] Means within a column for a main effect or interaction response that do not share common superscripts differ ($P \leq 0.1$).

TABLE 3.3. Impact of Adrenocorticotropin (ACTH) Treatment and Hematocrit (Hct) Level on Broiler Blood Electrolytes

ACTH	Hct Level	Na^+ (mEq/L)	K^+ (mEq/L)	Ca^+ (mEq/L)	Cl^- (mEq/L)
Control		137	5.2	2.72	102
ACTH		139	5.3	2.77	102
SEM		1.1	0.12	0.044	1.4
	Low	144^x	5.2	2.79	111^x
	High	132^y	5.3	2.70	93^y
SEM		1.1	0.12	0.044	1.4
CV		3.53	9.91	7.01	6.01
Source of Variation					
ACTH		0.199	0.691	0.489	0.885
Hct Level		0.001	0.843	0.132	0.001
ACTH x Hct Level		0.879	0.353	0.808	0.807

$^{x-y}$ Means within a column for a main effect response that do not share common superscripts differ ($P \leq 0.1$).

TABLE 3.4. Impact of Adrenocorticotropin (ACTH) Treatment and Hematocrit (Hct) Level on Blood pH and Anion Gap

ACTH	Hct Level	pH	(Anion Gap) Cation-Anion
Control		7.31	14.3
ACTH		7.30	13.4
SEM		0.018	0.53
	Low	7.31	14.8[x]
	High	7.30	12.9[y]
SEM		0.018	0.53
CV		1.09	16.71
Source of Variation			
ACTH		0.681	0.217
Hct Level		0.716	0.020
ACTH x Hct Level		0.310	0.289

[x-y] Means within a column for a main effect response that do not share common superscripts differ ($P \leq 0.1$).

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CHAPTER IV

EVALUATION OF THE EFFECTS OF STRESS ON BLOOD GASES AND IMMUNOLOGICAL PARAMETERS IN BROILERS WITH NON-SELECTED HEMATOCRIT LEVELS

Abstract

An experiment was conducted to evaluate how a broiler physiologically adapts to stress. The experiment was a completely randomized design to evaluate the effects of ACTH on broilers with average hematocrit levels. Birds were evaluated for pH, partial pressure CO_2 (pCO_2), partial pressure O_2 (pO_2), hematocrit, hemoglobin, electrolytes (Na^+, K^+, Ca^+, Cl^- and HCO_3^-), anion gap, and corticosterone (CS) levels, as well as leukocyte differential counts, total white blood cell counts, total red blood cell counts, and erythropoietin levels. Birds were statistically equivalent prior to treatment. Additionally organ hemoglobin levels were evaluated on d 39. On d 39, ACTH treatment significantly ($P < 0.1$) increased pCO_2, bicarbonate, K^+, total white blood cell count, monocytes, heterophils and corticosterone. Significant ($P < 0.1$) decreases due to ACTH were observed for pO_2, Na^+, Ca^+, Cl^-, bone marrow hemoglobin, spleen hemoglobin, lymphocytes, and erythropoietin. During stress birds maintain acid base balance through shifts in blood gas and electrolyte levels and regain oxygen carrying capacity by increased release and maturation of red blood cells from storage sites. However, accelerated new red blood cell production appears to not contribute to the rise in oxygen carrying capacity as indicated by a decline in erythropoietin.

Introduction

Stress plays an important role in maintaining a normal homeostatic state in the body of an animal. Although it often seems to have a detrimental effect on the well being and performance of an animal, these changes are necessary for survival and adaptation and are not always negative effects. Changes in the body due to stressors were first described by Walter B. Cannon in the early 1900's through the term "Fight or Flight". Characteristics of fight or flight were described as specific response such as release of hormones like adrenaline and symptoms like dilation of the eyes, sweating, shivering and increasing heart rate and blood pressure depending on the stressor. These changes by the body allow the animal to adapt to the stressor presented. For instance, animals adapt to cold stress by shivering and constriction of blood vessels to conserve heat. If an animal is unable to adapt, homeostasis cannot be maintained (Cannon, 1935). Hans Selye (1976) has described stress slightly differently as a non-specific response to a demand or specific stimulus. He developed the concept of the general adaptation syndrome: alarm, resistance and fatigue. The alarm stage, a neurogenic stage (Olanrewaju et al., 2006), is characterized by initial stimulation of the hypothalamus to release corticotrophin releasing factor. This leads to release of pro-opimelanocortin (POMC) followed by adrenocorticotropin (ACTH) by the anterior pituitary. This in turn stimulates the adrenal cortex to release the glucorticoid, corticosterone (CS). Corticosterone will then signal metabolic changes such as an increase in gluconeogenesis, as well as, immunological changes to try to maintain a state of homeostasis. This increase in gluconeogenesis leads into the stage of resistance. The resistance stage is defined by the changes in the body to resist the effects of the stressor. During this time changes in blood gases, blood pH and

acid base balance are observed. If the bird is unable to resist the effects of the stressor the result will often times be death from fatigue, the final stage of the general adaptation syndrome.

A model has been developed to observe physiological adaptive responses in broilers and layers under stressful conditions using a continuous infusion mini-osmotic ACTH pump (Puvadolpirod and Thaxton 2000 a,b,c,d). Responses observed in the model experiment included increased CS, glucose, cholesterol, triglycerides, lipoproteins, and heterophil/lymphocyte ratio, however blood gases and acid base balance were not evaluated. Using this model, research has been conducted to evaluate acid base balance and blood gas changes in broilers. The ACTH induced broilers had increased hematocrit, partial pressure of CO_2 (pCO_2), anion gap, CS, mean corpuscular hemoglobin concentration, blood hemoglobin, and HCO_3^-, but reduced partial pressure of O_2 (pO_2), Na^+, K^+, and Cl^-. The pH was not affected by ACTH, indicating a balance of acid and base (Olanrewaju et al., 2006).

Stress is known to effect blood cell numbers in the body of animals and humans. Research suggests that overall immune responses are enhanced during acute stress but suppressed during chronic stress (Dhabhar, 2003). It is believed that adrenal hormones prepare the immune system for antigen invasion through the skin just as it prepares the musculoskeletal system and cardiovascular system for fight or flight. Without the adrenal glands it has been shown that delayed type hypersensitivity responses are not enhanced by stress (Dhabhar, 2003). Students reacting to the psychological stress of testing had a significant increase in the number of neutrophils, monocytes, $CD8^+$, $CD2^+CD26^+$, and $CD2^+$HLA-DR^+ T cells and $CD19^+$ B cells, and significant reductions

in the $CD4^+/CD8^+$ T cell ratio were observed. It has been observed that lymphocytes decrease (Elrom, 2000), while heterophils increase in the presence of stress and rising CS levels in chicken feed, indicating a rise in heterophil/lymphocyte ratio in the presence of stress (Gross and Siegel, 1983; Puvadolpirod and Thaxton, 2000c). Erythropoiesis, red blood cell formation, is thought to increase to produce O_2 needed to support energy production through gluconeogenesis.

Little research has investigated the up regulation of blood cells from storage sites and erythropoietin (EPO) levels in broilers when stress is present. Storage sites for red blood cells could be vital when oxygen carrying capacity is low and oxygen is needed to support gluconeogenesis. Therefore, the objectives of this research were to look at the physiological changes in the blood gases, electrolytes, immune cells counts, EPO, CS, hematocrit and hemoglobin levels and changes in possible red blood cell storage sites in broilers induced with stress via a mini-osmotic pump of ACTH. It is important to understand how all these parameters work together to maintain homeostasis in the body.

Materials and Methods

Treatment

Mini-osmotic pumps were loaded with porcine pituitary ACTH dissolved in 0.85% saline solution, and subcutaneously implanted on the backs of broilers, above the scapula bone. The interscapular tract skin was sterilized with betadine, anesthetized locally by infusing with Lidocain HCl, and a small incision (10mm) was made. A mini-pump was inserted between the skin and the muscle, and the incision site was closed with

one to two surgical staples. The delivery rate of the pump was 1µL/h for 7 consecutive d; dosage was adjusted to 8 IU/kg BW/d.

Blood Sample Preparation

Blood samples were collected via a wing vein. Blood corticosterone (CS) levels increase if broilers are restrained for periods exceeding 45 s (Beuving and Vonder, 1978) therefore; sampling time never surpassed 45 s. Samples were taken between 7:30 am and 12:00 pm to avoid interfering with varying hormone levels of erythropoietin (EPO) throughout the day. Samples of 3 to 5ml of blood were collected into a syringe with lithium-heparin, EDTA, or into a tube containing no additive. Whole blood samples were centrifuged at 4,000 x g for 15 min and plasma samples were collected. Serum samples were collected after blood coagulation. Serum and plasma samples were stored at -20 C for later analysis. Whole blood was used for differential blood smears, heparinized blood was used for evaluation of blood gases and white and red blood cell counts, plasma was used for analysis of CS, and serum was used for analysis of EPO.

Analysis of Blood by ABL 77

A 70µl sample of whole blood was analyzed using the ABL 77 blood gas analyzer[1]. Parameters evaluated included pH, pCO_2, pO_2, hematocrit, hemoglobin, electrolytes (Na^+, K^+, Ca^+, Cl^-). Blood gases and pH were corrected for temperature according to the ABL 77 formulations. Bicarbonate and anion gap (cation-anion) were also calculated.

[1] Radiometer America, Inc., Westlake, OH 44145

Organ Preparation for Hemoglobin Analysis

A bone marrow sample from the tibia and the spleen were removed from each bird and weighed until appropriate sample size was reached for each parameter. Bone marrow sample size was 0.02 g and spleen sample size was 0.045. The sample was homogenized then combined with 5ml of cynamet and read on spectrophotometer (Thaxton, 2006).

Enzyme Linked Immunoassay and Immunosorbant Assay Kit Analysis

Plasma CS concentrations were determined by an enzyme linked immunoassay kit (ELISA) as described by Odhiambo (2004). To assay, 25ul of each plasma sample was transferred into an Eppendorf tube and 25ul of steroid displacement buffer (20ul steroid displacement reagent + 980 ul Tris buffer) was added and then vortexed. All samples were brought to a 1:10 dilution using Tris buffer solution with sodium azide before application on the 96 well plate. Standard curves and sample concentrations were calculated using KC Junior data analysis software.

