

ABSTRACT

LOWMAN, ZACHARY. Actigen Influence on the Gene Expression of Heat Shock Proteins in Ross 708 Broiler Chickens. (Under the direction of Christopher Ashwell).

Actigen (Alltech, Lexington, KY.), a concentrated mannan oligosaccharide (MOS) product is composed of extracts from the yeast *Saccharomyces cerevisiae* outer cell wall. It has been shown in chickens and other animals to improve gut health and function, promote beneficial bacteria, and decrease potentially pathogenic bacterial loads. It has been suggested that MOS plays some role in the regulation of the heat stress response, possibly via modification of Heat Shock Protein (HSP) production. HSPs are a group of highly conserved proteins that are produced in response to stressors such as high ambient temperatures. The HSPs function as molecular chaperones by aiding the folding of newly synthesized proteins, refolding of misfolded proteins, and degradation of denatured proteins.

The goals of the current study were first, to determine if Actigen feeding influenced gender-related HSP response in broiler chickens exposed acutely to elevated ambient temperature, and second, to determine if the addition of Actigen to broiler diets influenced HSP production. Broiler chicks were divided by gender and fed either a control diet or Actigen supplemented diet. Birds from each gender and diet were subjected to either acute heat stress at 41°C for one hour or held at room temperature. The liver and ileum from each bird were collected for gene expression using Real Time PCR, or flash frozen for protein extractions. Significant differences in gene expression due to gender were found in the ileum for HSP 90A (P=0.0001), 90AA (P=0.0014), and 90 B (P=0.0002). No differences due to sex were found for HSP mRNA expression in the in the liver tissues. There was an increase in expression levels of all genes HSP 90A, HSP 90 AA, HSP 90B, HSP 70, and HSP 60, in

the heat stressed birds in both the liver and ileum. There were only significant changes in expression levels due to diet in the liver for HSP 90AA (P=0.004) and HSP 90 B (P=0.0038) suggesting that Actigen caused an improved thermotolerance.

Actigen Influence on the Gene Expression of Heat Shock Proteins in Ross 708 Broiler
Chickens

by
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Literature Review

Actigen

Actigen (Alltech, Lexington, KY) is a second generation yeast outer cell wall derivative from *Saccharomyces cerevisiae* and provides 2.5 fold more concentrated mannan oligosaccharide (MOS) than its predecessor, Bio-Mos (Hooge, and Connolly, 2001). MOS has been shown to improve gut health in broilers, which was indicated by a significant increase in poult bodyweight associated with increased villi length and area, decreased number of goblet cells (Bradley et al., 1994), increased populations of beneficial bacteria, such as the Lactobacilli and Bifido strains, decreased populations of undesirable bacteria such as the pathogenic Enterobacteria and *Escherichia coli* and *Salmonella* (Baurhoo et al., 2007; Sims et. al., 2004). MOS has been shown to improve body weights in laying hens subjected to chronic high ambient temperatures (Bozkurt et. al. 2011), and it has been shown to help combat some of the oxidative damage that results from heat stress in broiler chicks (Sohail, 2011). The mechanism of this beneficial effect is not yet clear, but it is believed to be associated with increased numbers of gut microbes, which potentially release a bioactive substance that could prevent oxidative damage (Sohail, 2011). The actual pathway by which MOS works is still unknown. A study by Xiao et al. (2012) reported that MOS plays a significant role in many signaling pathways including protein synthesis, carbohydrate metabolism, molecular transport, protein trafficking, RNA trafficking, protein synthesis, RNA post transcriptional modifications, nucleic acid metabolism, energy production, free radical scavenging, and cell death (Xiao et. al. 2012). Some research indicates that MOS binds directly to transmembrane receptors in the small intestine and causes the induction of a response (Cheled-Shoval, 2011),

but other work suggests that MOS interacts with carbohydrate receptors in the intestinal epithelial cells (Seifert and Watzl, 2007).

Physiological responses to heat stress

One of the first responses to heat stress is a sudden increase in corticosteroid levels until a critical body temperature of around 44.5 °C is reached which is then followed by a sudden drop in corticosteroid levels. Immediately before hyperthermic induced death of a chicken, there is very little corticosteroid in the blood plasma (Edens, 1977). Sodium appears to be involved in the emperature regulation of birds. Denbow and Edens (1980) were able to induce hyperthermia in broiler cockerels by injections of sodium. When birds are exposed to ambient temperatures at 45°C and above, sodium begins to be excreted from the kidneys and levels immediately drop in an effort to decrease the body temperature. Potassium levels in the blood increase during heat stress; this is caused by cell death and the leakage of potassium from the dying cells into the blood stream. Increases in potassium have shown to slow heart function, and is thought to be one of the major causes of heat exhaustion (Navarro and Vaquero, 2003). Blood calcium levels decrease as birds are exposed to heat. The calcium is thought to be excreted from the bird, or forced in to soft tissue such as cardiac or skeletal muscle. (Edens, 1978; Edens 1976, Edens 1977). Phosphate levels also drop during heat stress via excretion in urine. Since sodium and phosphate are both excreted during heat stress situations, acid base balances are also disrupted. This disruption in the acid base balance changes the blood pH causing an increase, but with continued exposure to heat, the pH sharply drops due to loss of CO₂ and bicarbonate. The increase in acidity of the blood results in coma and death. One of the most physically noticeable results of heat stress is the

increase in respiration rates. Respiration rates are very important in maintenance of body temperature in birds, but can also be the cause of the bird's loss of regulatory capacity which is crucial for blood buffering systems. CO_2 and HCO_3^- are very important in maintaining blood pH. There is an inverse relationship between pCO_2 and blood pH. When respiration rates increase, CO_2 levels decrease. Since HCO_3^- is dependent on CO_2 , the decrease in CO_2 levels causes an increase in blood pH. This is commonly called respiratory alkalosis and when severe enough, results in death of the bird (Edens, 1977).

Genomics of Heat stress

Horowitz (2004) performed a large scale study in mice studying the effects of heat stress and the genomic responses of mice to heat acclimation, and reportedly that 95 different genes underwent significant changes in expression levels in heat stressed animals. Genes that are involved in maintaining DNA integrity and for free radical scavenging were typically up-regulated. There were several genes that only showed only transitory expression, but were not affected during the acclimation period. The authors suggested that heat stress and heat acclimation resulted in two independent responses. Horowitz et al. (2004) suggested that damaged DNA and damaged proteins were the actual inducers of the acclimation response (Horowitz et al. 2004). In a similar study in broilers, there were 110 differentially expressed genes between chronically heat stressed birds and controls (Li, 2011). The function of the differentially expressed genes were traced to 8 different categories including cell structure and motility, carbohydrate metabolism, nucleoside and nucleic acid metabolism, protein metabolism and modification, signal transduction and transport, lipid metabolism, cell cycle, and unclassified genes (Li et al., 2011).

Heat Shock Proteins

Heat Shock Proteins (HSP) are found in both prokaryotes and eukaryotes and belong to a family of highly conserved proteins within all organisms (Lindquist, 1986). The HSPs were first reported by Ritossa in (1963) as puffs on salivary gland chromosomes from heat distressed *Drosophila*. The actual heat shock proteins, that were associated with the chromosome puffs, were later described by Mitchell (1974). These proteins have been found within every cell type of the body. However, they are predominantly in the cytoplasm and endoplasmic reticulum. They have also been found on cell surfaces of a wide variety of cells including tumor cells, spermatogenic cells, endothelial cells in arteries, smooth muscle cells, and even neural stem cells. These cell surface HSPs are thought to act as virus receptors for various viral diseases (Mayer, 2005; Zhu, 2011; Kim,2012). Both prokaryotic and eukaryotic organisms respond to specific stressors, especially heat distress, similarly showing elevated HSP expression. This response has been classified as the heat shock response and is defined as the rapid expression and synthesis of these of highly conserved polypeptides called Heat Shock Proteins (Lindquist, 1986). HSPs are categorized with respect to their relative molecular weights in kilo Daltons (eg. 70 kDa, 60 kDa, 90 kDa etc). HSPs have been broken down into five different groups based upon their molecular weight and structures: 100-110 kDa, 90 kDa, 70 kDa, 60 kDa, and the small HSPs 15-30 kDa (Givisiez, 2000; Kim, 2011, Mahmoud, 2000). Within HSP families, all members of the group have similar nucleotide sequences (Hightower, 1993). Within each group, there are 2 forms of the protein-an inducible form that is known as the HSP, and a heat shock cognate form (HSC). HSCs are expressed at low levels throughout the organism even when it is not exposed to a stressor,

and they are not induced during heat stress. The HSPs are easily induced and are produced very rapidly during times of stress (Pauli and Tissieres, 1990; Fayet et. al., 1989). The increase in expression of HSPs indicate that the organism is undergoing some type of stressor, with heat stress being the main inducer. However, there are numerous methods that have been shown to be effective in the induction of HSPs, and these range from exposure to Zn, amino acid analogues of proline, arsenic, cadmium, ethanol, hypoxia, and glucocorticoids (Wang, 1998; Fisher, 1986; Li, 1985; Li, 1982; Tomoasovic, 1983).