Serum EPO concentrations were determined by an ELISA kit. Calibrators and controls were prepared as described by MD Biosciences in the EPO ELISA.[2] To assay, 200ul of each serum sample, calibrator, and control were used in duplicate on a 96 well plate. Following sample application, 25 ul of reagent 1 (biotinylated antibody) and 25 ul reagent 2 (enzyme labeled antibody) were transferred into each well. The plate was then incubated on a shaker for 2 h at room temperature. The wells were then washed and

[2] MD Biosciences, Inc., St. Paul, MN 55114

aspirated five times using an ELx50 auto strip washer[3]. Then 150ul of substrate was added to each well and incubated at room temperature for 30 min on a shaker. Stop solution, 100ul, was added prior to reading at 450 nm (adjusted 405) using Bio-Tek µQuant Universal Microplate Spectrophotometer[4]. Standard curves and sample concentrations were calculated using KC Junior data analysis software.

Experimental Facility.

The experiment was conducted in a facility containing floor pens on a concrete pad with side wall curtains. All floor pens measured 1.5 x 2.9m. Each pen contained one tube feeder, one bell type drinker, and used litter supplemented with new shavings. The facility temperature was maintained between a low of 70°F and a high of 80°F using curtain ventilation. The lighting program consisted of 22 h of light throughout the trial period.

Experimental Design, Bird Husbandry, Treatment and Diets.

One strain of commercial broilers was obtained from a local hatchery after being set and hatched in a common incubator. The vaccination program consisted of Marek's vaccination *in ovo*. Twenty broiler chicks were distributed into two pens, each containing ten birds. All birds were fed a common diet throughout the trial period. On d 35 pumps were inserted in the treatment group with the remaining group being used as a control in our completely randomized design with ten replications per treatment.

[3] Bio-Tek Instruments, Inc., Winooski, VT 05404
[4] Bio-Tek Instruments, Inc., Winooski, VT 05404

Hematology and Hormone Measurements.

Blood from all broilers were evaluated for leukocyte differential counts, total white blood cell counts and total red blood cell counts, hematocrit, hemoglobin, electrolytes (K^+, Na^+, Cl^-, Ca^+, HCO_3^-), pCO_2, pO_2, pH, EPO and CS as a baseline. After 4 d of continuous infusion of ACTH blood was taken and evaluated for hematocrit, hemoglobin, electrolytes (K^+, Na^+, Cl^-, Ca^+, HCO_3^-), pCO_2, pO_2, pH, leukocyte differential counts, total white blood cell counts, total red blood cell counts, EPO, organ hemoglobin levels and CS. Anion gap was calculated from anions and cations. The pH, pCO_2 and pO_2 were corrected for average (41.5 C) bird temperature. Bicarbonate was calculated from the pH and pCO_2.

Statistical Analysis

A completely randomized design was employed in the experiment. Data were analyzed by a general ANOVA (Statistix, 2003). Means were compared for significant ($P \leq 0.1$) differences by using the means and all-pair wise comparison options of Statistix (Statistix, 2003). Statements of significance are based on $P \leq 0.1$ unless otherwise noted.

Results and Discussion

The experiment presented evaluates the effects of stress induced by ACTH on broiler physiology. Because of the importance of broiler welfare and performance, many stress studies have occurred to try to understand the effects stressors can have on the physiology of the bird, by observing changes in blood gases, CS levels and some immune parameters. Our objective in this study was to not only observe previously investigated

parameters, but to also take an in depth look at new parameters, such as spleen and bone marrow hemoglobin and erythropoietin, that may explain how a bird adapts to changes around them in their attempt to maintain homeostasis.

Prior to 2000, many broilers were observed while adapting to natural states of stress: heat, cold, altitude changes (Thaxton and Siegel, 1970, 1972, 1973) and feed deprivation (Beuving et al., 1989). However in 2000 a new model for observing broilers under stress was created using mini-osmotic pumps loaded with ACTH, a direct stimulator of CS (Puvadolpirod and Thaxton 2000 a,b,c,d). These researchers discovered that using a controlled, direct method of stimulation would cause the similar effects as say, a temperature trial (Borges et al., 2004). Previous research has demonstrated that ACTH induced stress can increase hematocrit, pCO_2, anion gap, CS (Olanrewaju et al., 2006; Puvadolpirod and Thaxton, 2000a,b,c,d), mean corpuscular hemoglobin concentration, blood hemoglobin, HCO_3^- (Olanrewaju et al., 2006), glucose, cholesterol, triglycerides, lipoprotein, and heterophil:lymphocyte ratio (Puvadolpirod and Thaxton, 2000a,b,c,d). It may reduce pO_2, plasma K^+, Na^+, and Cl^- with no effect on pH or plasma Ca^+. The balance in pH suggests birds are capable of maintaining acid base balance even in the presence of stress. Stress from heat has been shown to have similar effects on electrolytes (HCO_3^-, K^+, Na^+, and Cl^-) but an opposite effect on pCO_2, reducing it (Borges et al., 2004; Sandercock et al., 2001). One researcher found heat stress has no effect on hematocrit levels (Altan, 2000).

Present research indicates similar effects for the ACTH model. In agreement with previous research, ACTH treated birds had significantly increased ($P < 0.1$) pCO_2 and HCO_3^-. A significant decrease in pO_2 was noted for ACTH treated birds (Table 4.1).

Hematocrit and hemoglobin levels were not significantly ($P > 0.1$) different during stress in these unselected birds (Table 4.2), indicating these birds do not have the same adaptive advantage of maintaining a higher oxygen carrying capacity during stress, as has been shown by hematocrit selected birds in the previous study (Chapter 3). In the present study, ACTH significantly ($P < 0.1$) decreased Ca^{+}, Cl^{-}, and Na^{+} levels in birds but increased K^{+} levels (Table 4.3). Electrolytes are constantly changing in order to keep an electrolyte balance; this may explain why the significant increase in K^{+} and significant change in Ca^{+} contradicts previous research (Olanrewaju et al., 2006; Puvadolpirod and Thaxton, 2000a,b,c,d). Rapidly shifting electrolyte levels may also explains why the results of anion gap, calculated from anions and cations, are not in agreement with previous research (Olanrewaju et al., 2006). Stress due to ACTH significantly decreased on anion gap, with no effect noted for pH (Table 4.4). The significant ($P < 0.1$) increase in CS, which agrees with previous research using this model, can be observed in Table 4.4. Red blood cell counts were not affected by ACTH, but white blood cells were increased significantly (Table 4.5), suggesting initiation of an inflammatory response. Table 4.6 shows the effect of ACTH on blood leukocytes in experimental broilers. The percent of monocytes and heterophils in the blood significantly ($P < 0.1$) increased in treated broilers, however lymphocyte numbers decreased. There was no significant effect on percent basophils or eosinophils. The increase in heterophils in addition to the decrease in lymphocytes indicates an increase in heterophil: lymphocyte ratio, in agreement with results found by Puvadolpirod and Thaxton (2000a,b,c,d).

As stated earlier some important parameters that have not been observed in previous research are levels of EPO and organ hemoglobin levels in broilers. Data in

Tables 4.2 and 4.4 show a significant ($P < 0.1$) decrease in EPO, spleen hemoglobin and bone marrow hemoglobin levels due to ACTH. Previous research in rats has shown that in the presence of ACTH, serum EPO decreases while hematocrit and hemoglobin increases (Zhang et al., 2004), indicating stress causes a need for mature red blood cells but tends to suppress red blood cell production. It appears ACTH also suppressed red blood cell production in chickens as indicated by the EPO decrease. The decrease in organ hemoglobin levels indicate the birds are trying to meet their need for oxygen carrying capacity by increasing release of red blood cells from the suggested storage sites. It is suspected that birds first release red blood cells from the spleen and bone marrow decreasing the organ hemoglobin levels but eventually increasing the blood hemoglobin levels. Maturation of present red blood cells may then in turn may play a role in increased hemoglobin and hematocrit levels in the blood in days to follow.

Research herein suggests broilers undergoing stress have the ability to maintain acid base balance and pH through constant shifts in electrolyte levels and blood gases. Birds appear to regain oxygen carrying capacity via increased release of red blood cells from storage sites, however, red blood cell formation stimulated by erythropoietin appears to not be playing a role in this increase in oxygen carrying capacity by 4 d post treatment.

TABLE 4.1. Impact of Adrenocorticotropin (ACTH) Treatment (Trt) on Broiler Blood Gases

Trt	pO_2	pCO_2	HCO_3[1]
	----------(mm/Hg)----------		
Control	75[a]	54[b]	24[b]
ACTH	53[b]	75[a]	30[a]
SEM	4.4	2.8	0.9
CV	21.12	13.38	10.11
Source of Variation			
Trt	0.002	0.001	0.001

[1]HCO_3 is calculated from pH and pCO_2.
[a-b] Means within a column for a main effect response that do not share common superscripts differ ($P \leq 0.1$).

TABLE 4.2. Impact of Adrenocorticotropin (ACTH) Treatment (Trt) on Broiler Hemoglobin and Hematocrit

Trt	Blood Hemoglobin	Hematocrit	Spleen Hemoglobin	Bone Marrow Hemoglobin
	g/dL	%	g/dL	g/dL
Control	7.3	23	49[a]	29[a]
ACTH	7.5	23	31[b]	19[b]
SEM	0.42	1.2	4.2	2.2
CV	17.31	16.63	32.36	28.51
Source of Variation				
Trt	0.818	0.809	0.008	0.005

[a-b] Means within a column for a main effect response that do not share common superscripts differ ($P \leq 0.1$).

TABLE 4.3. Impact of Adrenocorticotropin (ACTH) Treatment (Trt) on Broiler Electrolytes

Trt	Na^+	K^+	Ca^+	Cl^-
	(mEq/L)	(mEq/L)	(mEq/L)	(mEq/L)
Control	146 [a]	5.3 [b]	2.95[a]	114[a]
ACTH	137 [b]	5.8 [a]	2.84 [b]	101[b]
SEM	1.1	0.16	0.051	1.2
CV	2.37	8.89	32.36	3.56
Source of Variation				
Trt	0.001	0.028	0.008	0.001

[a-b] Means within a column for a main effect response that do not share common superscripts differ ($P \leq 0.1$).