HSP 70

The HSP 70 family protein is the most studied of the HSPs in poultry. The N-terminal portion of HSP 70 is highly conserved with identical repeating amino acid sequences (Boorstein, 1994, Schlesinger, 1990). The N-terminus is considered the ATPase domain (Flaherty, 1991) whereas, the C-terminus of the protein is considered the peptide-binding domain (Zhu,1996). HSP 70 proteins are found in the nucleus, mitochondria, endoplasmic reticulum (ER) and chloroplast. The HSP 70 group aids in importation of several crucial proteins into the ER and nucleus (Schlesinger, 1990). Within the HSP 70 family there are several different subtypes of HSP 70 proteins; HSP 70,72, and 78 (Barral, 2004; Amici, 1991). HSP 70 is the main product; HSP 72 is known to be the main inducible product (Kim, 2011). Hightower suggested that the HSP 70 group members may be capable of auto-phosphorylation under the correct conditions (Hightower, 1991). In fact, Barral (2004) stated that all HSP 70 functions are said to be controlled by ATP-regulated substrate binding and release. The hydrolysis of ATP to ADP causes the stabilization of the chaperone-substrate complex (Mahmoud, 2000). The N-terminal portion of HSP 70 binds to peptides,

the rate at which HSP 70 binds and releases the peptides is determined by whether ATP or ADP is present (Schmid et al. 1994, Barral, 2004). The HSP 70 proteins appear to be the most responsive to heat stress within an organism (Barral, 2004), and HSP 70 has been shown to be very important to the development of thermotolerance. Cultured cells that were injected with anti-HSP70 antibodies died when faced with a heat stress, whereas cells injected with control HSP 70 antibodies resulted in normal growth (Riabowol, 1998). HSP 70 can also act as a neuro-protectant in animals and is induced in epilepsy, cerebral ischemia, and various types of trauma (Turturici et al., 2011). Genes that control HSP 70 family members in humans have been traced to chromosomes 6, 14, and 21 (Schlesinger, 1990). The gene that encodes HSP 70 production in the chicken is located on chromosome 5 (Morimoto et al., 1986).

HSP 90

The HSP 90 family of proteins has two major functions, cell signaling and de-novo synthesis of certain proteins (Nathan et al., 1997). HSP 90 exists in several forms HSP 90 A, and HSP 90 B, as well as a newly discovered HSP 90 AA, which is an inducible form of HSP 90 A. Meng et al. (1993) reported that HSP 90 A and HSP 90 B genes appeared as a gene duplication that occurred during the emergence of vertebrates. HSP 90 proteins are considered to be cytoplasmic proteins (Mahmoud, 2000), but HSP 90 proteins are also found in the endoplasmic reticulum (ER) of higher eukaryotes (Kang et al., 1994) HSP 90 AA plays an important role in the regulation of certain target proteins as well as interact with cell control and signal transduction (Binart et. al., 1989) The HSP 90 A and HSP 90 AA are located on chromosome 14 and HSP 90 B is located on chromosome 6 in humans (Urban,

2011). HSP 90 AA is found on chromosome 5 in the chicken, and HSP 90 B is found on Chromosome 1 in the chicken. All of the HSP 90 isoforms, as well as many other HSPs, have the same three main regions, which contain an ATP-Binding N-terminal domain, a middle substrate binding domain, and a carboxyl terminal domain. (Urban et al., 2011, Prodromou, 1997, Meyer et al., 2003)