TABLE 4.4. Impact of Adrenocorticotropin (ACTH) Treatment (Trt) on Broiler Physiology

Trt	Anion Gap Cation-Anion	pH	Corticosterone pg/mL	Erythropoietin pg/mL
Control	13.8	7.3	248[b]	27[a]
ACTH	11.8	7.3	8011[a]	17[b]
SEM	0.76	0.019	938.2	3.0
CV	18.10	0.79	57.16	43.88
Source of Variation				
Trt	0.080	0.140	0.001	0.028

[a-b] Means within a column for a main effect response that do not share common superscripts differ ($P \leq 0.1$).

TABLE 4.5. Impact of Adrenocorticotropin (ACTH) Treatment (Trt) on Broiler Total Blood Counts

Trt	Red Blood Cells	White Blood Cells
	million	thousand
Control	10	10[b]
ACTH	9	13[a]
SEM	1.1	8.2
CV	38.07	20.85
Source of Variation		
Trt	0.735	0.010

[a-b] Means within a column for a main effect response that do not share common superscripts differ ($P \leq 0.1$).

TABLE 4.6. Impact of Adrenocorticotropin (ACTH) Treatment (Trt) on Broiler Leukocyte Counts

Trt	Monocyte	Lymphocyte	Heterophil	Basophil	Eosinophil
	----------% of total leukocytes----------				
Control	11[b]	79[a]	7[b]	1.1	1.8
ACTH	19[a]	56[b]	20[a]	1.2	3.1
SEM	2.6	2.6	2.6	0.45	0.82
CV	53.22	11.84	61.27	119.33	104.34
Source of Variation					
Trt	0.047	0.001	0.002	0.850	0.274

[a-b] Means within a column for a main effect response that do not share common superscripts differ ($P \leq 0.1$).

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CHAPTER V

EVALUATION OF THE EFFECTS ON BLOOD GASES AND IMMUNOLOGICAL PARAMETERS OF VARIOUS HEMATOCRIT LEVELS ON BROILERS DURING STRESS

Abstract

This experiment was conducted to confirm that high hematocrit levels improve a bird's ability to increase oxygen carrying capacity and adapt to stress and to evaluate the physiological mechanisms by which this occurs. The experiment was a 2(ACTH)x 3(hematocrit level) factorial arrangement in a completely randomized design with twenty-one replications per treatment. An initial evaluation separated the broilers into three groups, low (18 to 21%), high (24 to 27%), and non-selected based on hematocrit level. Birds were evaluated for pH, partial pressure CO_2 (pCO_2), partial pressure O_2 (pO_2), hematocrit, hemoglobin, electrolytes (Na^+, K^+, Ca^+, Cl^- and HCO_3^-), anion gap, and corticosterone (CS) levels. Additionally leukocyte differential counts, total white blood cell counts, total red blood cell counts, and organ hemoglobin levels were evaluated 4 d after treatment was applied. Values prior to treatment were not significantly different for any parameter except the expected rise in hemoglobin and hematocrit levels. Four d after applied treatment, ACTH significantly ($P < 0.1$) increased CS, heterophils, basophils, monocytes, bicarbonate, pCO_2, hemoglobin, and hematocrit levels. ACTH decreased ($P<0.1$) bone marrow hemoglobin, lymphocyte, Cl^-, Na^+, and anion gap. There was an increase in heterophil level for the high and non-selected hematocrit groups compared to

the low hematocrit group. Hematocrit and hemoglobin were significantly higher in the high group over the low hematocrit group. Treatment by hematocrit level interactions ($P < 0.1$) occurred for pO_2, hematocrit, hemoglobin, lymphocytes and K^+. ACTH induced stress, increased hemoglobin and hematocrit only in birds with low or non-selected hematocrit, suggesting that birds with high hematocrit prior to stress have an adaptive advantage during stress. Higher hematocrit prior to stress apparently provides ample oxygen carrying capacity during stress and promotes more available oxygen to be utilized by the tissue. High hematocrit birds appear less susceptible to stress effects by maintaining leukocytes at a constant level, while in non-selected birds the percent of lymphocytes drop.

Introduction

Understanding stress and the mechanisms that can prevent stress or allow animals to adapt is vital. Some growers view stress negatively due to the negative effects it has on the growth and physiology of the bird. They may observe changes in blood gases or acid-base balance, immunosuppression and an increased need for oxygen by the body.

Hans Selye (1976) developed a concept to describe how these changes occur in the body; the general adaptation syndrome: alarm, resistance and fatigue. The alarm stage, a neurogenic stage (Olanrewaju et al., 2006), is characterized by initial stimulation of the hypothalamus to release corticotrophin releasing factor. The stimulation can consist of many stressors, environmental or climatic changes (Thaxton and Siegel, 1970, 1972, 1973), feed deprivation (Beuving et al., 1989), restraint (Freeman and Manning, 1976), ingestion of a toxic substances (Thaxton et al., 1974, 1975, 1982) even mediation

of the adrenal cortex by ACTH administration (Thaxton et al., 1982; Gould and Siegel 1980, 1981) that lead to the adaptive changes that are described in the following. This leads to release of pro-opimelanocortin (POMC) followed by adrenocorticotropin (ACTH) by the anterior pituitary. This in turn stimulates the adrenal cortex to release the glucorticoid, corticosterone (CS). Corticosterone will then signal metabolic changes such as an increase in gluconeogenesis, as well as, immunological changes to try to maintain a state of homeostasis. This increase in gluconeogenesis leads into the stage of resistance. The resistance stage is defined by the changes in the body to resist the effects of the stressor. During this time changes in blood gases, blood pH and acid base balance are observed. If the bird is unable to resist the effects of the stressor the result will often times be death from fatigue, the final stage of the general adaptation syndrome.

In the series of experiments herein, two initial studies have been conducted using a model (Puvadolpirod and Thaxton 2000 a,b,c,d) to observe physiological adaptive responses in broilers under stressful conditions using a continuous infusion mini-osmotic ACTH pump. These experiments evaluated normal broilers and broilers with various hematocrit levels introduced to stress by ACTH pump. The importance of looking at normal or average birds is to set a base for how the birds respond without selective breeding for hematocrit levels. The importance of looking at birds with high or low hematocrit levels allows the researcher to observe whether selective breeding would allow birds to adapt more easily and rapidly to the lack of oxygen carrying capacity needed for energy production.

Glucocorticoids stimulate gluconeogenesis, leading to new glucose production from muscle, lymphoid or connective tissue proteins. This occurs at the expense of

available oxygen for the body or cells, as suggested by reductions in pO_2 (Olanrewaju et al., 2006, 2007) levels in the blood. Therefore, during stress birds must increase erythropoiesis to increase oxygen carrying capacity and fulfill oxygen needs. Increased hematocrit and blood hemoglobin levels in present and previous research (Olanrewaju et al., 2006, 2007; Muldoon et al., 1995; Chapter 3) support the previous statement.

A known practice in some athletes is to use erythropoiesis stimulating drugs (Jelkmann, 2007; Kois, 2004) to increase hematocrit levels, as higher hematocrits should allow for higher oxygen carrying capacity. Research in rats have shown that in the presence of ACTH, serum EPO decreases while hematocrit and hemoglobin increases (Zhang et al., 2004), indicating stress causes a need for mature red blood cells but tends to suppress red blood cell production. So, if broilers could be selected for higher hematocrit levels would this allow them to adapt more readily without having a sudden depletion in red blood cell storage sites and erythropoietin to support erythropoiesis? Therefore the objectives in this study are to confirm that high hematocrit levels improve a bird's ability to increase oxygen carrying capacity and adapt to stress and to evaluate the physiological mechanisms by which this occurs.

Materials and Methods

Treatment

Mini-osmotic pumps were loaded with porcine pituitary ACTH dissolved in 0.85% saline solution, and subcutaneously implanted on the backs of broilers, above the scapula bone. The interscapular tract skin was sterilized with betadine, anesthetized

locally by infusing with Lidocain HCl, and a small incision (10mm) was made. A mini-pump was inserted between the skin and the muscle, and the incision site was closed with one to two surgical staples. The delivery rate of the pump was 1µL/h for 7 consecutive d; dosage was adjusted to 8 IU/kg BW/d.

Blood Sample Preparation

Blood samples were collected via a wing vein. Blood corticosterone (CS) level increase if broilers are restrained for periods exceeding 45 s (Beuving and Vonder, 1978) therefore; sampling time never surpassed 45 s. Samples of 3 to 5ml of blood were collected into a syringe with lithium-heparin, or EDTA. Whole blood samples were centrifuged at 4,000 x g for 15 min and plasma samples were collected. Plasma samples were stored at -20 C for later analysis. Whole blood was used for differential blood smears, heparinized blood was used for evaluation of blood gases and white and red blood cell counts, and plasma was used for analysis of CS.

Analysis of Blood by ABL 77

A 70µl sample of whole blood was analyzed using the ABL 77 blood gas analyzer[1]. Parameters evaluated included pH, pCO_2, pO_2, hematocrit, hemoglobin, electrolytes (Na^+, K^+, Ca^+, Cl^-). Blood gases and pH were corrected for temperature according to the ABL 77 formulations. Bicarbonate and anion gap (cation-anion) were also calculated.

[1] Radiometer America, Inc., Westlake, OH 44145

Organ Preparation for Hemoglobin Analysis

A bone marrow sample from the tibia and the spleen were removed from each bird and weighed until appropriate sample size was reached for each parameter. Bone marrow sample size was 0.02 g and spleen sample size was 0.045. The sample was homogenized then combined with 5ml of cynamet and read on spectrophotometer (Thaxton, 2006).

Enzyme Linked Immunoassay and Immunosorbant Assay Kit Analysis

Plasma CS concentrations were determined by an enzyme linked immunoassay kit (ELISA) as described by Odhiambo (2004). To assay, 25ul of each plasma sample was transferred into an Eppendorf tube and 25ul of steroid displacement buffer (20ul steroid displacement reagent + 980 ul Tris buffer) was added and then vortexed. All samples were brought to a 1:10 dilution using Tris buffer solution with sodium azide before application on the 96 well plate. Standard curves and sample concentrations were calculated using KC Junior data analysis software.

Experimental Facility

The experiment was conducted in a facility containing floor pens on a concrete pad with side wall curtains. All floor pens measured 1.5 x 2.9m. Each pen contained one tube feeder, one bell type drinker, and used litter supplemented with new shavings. The facility temperature was maintained at an average low of 71°F and a high of 88°F using curtain ventilation. The lighting program consisted of 22 h of light throughout the trial period.

Experimental Design, Bird Husbandry, Treatment and Diets.

One strain of commercial broilers was obtained from a local hatchery after being set and hatched in a common incubator. The vaccination program consisted of Marek's vaccination *in ovo*. One-hundred twenty six broiler chicks were distributed into eighteen pens, each containing seven birds.

All birds were fed a common diet throughout the trial period. On d 30, birds were sorted by hematocrit level into three groups of high (24 to 27%), low (18 to 21%), hematocrit and non-separated hematocrit levels. The high and low hematocrit treatments were roughly based on the upper and lower quartiles of the hematocrit population distribution, respectively (Figure 5.1). Approximately 20% (8 birds) of the birds with a hematocrit of 21% were needed to provide enough animals to compose the low hematocrit group. Therefore, the majority (80%) of the low hematocrit group was compiled of birds with hematocrits from 18-20%. Pumps were surgically inserted into half the birds on d 31, giving a 2(ACTH treatment) x 3(hematocrit level) factorial arrangement in a completely randomized design with twenty-one replications per treatment.

Hematology and Hormone Measurements.

Blood from all broilers were evaluated for hematocrit, hemoglobin, electrolytes (K^+, Na^+, Cl^-, Ca^+, HCO_3^-), anion gap, pCO_2, pO_2, pH, and CS as a baseline on d 30. After 4 d of continuous infusion of ACTH, d 35, blood was taken and evaluated for hematocrit, hemoglobin, electrolytes (K^+, Na^+, Cl^-, Ca^+, HCO_3^-), pCO_2, pO_2, pH, and CS

in all experiments. Leukocyte differential counts, total white blood cell counts, total red blood cell counts, and organ hemoglobin levels were also evaluated. Anion gap was calculated from anions and cations. The pH, pCO_2 and pO_2 were corrected for average (41.5 C) bird temperature. Bicarbonate was calculated from the pH and pCO_2.

Statistical Analysis

A 2x3 factorial arrangement of treatments in a completely randomized design was employed in the experiment. Data were analyzed by a general ANOVA (Statistix, 2003). Means were compared for significant ($P \leq 0.1$) differences by using the means and all-pair wise comparison options of Statistix (Statistix, 2003). Statements of significance are based on $P \leq 0.1$ unless otherwise noted.

Results

The impact of ACTH treatment and hematocrit level on broiler blood gas levels are presented in Table 5.1. Treatment with ACTH significantly increased ($P \leq 0.1$) HCO_3^- and pCO_2 (Table 5.1) with no significant effects observed for pO_2. No blood gas parameters were significantly ($P \leq 0.1$) changed by hematocrit level, however there was a significant interaction for pO_2 between hematocrit level and ACTH treatment. The control low hematocrit and non-selected broilers were not significantly different from the ACTH treated low hematocrit and non-selected, but the control high hematocrit birds were significantly lower than the high hematocrit birds receiving ACTH treatment.

The impact of ACTH and hematocrit level on broiler blood and organ parameters is noted in Table 5.2. Hematocrit and blood hemoglobin were significantly ($P \leq 0.1$)

increased by ACTH treatment, while bone marrow hemoglobin was decreased. Hematocrit levels were elevated in high vs. low hematocrit broilers. A significant ($P \leq 0.1$) interaction between ACTH treatment and hematocrit level was noted for hematocrit and blood hemoglobin. Hematocrit and hemoglobin levels significantly ($P \leq 0.1$) increased in low hematocrit and non-selected broilers treated with stress in comparison to low and non-selected control broilers. High hematocrit broilers did not have a significant ($P > 0.1$) increase in hemoglobin or hematocrit level when treated with ACTH.

Table 5.3 represents the effects of the ACTH treatment and hematocrit level on blood electrolytes. ACTH significantly ($P \leq 0.1$) decreased Cl^- and Na^+. No significant ($P \leq 0.1$) effects were noted for any electrolytes between the hematocrit levels. An interaction was observed between ACTH and hematocrit level for K^+. ACTH high hematocrit and control non-selected broilers had significantly higher K^+ levels than control low hematocrit broilers.

Table 5.4 represents the effects of ACTH and hematocrit level on CS, anion gap and blood pH. Treatment by ACTH significantly ($P \leq 0.05$) increased CS and decreased anion gap in treated birds. No ACTH and hematocrit level interactions occurred and no effects due to the hematocrit levels were noted for CS, pH and anion gap.

Tables 5.5 and 5.6 represent effects of ACTH and hematocrit level on total blood counts and blood differential counts. Increases due to the ACTH treatment were noted for heterophil, basophil, and monocyte percents (Table 5.6) while lymphocyte percent was reduced. Results showed an increase in heterophil level for the high and average hematocrit groups compared to the low hematocrit group. An interaction occurred between ACTH and hematocrit level for percent lymphocytes only. Non-selected

broilers treated with ACTH had a significant ($P < 0.1$) decrease in lymphocyte percent compared to the non-selected control broilers. High and low hematocrit broilers did not significantly ($P > 0.1$) differ between control and ACTH treatment.

Discussion

A great deal of research has been conducted using the ACTH mini-osmotic pump model previously described. Results from previous experiments have indicated ACTH induced broilers have increased hematocrit, pCO_2, CS, blood hemoglobin, HCO_3^-, (Chapter 3 and 4) mean corpuscular hemoglobin concentration, anion gap, (Olanrewaju et al., 2006) glucose, cholesterol, triglycerides, high density lipoprotein, total protein, heterophil/lymphocyte ratio, liver weight and lipid content (Puvadolpirod and Thaxton 2000, a,b), but reduced pO_2, Na^+, Cl^- (Chapter 4), K^+ (Olanrewaju et al., 2006), body weight, relative spleen, thymus and bursa (Puvadolpirod and Thaxton, 2000 a,b). In the previous research pH was shown to not be affected by ACTH, indicating birds have the ability to balance acid and base (Olanrewaju et al., 2006).

In agreement with previous research, (Olanrewaju et al., 2006; Olanrewaju et al., 2007; Puvadolpirod and Thaxton, 2000, a,b,c,d) the research herein resulted in a significant increase in CS stimulated by the ACTH mini-osmotic pump and a further result of induction of physiological stress in broiler chickens. Stressed chicks often exhibit elevated levels of CS, as observed using various research methods such as treatment by ACTH (Davis and Siopes, 1989; Olanrewaju et al., 2006; Olanrewaju et al., 2007; Puvadolpirod and Thaxton 2000 a,b,c,d), CS (Post et al., 2003, Donker and Beuving, 1989), and feed restriction (Weber et al., 1990; Mench, 1991; Rajman et al.,

2006). One of the first physiological processes to occur as glucocorticoids increase in the body is a stimulation of gluconeogenesis. Glucocorticoids have a great impact on bird metabolism by stimulating the breakdown of muscle, lymphoid and connective tissue proteins for the production of new glucose which will be utilized by body cells especially in times of stress. This muscle myopathy is commonly observed in poultry due to the increased selection for high growth rates and muscle yield in the commercial broiler (Soike and Bergmann, 1998). The process of gluconeogenesis is responsible for increases in circulating levels of glucose and cholesterol (Olanrewaju et al., 2006) and an increase in heterophil/lymphocyte ratio (Siegel, 1971) observed in present data. The increases in hematocrit, blood hemoglobin, and decreases in bone marrow hemoglobin suggest an upregulation of red blood cells from storage sites and an increase in erythropoiesis are required to meet metabolic energy demands during stress. Previous research (Olanrewaju et al., 2006; Chapter 4) has proven that during gluconeogenesis pO_2 is depleted while supporting the process of new energy production, therefore leading to the up regulation of cells previously described. Significantly increased effects on pCO_2 and HCO_3^-, noted in the current study with ACTH treatment, typically parallel this pO_2 depletion (Olanrewaju et al., 2006; Chapter 4). The increase in pCO_2 may lead to H^+ accumulation resulting in respiratory acidosis (Wideman and Tackett, 2000; Wideman et al., 2000, 2002). As stress increases, extracellular fluid volume decreases and body fluids tend to be lost, leading to shifts in electrolytes as noted by the significant decrease in anion gap, Na^+, and Cl^-.

The process of erythropoiesis and bone marrow production of erythroblasts controlled by erythropoietin production has been previously investigated in the chicken

(Pain et al., 1991; Wickeramasinghe et al., 1994). As previously discussed above, the increase in hematocrit and blood hemoglobin with stress, indicate erythropoiesis increases as glucocorticoid levels increase (Bauer et al., 1999). Olanrewaju and coworkers (2006) suggest that this increase in hematocrit and hemoglobin may be a permanent adaptation.

In addition to the increase in heterophil/lymphocyte ratio, (Gross and Siegel, 1983; McFarlane and Cutris, 1989; Altan et al., 2000) or the increase in heterophils paralleled by the decrease in lymphocytes, there is a marked increase in basophils (Mitchell et al., 1992; Maxwell et al., 1992; Altan, 2000) and monocyte numbers observed during stress. Previous research (Maxwell et al., 1992; Maxwell1993); suggests that increases in heterophil/lymphocyte ratio are indicative of mild stress while increases in basophil numbers are indicative of extreme stress responses.

Very little research has been conducted to evaluate the differences in physiological parameters for birds with varying hematocrit levels. Data for the main effect of hematocrit treatment in the present study indicates an increase in percent heterophils for the high and average hematocrit groups compared to the low hematocrit group and an increase in hemoglobin and hemactocrit for the high hematocrit group over the low group. These results are in contrast to previous data showing significant differences in pCO_2, HCO_3^-, Cl^-, Na^+, anion gap levels between low and high hematocrit groups (Chapter 3). However, ACTH by hematocrit interactions did exist in the current study complicating main effect results.