The HSP 90 proteins function downstream to the HSP 60 and HSP 70 proteins. (Miska, 2005; Barral et al., 2004; Lin, 2007) There is an abundance of research describing many different cells in which the HSP 90 proteins act as cellular receptors or are part of the cellular receptor complex (Lin, 2007; Barral et al., 2004; Schlesinger, 1990; Hightower, 1991). HSP 90 interacts with steroid receptor pathways such as aldosterone, androgen, estrogen and progesterone (Hightower, 1991, Borrelli, 1996). HSP 90 is directly involved in the glucocorticoid receptor pathway (Pratt and Toff, 1997). In addition, C-terminal amino acids 604-659 from HSP 90 in mice are required for glucocorticoid receptor production (Dalman, 1991). HSP 90 has been shown to bind directly to estrogen receptors in the hormone binding domain. (Pratt and Toff, 1997). HSP 90 is a crucial component of the hetero-complex and HSP 90 interacts directly with the hormone binding domain (HBD) of the receptors where it facilitates the folding of the HBD of the receptors resulting in a high affinity steroid binding complex (Pratt and Toff, 1997). HSP 90 has been found to be crucial to estrogen, progesterone, androgen, and glucocorticoid receptors binding (Pratt and Toff, 1997). Sullivan and Toft (1993) demonstrated that the C-terminus domain of HSP 90 is crucial for receptor binding (Sullivan and Toft, 1993). The carboxyl terminal is the main site of HSP 90 dimerization (Meyer, 1999). The dimerization site consists of the 191 amino

acids at the C- terminus portion of HSP 90 except for the very last 35 amino acids (Nemoto et. al. 1995). The substrate binding region which connects the C-terminus and N- terminus ends of the HSP 90 is a highly charged region. The length of this region can vary from 34 to 52 acids in length (Scheibel and Buchner, 1997) The substrate binding region is present in all eukaryotic cells but is not present in any of the HSP 90 families produce from *E. coli* (Scheibel and Buchner, 1997). The N- terminus end is composed of 380 amino acids, and has been shown to be less important in binding activity. When the N- terminus portion was removed, there were no significant differences in the receptor binding activity (Sullivan and Toft, 1993).

HSP 60

The HSP 60 proteins are one of the two major subgroups within the chaperonin family. Chaperonins are considered to be a family of related proteins, all around 60 kDa in size. They form double ring complexes and enclose a central cavity of each ring (Barral et al., 2004). The chaperonin family is composed of two groups with both groups having a molecular weight of approximately 60 kDa and have similar structures. However, they can be differentiated based upon their gene sequence. HSP 60 from group II is found in the cytosol of animal cells and the group I HSP 60 is found in mitochondria and chloroplasts of bacterial and plant cells and are located on chromosome 7 in the chicken (Morimoto et al., 1986). HSP 60 is quite unique compared to the other HSPs. Other HSPs try to help proteins avoid improper folding whereas HSP 60 shields proteins during conformational folding (Bukau and Horwich, 1998). HSP 60 forms double ringed complexes that are composed of 14 different proteins and form two separate central cavities within the ring (Barral et al.,

2004, Bukau and Horwich, 1998). HSP 60 functions in combination with HSP 10, which are believed to act as “lids” to cover the HSP 60 cylindrical cavities (Barral et al., 2004). The opening and closing of HSP 10 (the lid), and the entrance and folding of unfolded protein are all controlled by the hydrolysis of ATP (Bukau and Horwich, 1998). Many studies have linked HSP 60 to issues related to cardiovascular disease in humans including coronary heart disease, vascular diseases, hypertension, and atherosclerosis (Wu, 2006; Pockley, 2002). HSP 60 is an immune-dominant molecule, and increased levels of anti-HSP 60 have been closely associated with the diagnosis and progression of vascular diseases (Pockley, 2002). Wu et al. (2006) suggested the possibility of using HSP 60 levels or HSP 60 antibodies as a possible diagnostic method for cardiovascular disease and potentially as a method of treatment.

Heat stress has been shown to increase the concentrations of HSP 60 in the heart of broilers, and this has been explained by considering HSP 60 as a protective mechanism that allows an organism to maintain protein synthesis along with myocardial function (Yan et al., 2009; Yu, 2008).