All cells come from the same progenitor hematopoietic stem cells. These cells differentiate into three different lineages, erythroid, myeloid, or lymphoid cells. It

appears no matter the margin of difference in hematocrit level, as hematocrit levels increase there is a shift in hematopoietic stem cells to differentiate toward the erythroid, then myeloid progenitors away from the lymphoid progenitors. As more red blood cells are needed to meet the increasing hematocrit level, the body shifts toward making red blood cells (erythrocytes) and heterophils (myeloid), explaining the increase in heterophils in the higher hematocrit group over the lower group.

There were five ACTH by hematocrit level interactions, hematocrit, hemoglobin, oxygen, percent lymphocytes and K^+. Control birds with low and average hematocrit levels had significantly lower blood hemoglobin and hematocrit levels than control birds with high hematocrit or ACTH treated birds with high, non-selected or low hematocrit levels. This indicates that as stress increases, the low and non-selected birds must raise their hematocrit level to produce more hemoglobin, thereby increasing oxygen carrying capacity, while high hematocrit birds already have an adaptive advantage and can maintain their oxygen carrying capacity. The higher oxygen carrying capacity promoted by the higher hematocrit group, creates an opportunity for more oxygen to bind and be utilized by the tissues during stress as revealed by the significant increase in pO_2 in the high hematocrit broilers presented with stress. The non-selected and low hematocrit groups lack this adaptive advantage and therefore are unable to improve pO_2 level. High hematocrit birds appear less susceptible to stress effects by maintaining leukocytes at a constant level, while in non-selected birds lymphocyte numbers drop. Data shows that K^+ levels are significantly less in the low hematocrit control broilers in comparison to the average hematocrit control broilers and high hematocrit ACTH treated broilers. It appears in the control broilers the low hematocrit levels may cause lower K^+ levels.

Treatment with ACTH appears to have no significant effect between hematocrit groups. When comparing the low hematocrit level broilers, it appears ACTH has no effect on K^+ level, nor does ACTH have an effect on the average or high hematocrit birds when evaluating K^+ for each individual hematocrit group.

In conclusion, birds with normal hematocrit levels have the ability to maintain acid base balance, pH and regain oxygen carrying capacity through constant shifts in electrolyte levels and via increased red blood cell release from storage sites. Results suggest that birds with a high hematocrit prior to stress have an adaptive advantage during stress. Higher hematocrit prior to stress apparently provides ample oxygen carrying capacity during stress and promotes more available oxygen to be utilized by the tissue. High hematocrit birds appear to be less susceptible to stress effects by maintaining leukocytes at a constant level, while in non-selected birds, lymphocyte numbers drop.

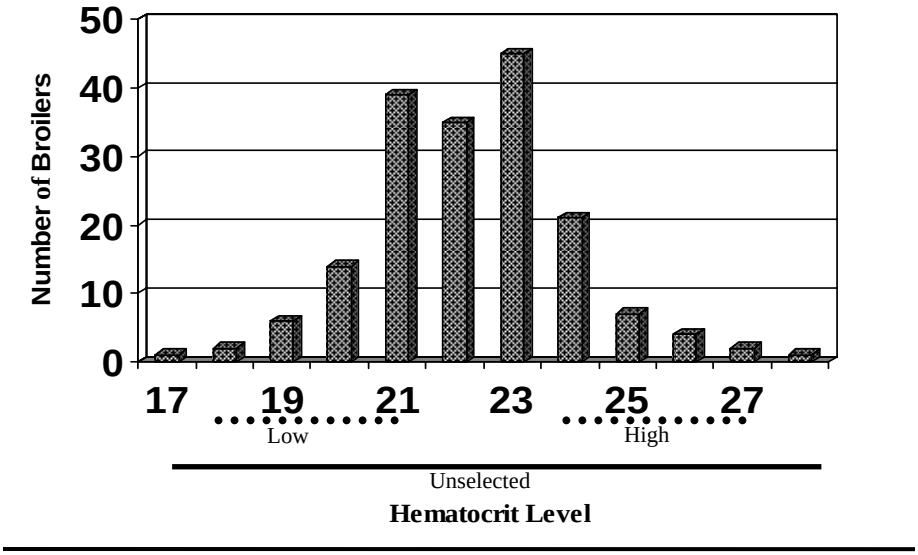

FIGURE 5.1

Distribution of Hematocrit Treatment

TABLE 5.1. Impact of Adrenocorticotropin (ACTH) and Hematocrit (Hct) Level on Broiler Blood Gases

ACTH	Hct Level	pO_2	pCO_2	HCO_3[1]
		----------(mm/Hg)----------		
Control		74	59[b]	23.6[b]
ACTH		78	67[a]	25.6[a]
SEM		3.1	1.8	0.46
	Low	73	64	25.1
	Average	78	60	23.9
	High	76	66	24.8
SEM		3.8	2.2	0.57
Control x Low		76[ab]	57	24
Control x Non-selected		79[ab]	57	23
Control x High		68[b]	64	24
ACTH x Low		70[ab]	70	26
ACTH x Non-selected		78[ab]	63	25
ACTH x High		85[a]	68	26
SEM		5.4	3.1	0.81
CV		31.25	21.75	14.37
Source of variation				
ACTH		0.433	0.003	0.002
Hct Level		0.641	0.161	0.288
ACTH x Hct Level		0.100	0.362	0.981

[1]HCO_3 is calculated from pH and pCO_2.

[a-b,] Means within a column for a main effect or interaction response that do not share common superscripts differ ($P \leq 0.1$).

TABLE 5.2. Impact of Adrenocorticotropin (ACTH) and Hematocrit (Hct) Level on Broiler Blood and Organ Parameters

ACTH	Hct Level	Hemoglobin (Hb)	Hct	Spleen Hb	Bone Marrow Hb
		g/dL	%	g/dL	g/dL
Control		6.6[b]	20.6[b]	8	6.3[a]
ACTH		6.9[a]	21.7[a]	6	5.4[b]
SEM		0.11	0.32	1.1	0.29
	Low	6.6[n]	20.7[n]	6	6.4
	Average	6.7 [mn]	20.9[mn]	6	5.7
	High	7.0 [m]	21.9[m]	9	5.4
SEM		0.13	0.40	1.3	0.36
Control x Low		6.3[y]	19.8[y]	6	6.6
Control x Non-selected		6.2[y]	19.6[y]	6	6.4
Control x High		7.2[x]	22.4[x]	11	5.9
ACTH x Low		6.9[x]	21.6[x]	6	6.2
ACTH x Non-selected		7.1[x]	22.1[x]	7	5.0
ACTH x High		6.8[x]	21.4[x]	7	4.9
SEM		0.19	0.56	2.0	0.50
CV		12.17	11.70	118.95	37.92
Source of Variation					
ACTH		0.015	0.015	0.295	0.031
Hct Level		0.085	0.078	0.234	0.108
ACTH x Hct Level		0.008	0.008	0.361	0.628

[a-b, m-n, x-y] Means within a column for a main effect or interaction response that do not share common superscripts differ ($P \leq 0.1$).

TABLE 5.3. Impact of Adrenocorticotropin (ACTH) and Hematocrit (Hct) Level on Broiler Blood Electrolytes

ACTH	Hct Level	Na^+ (mEq/L)	K^+ (mEq/L)	Ca^+ (mEq/L)	Cl^- (mEq/L)
Control		145.9[a]	6.1	3.03	110[a]
ACTH		140.4[b]	6.3	3.01	106[b]
SEM		0.65	0.15	0.022	1.4
	Low	143.1	6.0	3.04	109
	Average	143.0	6.3	3.01	109
	High	143.4	6.4	3.01	106
SEM		0.80	0.18	0.027	1.8
Control x Low		146	5.8[y]	3.04	112
Control x Non-selected		146	6.6[x]	3.03	113
Control x High		146	6.0[xy]	3.04	106
ACTH x Low		141	6.2[xy]	3.05	106
ACTH x Non-selected		141	6.0[xy]	2.99	105
ACTH x High		140	6.7[x]	2.99	107
SEM		1.1	0.26	0.039	2.5
CV		3.43	18.03	5.58	10.08
Source of Variation					
ACTH		0.001	0.422	0.509	0.032
Hct Level		0.924	0.433	0.639	0.455
ACTH x Hct Level		0.865	0.030	0.696	0.207

[a-b, x-y] Means within a column for a main effect or interaction response that do not share common superscripts differ ($P \leq 0.1$).

TABLE 5.4. Impact of Adrenocorticotropin (ACTH) and Hematocrit (Hct) Level on Broiler Physiology

ACTH	Hct Level	pH	(Anion Gap) Cation-Anion	Corticosterone pg/mL
Control		7.26	18[a]	2254[b]
ACTH		7.25	15[b]	12104[a]
SEM		0.010	1.3	1183.2
	Low	7.25	15	5887
	Average	7.26	16	6981
	High	7.24	19	8670
SEM		0.013	1.6	1457.9
CV		1.07	58.57	125.51
Source of Variation				
ACTH		0.443	0.098	0.001
Hct Level		0.596	0.266	0.366
ACTH x Hct Level		0.201	0.243	0.187

[a-b] Means within a column for a main effect response that do not share common superscripts differ ($P \leq 0.1$).

TABLE 5.5. Impact of Adrenocorticotropin (ACTH) and Hematocrit (Hct) Level on Broiler Total Blood Cell Counts

ACTH	Hct Level	Red Blood Cells	White Blood Cells
		million	thousand
Control		3.8	2.6
ACTH		3.8	2.6
SEM		0.23	2.66
	Low	3.6	2.9
	Average	3.9	2.2
	High	3.9	2.7
SEM		0.28	3.26
CV		30.94	52.98
Source of Variation			
ACTH		0.834	0.829
Hct Level		0.647	0.217
ACTH x Hct Level		0.655	0.284

TABLE 5.6. Impact of Adrenocorticotropin (ACTH) and Hematocrit (Hct) Level on Broiler White Blood Cell Counts

ACTH	Hct Level	Monocyte	Lymphocyte	Heterophil	Basophil	Eosinophil
		----------% of total leukocytes----------				
Control		6[b]	74[a]	14[b]	2.6[b]	0.8
ACTH		11[a]	63[b]	22[a]	4.0[a]	0.7
SEM		0.82	2.7	1.8	0.52	0.19
	Low	7	72	13[y]	3.6	0.6
	Average	9	67	20[x]	3.3	0.6
	High	9	66	21[x]	3.1	1.0
SEM		0.99	3.3	2.1	0.64	0.23
Control x Low		5	72[xy]	10	3.1	0.6
Control x Non-selected		5	78[x]	14	2.1	0.6
Control x High		7	71[xy]	17	2.5	1.1
ACTH x Low		9	71[xy]	15	4.1	0.6
ACTH x Non-selected		13	56[z]	25	4.4	0.7
ACTH x High		11	60[yz]	25	3.6	0.9
SEM		1.4	4.7	3.0	0.91	0.33
CV		51.03	20.57	51.30	132.31	82.61
Source of Variation						
ACTH		0.001	0.005	0.003	0.055	0.897
Hct Level		0.299	0.420	0.014	0.825	0.400
ACTH x Hct Level		0.198	0.082	0.533	0.723	0.854

[a-b, x-z] Means within a column for a main effect or interaction response that do not share common superscripts differ ($P \leq 0.1$).

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CHAPTER VI

PROTEOMIC EVALUATION OF AVIAN PERIPHERAL BLOOD MONOCYTES FOR FUNCTIONAL PROTEINS

Abstract

Monocytes as well as other professional antigen presenting cells (APC), dendritic cells (DC) and macrophages, play a critical role in adaptive and innate immune responses. A differential detergent fractionation (DDF) analysis was conducted on avian monocytes to reveal proteins related to cell adhesion, uptake and antigen presentation to lymphocytes, receptor proteins, proteases and cytokines. We identified a total of 3229 proteins with 46 of these involved in the functions of professional APC. Of these proteins fourteen were receptor proteins, four were related to antigen presentation (including MHC Class I), six to antigen uptake, ten to cell adhesion, two toll-like receptor (TLR 4 and 15), and nine protease proteins were identified. We have shown that the DDF approach provides meaningful, interpretable, functional, information concerning protein expression profiles associated with monocytes activation and differentiation into macrophages and/or immature DC in avian species. This data will be instrumental in future experiments evaluating protein expression of monocytes in stressed broilers.

Introduction

Monocytes (CD14+cells), that originate in the bone marrow from a specialized progenitor, make up 5 to 10% of the circulating leukocytes in humans (Seta and Kuwana, 2007). These cells are important players in immune defense due to their ability to phagocytose foreign material, present antigens to immunocompetent cells and produce an array of cytokines after stimulation with bacterial products via TLR (Seta and Kuwana, 2007 and Lee et. al., 2006). Mammalian monocytes serve as professional antigen presenting cells, as they express high levels of both MHC class I and II molecules, co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86), endocytic and phagocytic specific receptors, and adhesion molecules (Kruger et. al., 2003). Monocytes typically circulate in the blood for 10 to 20h before migrating from the capillaries to the tissues. Once in the tissue, under influence of cytokines, monocytes differentiate into macrophages or dendritic cells (DC) (Kruger et. al., 2003). Recent studies suggest that monocytes are not only precursors for macrophages and DC, but also for osteoclasts, microglia of the central nervous system and Kupffer cells of the liver (Seta and Kuwana, 2007).

Research indicates that avian monocyte development, much like its mammalian counterparts, is influenced by colony stimulating factors (Qureshi, 2003). Approximately 3 d after their arrival into the bloodstream, avian monocytes will seed various tissues and organs (Qureshi, 2003). Blood monocytes are responsible for 30% of avian alveolar macrophages and nearly 100% of avian Kupffer cells of the liver (Qureshi, 2003). Unlike mammals, chickens have no residential macrophages present in their abdominal cavity. However, monocytes can be recruited into the tissue in response to an active inflammatory signal at the tissue site (Qureshi, 2003). One known function of avian

monocytes is their chemotactic ability, the migration toward an inflammatory gradient. Chemotactic signals come from bacterial products, synthetic peptides, complement or certain products of an immune reaction, and factors released by damaged cells and extracellular matrix (Qureshi, 2003). Genetic origin and differences in major histocompatability complex (MHC) have been shown to play a role in avian monocytes' chemotactic potential and that avian monocytes typically have an active response to chemotactic signals (Qureshi et. al., 1988).

Our long-term goal is to understand the effects of stress on innate immunity in chickens. In the presence of some stress monocytes numbers are typically increased (Landmann et al., 1984), however monocytes function and cytokine release by monocytes is often decreased (Joyce et al., 1997). To this end, we need a basic measure of proteins expressed in monocytes, in order to go forward with our interests in understanding the effects of adrenal hormones, like corticosterone, on influencing the proliferation and migration of monocytes during stress. At present there is little information concerning protein expression profiles in avian monocytes. Therefore, we used proteomics methodology to obtain interpretable and meaningful information on the proteins expressed in normal chicken monocytes.

Materials and Methods

Experimental Facility

The experiments were conducted in a facility containing floor pens on a concrete pad with side wall curtains. All floor pens measured 1.5 x 2.9m. Each pen contained one

tube feeder, one bell type drinker, and used litter supplemented with about 5mm of new shavings. The facility temperature was maintained at a low of 69° F to a high of 80° F using curtain ventilation. The lighting program consisted of 22 h of light throughout the trial period.

Experimental Design, Bird Husbandry, and Diets

One strain of commercial broilers was obtained from a local hatchery after being set and hatched in a common incubator. The vaccination program consisted of Marek's vaccination *in ovo*. Fifty broiler chicks were distributed into five pens in the experimental facility, each containing ten birds. All birds were fed a common diet throughout the trial period.

Monocyte Isolation and Magnetic Separation

Peripheral blood was collected into an EDTA anti-coagulation tube from twenty-five broilers bled via cardiac puncture. Monocytes were isolated according to methods used by Lee and coworkers (2007) with minor revisions. Peripheral blood mononuclear cells were isolated using Percoll (1083) gradients and separation occurred in tubes rather than Petri dishes. CD 14^+ monocyes were positively selected by using magnetic cell separation methodology (Lee et. al., 2007). The total monocyte cell count was 4.2×10^7.

Protein Extraction, Trypsin Digestion and 2D-LC ESI MS^2

Proteins were isolated using DDF methodology as described by McCarthy and co-workers (2005). A series of detergents were used to extract proteins from cellular

compartments. Repeated washes with digitonin buffer were used for cytosolic protein isolation. This is followed by isolation of the membrane, nuclear and cytoskeletal proteins by triton X-100 (TX), deoxycholate (DOC), Tween 40, and SDS buffers. Protein samples (1%) were compared to evaluate quality of isolated proteins, using 10% SDS-PAGE. For each of the detergent fractions, equal amounts of protein were precipitated with 25% tricholoracetic acid to remove salts and detergents. Protein pellets were solubilized and then digested with 100ng trypsin (50:1 ratio of substrate to enzyme) overnight at 37°C. Peptides were desalted using a peptide microtrap (Michrom, BioResources, Inc.) and eluted by 0.1% triflouroacetic acid, 95% acetonitrile solution. Desalted peptides were dried and resuspended in 0.1% formic acid.

A 2D LC ESI MS^2 was done as described by McCarthy and co-workers (2005). Liquid chromatography (LC) analysis was accomplished by strong cation exchange (SCX) followed by reverse phase (RP) LC coupled directly inline with electrospray (ESI) ion trap MS. Samples were loaded into a LC gradient ion exchange system including a Thermo Separations P4000 quaternary gradient pump (ThermoElectron Corporation) coupled with a 0.32 x 100 mm BioBasic SCX column. A flow rate of 3µL/min was used for both SCX and RP columns. A salt gradient was applied in steps of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 57, 64, 71, 79, 90, 110, 300, and 700mM ammonium acetate in 5% acetonitrile, 0.1% formic acid and the resultant peptides were loaded directly into the sample loop of a 0.18 x 100 mm BioBasic C18 RP LC column of a Proteome X workstation (ThermoElectron). The RP gradient used 0.1% formic acid in acetonitrile and increased the acetonitrile concentration in a linear gradient from 5% to 30% in 30 min and then 30% to 65% in 9 min followed by 95% for 5 min and 5% for 15 min. The

spectrum collection time was 59 min for every SCX step. The LCQ Deca ion trap mass spectrometer was configured to optimize the duty cycle length with the quality of data acquired by alternating between a single full MS scan followed by three tandem MS scans on the three most intense precursor masses from full scan. The collision energy was normalized to 35%. Dynamic mass exclusion windows were set at 2 min, and all of the spectra were measured with an overall mass/charge (m/z) ration range of 200-2000.

Protein Identification and Analysis

Mass spectra were analyzed as previously described (Lee et al., 2007). Briefly, the search term Gallus gallus was searched against the organism field of the National Center for Biotechnology Information to create a chicken-specific protein database. The mass spectra were compared to the chicken-specific protein database using the TurboSEQUEST (Bioworks Browser 3.1; ThermoElectron) at the MSU Life Sciences and Biotechnology Institute. Each peptide was considered genuine if Delta Cn values were >0.1, and the presence of a C-terminal lysine or arginine. Alignments of amino acid sequences were conducted with the Clustal W version 1.81 program (Thompson et al., 1994).

Results and Discussion

A total of 3229 proteins have been identified. The majority of these proteins (1265 or 39%) were found in the nuclear compartment of the cells. The membrane compartment contained 30% (978), with 26% (831) identified in the cytosolic and 5% (155) found in the cytoskeleton (Table 6.1). Results indicate that of the 3229 total

proteins identified, 46 were involved in immune functions of the professional APC. In Table 6.2, proteins were categorized into seven groups according to immune function. Of the proteins, two toll-like receptors, ten cell adhesion, nine proteases, six antigen uptake, four antigen presentation, and fourteen receptor proteins were identified and compared the a database. Comparison of the proteins to mammalian counterparts create basis for understanding the function of normal avian monocytes.

Toll-Like Receptor Proteins

TLR are the key component of a signaling pathway which serves as the frontline subsystem against invasive microorganisms for both innate and adaptive immune responses (Iwasaki and Medzhitov, 2004). These receptors, which are widely expressed by leukocytes and epithelial cells, function through recognition and interaction with conserved motifs expressed on the surface of pathogen associated molecular patterns, like lipopolysaccharides (Higgs et al., 2006). This TLR-LPS interaction allows the receptor to discriminate between microbial pathogens or their products and to initiate trans-membrane signaling (Laflamme and Rivest, 2001). There are over eleven identified TLR in mammals, which recognize a variety of ligands from pathogens to trigger immune responses (Oda and Kitano, 2006). Some TLR (1, 2, 6, 4, 5) are located on the surface of the plasma membrane, while others on not known to be located on the cell surface (TLR 3, 7, 9) (Akira and Takeda, 2004).

In the chicken, TLR 1, 2, 3, 4, 5, 7 have been shown to be expressed by heterophils (Kogut et al., 2005). Toll-like receptor 4 (Table 6.2), one of two TLR identified herein, is suspected to play a key role in the response to Gram-negative

bacteria during pathogen invasion (Laflamme and Rivest, 2001) through recognition of the LPS associated outer membrane (Poltorak et al., 1998). Expression of TLR-4 is upregulated by Gram negative bacteria, especially in granulocytes and monocytes. The other TLR identified, TLR-15 (Table 6.2), appears to be avian specific. This TLR has been found on chromosome 15 and is characterized by an archetypal toll-interleukin receptor, transmembrane domains and a distinctive arrangement of extracellular leucine rich regions. Researchers believe that TLR-15 plays an important role in the avian defense against bacteria, as it has been shown to be upregulated in the presence of Salmonella enterica (Higgs et al., 2006).

Cell Adhesion Proteins

Integrins are receptors for different extracellular matrix (ECM) components depending on different combinations of alpha and beta chains. When binding to the ECM, integrins transmit outside - in signals resulting in cytoskeletal rearrangements which facilitate cell motility.

We found the expression of the αV and α11 subunits and the β1 and β5 subunits (Table 6.2), which predict at least three integrin heterodimers, αVβ1, αVβ5, and α11β1 with specificity for the ECM components collagen, fibronectin and osteopontin (Humphreys et al., 2006).

Protease Proteins

Chicken monocytes express a number of surface and secreted proteases that may facilitate entry into the tissues form the bloodstream. Several members of the protease

family a disintegrin and metalloprotease (ADAM) along with the membrane metalloproteases (MMP) are surface expressed on leukocytes and recognize ECM components as substrates (Stefanidakis and Koivunen, 2006). The binding of monocyte integrins to ECM proteins induce outside-in signaling through integrins resulting in upregulation in expression of proteases for movement through the endothelial basement membrane (Sudhakaran et al., 2007).

Antigen Uptake Proteins

Chicken monocytes express a number of receptors which may be important for antigen uptake and clearance upon differentiation to macrophages. Peptides derived from the chicken homologue of the CD36 scavenger-type receptor typical of the myeloid lineage were identified (Table 6.2). CD36 is important in clearance of apoptotic cells (Bottcher et al., 2006) and for oxidized lipoproteins (de Villiers and Smart, 1999). The peptides for two members of the mannose receptor family were identified in the digitonin and Tween 40-deoxycholate detergent fractions (Table 6.2). Mannose receptors function as phagocytic pattern-recognition receptors in innate immunity with specificity for mannose and fucose residues on prokaryotic glycoproteins and glycolipids (McGreal et al., 2005). The predicted CD206 protein showed 48% amino acid homology in Clustal alignments with human C-type 1 mannose receptor (accession number NP_001009567). The predicted CD280 protein showed 72% amino acid homology in with human (accession number NP_006030) C-type 2 collagen receptor.

Antigen Presentation Proteins

The B-complex encodes the major histocompatibility complex class I (*BF*) and class II (*BL*) antigen-presenting glycoproteins (Pink et al., 1977; Vainio et al., 1984) that are linked to the highly polymorphic *BG* proteins (Kaufman et al., 1990; Miller et al., 1991). Also on the same chromosome are the *Y*-complex genes (Briles et al., 1993) which function similar to minor histocompatibility complex proteins (Thoraval et al., 2003).

Evidence for the expression of MHC class I in monocytes was obtained with the observation of a *BF*-specific peptide which is found in the α3 domain of the *BF*2 gene product (Guillemot et al., 1988; Hunt and Fulton, 1998), which is the predominantly expressed *BF* gene (Hunt and Fulton, 1998; Fulton et al., 1995).

Two *BG*-specific peptides were found in the dataset. The peptide identified from proteins in the digitonin detergent fraction is encoded in the cytoplasmic region of the bg17 transcript from the B21 haplotype (Miller et al., 1991). The peptide identified from proteins in the Tween 40-deoxycholate could have come from the product of the bg3 or bg14/8 genes. Both genes are expressed at the protein level in the B21 haplotype (Miller et al., 1991).

Previous studies have identified the expression of *YF* genes in various chicken tissues at the RNA and protein level (Afanassieff et al., 2001; Hunt et al., 2006). Two *YF*-specific peptides were identified from proteins isolated with the Tween 40-deoxycholate detergent. The peptide "ATLKRSVQPEVR" spans the border of the α2/α3 domains in the predicted amino acid sequence from the jungle fowl *YF* gene (accession number XP_415352). The other YF-specific peptide is found within the α3 domain of the *YF* genes reported by Thoraval et al., (2003).

Receptor Proteins

The tryptic peptides from 14 receptor type proteins were found in the different detergent fractions analyzed by mass spectrometry in this study (Table 6.2). These proteins have been shown to enhance the immunological functionality of mammalian monocytes.

The TNF family member CD40 and its ligand have been identified and characterized in the chicken (Tregaskes et al., 2005; Kothlow et al., 2008). Evidence shows that expression of CD40 by activated monocytes and dendritic cells or differentiated monocytes may be involved in enhanced cytokine production (Banchereau et al., 1994). Chicken monocytes produce nitric oxide when stimulated with a fusion protein consisting of chicken CD40L, mimicking the interaction of monocytes with T_H1 helper T-cells (Tregaskes et al., 2005).

In chickens, the receptor for leptin has been well characterized (Niv-Spector et al., 2005). The role of leptin in regulating feed intake in chickens has been well established (Denbow et al., 2000). In the immune system, the leptin receptor expressed in human peripheral blood mononuclear cells is responsible for mediating leptin activation of monocytes leading to production of pro-inflammatory cytokines (Zarkesh-Esfahani et al., 2001). Leptin can also stimulate production of reactive oxygen species as a result of monocyte activation (Sancehz-Pozo et al., 2003).

The role of the Notch family of proteins in the chicken hematopoietic system has only been described for B-cell development in the bursa of Fabricius (Morimura et al., 2001). It has been shown that mammalian monocytes express relatively high amounts of

Notch-1 and Notch-2 and through the immobilized extracellular domain of the Notch ligand, Delta-1, may induce apoptosis in peripheral blood monocytes cultured with macrophage colony-stimulating factor (M-CSF), but not granulocyte-macrophage CSF (GM-CSF). Data indicates a key role for Notch signaling is in regulating cell fate decisions by bi-potent macrophage/dendritic precursors (Ohishi et al., 2001).

The chicken IL-20Rα receptor protein identified by the peptide in the dataset (Table 2) shows 39% amino acid identity with the human IL-20Rα chain protein, termed IL-20R1 (accession number Q9UHF4). In mammals the IL-20 receptor consists of the IL-20R1 ligand-binding chain and the IL-20R2 signal transduction chain (Nagalakshmi et al., 2004). The IL-20 receptor belongs to the class II cytokine receptor family and is responsible for binding IL-20 which has been shown to be produced by monocytes during incubation with TNF-α or LPS (Wang et al., 2006; Wolk et al., 2002). Research shows that IL-20 functions as a key regulator of keratinocytes proliferation during skin inflammatory responses (Wang et al., 2006).

The predicted protein for the β-chain of the chicken oncostatin M (OSM) receptor showed 35% homology with the human OSM β-chain protein (accession number AAC50946). Oncostatin M (OSM), a 28-kd cytokine in the IL-6 family, has been shown to be produced by macrophages and activated T-cells at sites of major inflammation. One study revealed enhanced levels of OSM, in synovial fluid from rheumatoid arthritis lesions. Oncostatin M played a key role in chronic joint inflammation through regulation of TIMP-1, MMP-1, as well as monocyte chemotactic protein-1 in synovial fibroblasts in vitro (Langdon et al., 2000).

The predicted protein for chicken monocyte colony stimulating factor (M-CSF) showed 53% identity with the human M-CSF protein (accession number P07333) in Clustal alignments. In mammals M-CSF is responsible for differentiation of mononuclear phagocytes to macrophages. Activated M-CSF receptor associates with phosphatidylinositol 3-kinase (PI 3-kinase) and induces direct interaction of PI 3-kinase with SH2/SH3 adaptor protein Grb2. Saleem and coworkers (1995) show that M-CSF induces the formation of a PI 3-kinase-Grb2-Sos complex, supporting a potential role of PI 3-kinase in Ras signaling pathways in monocytes.

Somatostatin functions in the control of pituitary hormone release in the chicken (Geris et al., 2000). Bhathena and coworkers (1981) demonstrated binding sites for somatostatin on lymphocytes and monocytes suggesting a role for somatostatin receptors in these cells of the human immune system. Dalm and coworkers (2003) found in one study that during differentiation of monocytes to macrophages or dendritic cells, time-dependent, significantly increased mRNA levels of somatostatin receptor-2 and cortistatin, a somatostatin-like peptide.

Park and coworkers (2003) revealed that FAS (CD95) can activate proinflammatory cytokine responses in normal human monocytes and macrophages. Following Fas ligation, both monocytes and monocyte-derived macrophages released TNF-α and IL-8 and conditioned medium from Fas-activated monocytes and macrophages induced the directed migration of neutrophils in a chemotaxis assay. Fas-induced monocyte cytokine responses were associated with monocyte apoptosis, nuclear translocation of NF-κB, and cytokine protein expression and were blocked by caspase inhibition but not by inhibition of IL-1β signaling (Park et al., 2003).

In chickens the receptor for osteoprotegerin has only been observed in ovarian tissues, primarily the postovulatory follicle (Bridgham and Johnson, 2003). In mammals osteoprotegerin is also an important regulator of differentiation and activation of osteoclasts that also affects different cells of the immune system. It has been shown to significantly stimulate monocyte chemotaxis; however preincubation of monocytes with osteoprotegerin was shown to inhibit monocyte migration toward optimal concentrations of T-cell secreted monocyte chemotactic protein 1, and procalcitonin (Mosheimer et al., 2005). Seshasayee and coworkers (2004) found that osteoprotegerin ligand will up-regulate receptor activator of NF-κB (RANK) receptor expression on monocytes, regulate their effector function by inducing cytokine and chemokine secretion, activate antigen presentation through up-regulation of co-stimulatory molecule expression, and promote monocyte survival.

Summary

In conclusion our data, suggest that chicken monocytes express proteins required for migration into vascularized tissues and differentiation into macrophages or immature dendritic cells. Future studies will be required to confirm the expression of the products identified by mass spectrometry.

In the presence of stress, monocyte numbers are typically increased (Landmann et al., 1984), however monocyte function and cytokine release by monocytes is often decreased by stress (Joyce et al., 1997). Future studies with protein expression may be beneficial in determining why monocytic function is suppressed during stress.

TABLE 6.1. Sub-cellular Location of Identified Proteins

Cell Compartment	Number of Identified Proteins	% of Total Protein Profile
Cytosolic	831	26%
Membrane	978	30%
Nuclear	1265	39%
Cytoskeleton	155	5%

TABLE 6.2. Proteins Organized by Immune Function

Toll-like receptors

Protein	DDF	Accession number	Peptide
TLR4	T	XP_415518	NKHSMICHTPAYMK
TLR15	TD	XP_419294	TEENKTSPPAATLR

Cell adhesion proteins

Protein	DDF	Accession number	Peptide
αV integrin (CD51)	T	NP_990770	SHQWFGASVR VNAALEVNPTILNPENK EPVGTCYLFDGSK
	TD		ILACAPLYHWR ACSLADVKVSCFKVK GKLPNSLNFQVELLLDK
α11 integrin	TD	XP_413930	WSTSSCKGPFR
β1 (CD29)	D	XP_418572	RVLEDREVTNR
β5 integrin	TD	NP_989814	DSKNIIELIVK
CD47	T	XP_416623	IYRHESVPSANFLSK
Contactin 6	TD	XP_425170	RGMPHFER
Cell-adhesion	T, TD	XP_422207	TLPYHNKYYWIGIRK
(possible selectin)			
Protocadherin 18	T, D, and TD	XP_420404	FRAMQRGNSPLLVVR
cHz cadherin	T, S, and TD	XP_418340	RWHNIKIK
Hyaluronan receptor (CD168)	T, S, and TD	XP_414495	KMSSLCMELMKLR

Table 6.2. (continued)

Proteases

Protein	DDF	Accession number	Peptide
ADAM 8	D	XP_421552	GDCCQDCKVKAAGVLCR
ADAM 12	T, TD	XP_423549	KPLPADPLNK
ADAM 17 (TACE)	TD	XP_419944	MLLEQFSFDIAEK
ADAM 20	TD	XP_428276	GGSCLYQAPALGSYYTL
ADAMTS-5	TD	XP_425541	EKGLEVNR
ADAMTSL-3	D	XP_413844	LIGNDNRLIEPPNLR
MMP3	D	XP_417175	KIDAAVHDQNTK
MMP27	D, TD	NP_990331	EVVDKAIQK
Heparanase	S	NP_989498	FGGTSTDFLIFNPNK

Antigen uptake

Protein	DDF	Accession number	Peptide
ced-12/ELMO	T	XP_417479	AFEELFAICIQLLNK
MR CD206	D	XP_001235095	QNAKWENQACNQR
MR CD280	TD	XP_418071	MCSDYGSTLVTITNR
			WSDGLGFFYHNFDR
CD36	TD	XP_415975	NNFIQLLLNTWIK
Clathrin heavy chain	TD	XP_415878	IHEGCEEPATHNALAK

Antigen presentation

Protein	DDF	Accession number	Peptide
MHC class I	T, TD	AF013493	AHGFYPRPIVVSWLK
B-G	D	AAA48627	ILASKLMKQMEK
	TD	AAA48618	HFQNMYLSAGK
Rfp-Y	TD	XP_415354	ATLKRSVQPEVR
		AAP33136	WKHELGTVCVQNLR
Calnexin	TD	XP_414608	CGEDYKLHFIFR

Table 6.2. (continued)

Receptors

Protein	DDF	Accession number	Peptide
CD40	T	NP_989996	VKGTNTSDVICESSRR
IGF receptor	T	NP_990363	MCWQYNPKMR
Leptin receptor	T	NP_989654	MLIPSEMSISASQER
Notch	D	XP_415420	CEGDVNECLSNPCDAR
C-delta	S, TD	NP_990304	NEDSVKEEHGK
RORβ	D	XP_425885	QRNCLIDRTNR
Opioid growth factor receptor	D	XP_425708	KSEDACAAQAALLLSAGR
IL-20Rα	TD	XP_419723	LKMADTVDELLGKGR
Oncostatin M (β-subunit)	T, TD	XP_425020	DAELVMSFEIQVRR
M-CSF (CD115)	TD	XP_414597	EDSVLKVAVKMLK
Megalin	TD, S	XP_422014	QDLIKTK
Somatostatin	T	XP_426102	MRAVAQRVGWQQR
FAS	TD	XP_421659	LIHIDVDLTHHVPDIVR
Osteoprotegerin	TD	XP_418394	QVMCNQCPPGSYVK

Legend: DDF; Differential detergent fractionation, D; Digitonin, T; Triton X-100, TD; Tween 40-deoxycholate, S; Sodium dodecyl sulfate.

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CHAPTER VII

CONCLUSION

Many models have been developed in an attempt to understand the effects of stress on broiler physiology and well-being, however, a clear understanding of the effects of stress is lacking. There were three overall objectives in this research series. The first objective in this research was to determine if birds that have higher hematocrit levels would have a selective advantage in maintaining a higher oxygen carrying capacity during stress. The second objective was to determine if a mechanism exists that provides additional red blood cells, either through production or from storage sites, to increase oxygen carrying capacity during stress. The final objective was to evaluate cells at the molecular level, by identifying protein profiles in monocytes in unstressed broilers. This information would provide a data profile in which to compare monocyte proteins of stressed broilers to in future research.

The primary objective in Chapter III, was to determine the effects high or low hematocrit levels have on control broilers as well as stressed broilers. High hematocrit grouped broilers had significantly higher hematocrit, hemoglobin, pCO_2, and HCO_3^- levels and decreased Cl^-, Na^+, anion gap levels compared to the low hematocrit group. Corticosterone levels were not significantly different between low and high hematocrit birds. Adrenocorticotropin hormone treated birds had significantly increased ($P < 0.1$)

hematocrit, hemoglobin, pCO_2, HCO_3^-, and corticosterone levels. An ACTH x hematocrit response interaction was observed for hematocrit and hemoglobin. The results indicate birds are capable of maintaining pH and acid base balance through constant shifts in blood gas and electrolyte levels. High hematocrit birds exhibit a minimal increase in hematocrit and hemoglobin in an attempt to improve oxygen carrying capacity, making it easier to adapt to stress than low hematocrit birds.

The objective for Experiment 2, Chapter IV, was to observe the physiological changes in the blood gases, electrolytes, immune cells counts, EPO, CS, hematocrit and hemoglobin levels and the mechanisms responsible for increasing oxygen carrying capacity in broilers induced with stress via a mini-osmotic pump of ACTH. Results indicated ACTH treatment significantly increased pCO_2, HCO_3^-, K^+, total white blood cell count, monocytes, heterophils and corticosterone. Significant decreases were observed for pO_2, Na^+, Ca^+, Cl^-, anion gap, bone marrow hemoglobin, spleen hemoglobin, lymphocytes, and erythropoietin. During stress birds maintain acid base balance through shifts in blood gas and electrolyte levels and regain oxygen carrying capacity by increased release and maturation of red blood cells from storage sites. However, accelerated red blood cell production appears to not contribute to the rise in oxygen carrying capacity as indicated by the drop in erythropoietin.

Results from Experiments 1 and 2 set the standards for treatments applied and parameters tested in Experiment 3. The purpose of Experiment 3 was to confirm that high hematocrit levels improve a bird's ability to increase oxygen carrying capacity and adapt to stress and evaluate the physiological mechanisms by which this occurs. Treatment with ACTH significantly increased CS, heterophils, basophils, monocytes,

bicarbonate, pCO_2, hemoglobin, and hematocrit levels and decreased ($P<0.1$) bone marrow hemoglobin, lymphocyte, Cl^-, Na^+, and anion gap. This indicates normal birds adapt and maintain homeostasis through enhanced immune responses, constant shifts in electrolytes and blood gases and release of red blood cells from storage sites. Treatment by hematocrit level interactions ($P < 0.1$) occurred for pO_2, hematocrit, hemoglobin, lymphocytes and K^+. Stress induced via ACTH, increased hemoglobin and hematocrit only in birds with low or non-selected hematocrit, suggesting that birds with a high hematocrit prior to stress have an adaptive advantage during stress. Higher hematocrit prior to stress apparently provides ample oxygen carrying capacity during stress and promotes more available oxygen to be utilized by the tissue. High hematocrit birds appear less susceptible to stress effects by maintaining leukocytes at a constant level, while in non-selected birds the percent of lymphocytes drop.

Little research has been conducted to evaluate the immune system at the protein level for control and stressed broilers. Therefore, the purpose of the final experiment was to use proteomics methodology to obtain interpretable and meaningful information on the proteins expressed in normal chicken monocytes which will be used in future comparison experiments for stressed and unstressed broilers. A total of 3229 proteins were identified with 46 of these involved in immune functions of professional APC. Of these proteins, fourteen receptor, four antigen presenting, six antigen uptake, ten cell adhesion, two toll-like receptor, one cytokine and nine protease proteins were identified.

In overall conclusion, results of these studies indicate normal broilers with average hematocrit levels have the ability to maintain acid base balance during stress. Stress limits metabolic O_2 levels, but elevated hematocrit prior to stress and natural

adaptation, such as release of stored red blood cells from the bone marrow or spleen, give broilers the ability to maintain oxygen carrying capacity and physiological homeostasis.