Molecular Chaperone

One of the main HSP functions is their role as molecular chaperones within the cells. The molecular chaperone is involved in the tertiary folding of the newly synthesized poly peptide chains, to refold mis-folded proteins, and to aid in the degradation of denatured proteins (Hightower, 1991; Barral et al., 2004; Kim, 2011). Hendrick and Hartl (1993), after an extensive review of the literature, constructed the following definition for a molecular chaperone- “a protein that binds to and stabilizes and otherwise unstable conformer of

another protein and by controlled binding and release of the substrate protein, facilitates its correct fate *in vivo*: be it folding, oligometric assembly, transport to a particular sub-cellular compartment, or controlled switching between active/inactive conformation.” There is a considerable amount of research suggesting that HSP 90, HSP 70, and HSP60 do not all function independently of each other, but they actually interact to carry out the various tasks of their molecular chaperone function. In fact, there are several other helper proteins that assist the HSPs’ interaction to facilitate their function role (Hendrick and Hartl, 1993; Morimoto et al., 1994). HSPs acting as chaperones, present damaged proteins to proteases within the cell in order that they may be degraded and recycled (Macario, 1995). Morimoto et al. (1994) reported that HSPs might actually act as proteases and sometimes interact in protease systems. The important role of molecular chaperones is only now being appreciated. Illustrated by observations linking development of neurodegenerative diseases such as Alzheimer’s disease and cystic fibrosis in humans to improper protein folding, which prevents proper functioning of those proteins (Barral et al., 2004; Hightower, 1991). These improperly folded proteins become incapable of interacting with their receptors or substrates leading to the development of various neurodegenerative diseases (Barral et al., 2004).

Issues with HSP

One of the major problems with the production of HSPs is their synthesis is metabolically expensive when considering efficient production systems. When a bird is heat stressed there is a noted decrease in growth, attributed to the decreased rate of synthesis of structural proteins in animals that were chronically heat stressed (Mahmoud and Edens, 2005). Once heat shock protein production begins in the stressed cells, their synthesis becomes the

primary process, even to the exclusion of other proteins normally produced within the cell. The reason for this phenomenon is due to the fact that initiation of HSP production is in response to life threatening stressors exerting pressures upon the cell. Thus, HSP production takes precedence due to necessary protection of the cellular structure and genome. Therefore, the production of other proteins within the cell decrease, which then causes decreased growth and performance of the animal (Williams, 1982; Kelley, 1982).

Heat Shock Proteins and their corresponding antibodies have been linked to many autoimmune diseases, cancers, infectious diseases, cardio vascular diseases, and cerebral damage with both positive and negative effects (Wu and Tanguay, 2006). However, there is still much debate as to the precise role they play in these conditions. Wu and Tanguay (2006) have suggested that the antibodies to HSPs are the causative agents in these conditions. When an organism is under chronic stress there is a constant high production of HSP, and this constant high production of heat shock proteins can cause the production of HSP antibodies, which then can cause the various detrimental conditions within the organism (Wu and Tanguay, 2006). Ivanyi et al. reported a connection between HSP and diseases such as Leprosy, where the HSPs actually become antigen targets of the immune system, which then worsens the infection. The HSP alters the antigenic sites on the cells surface. The HSP then forms complexes with other proteins which makes it antigenically different. The HSP antibody is then produced by the infectious agent (Ivanyi et al. 1996)

Viral infection

Heat Shock proteins have also been found to play an important role in viral infection in both mouse neuroblastoma cells and in human cell lines (Das et al., 2009). HSP 70 has been

thought to be the putative receptor that allows the Japanese encephalitis Virus (JEV) entry into mouse neuroblastoma cells (Zhu et al, 2012). Zhu et. al (2012) found that binding of anti-HSP 70 to HSP 70 receptors could be prevented by incubating the cells in JEV, suggesting that the presence of HSP 70 is required in order for human cell lines to become infected with JEV. HSP 70 was shown to interact directly with the E protein on JEV, causing it to form a homodimer, which may play a role in virus entry into the cell (Zhu et al., 2012). Other HSPs also play important roles in viral infections. HSP 90 was found to be an important component of the infection process of Infectious Bursal Disease Virus in chicks. HSP 90 is not the actual receptor on the cell surface, but it is present on the cell surface and has been determined to be associated with the receptor component in the proposed receptor mediated endocytosis pathway of entry of IBDV in broiler chicks (Lin et al., 2007).

Heat Stress

Heat stress in today's commercial poultry industry is a problem associated with decreased egg production, shell quality, body weight gain, feed conversion and overall performance of the birds (Soleimani, 2011, Rozenbiom et al., 2007, Gonzalez-Esquerria and Leeson, 2005, Quinteiro-Filho et al., 2010). When birds are subjected to chronic levels of heat stress, they undergo cardiovascular collapse which results in death (Edens and Siegel, 1975). Mahmoud (2000) states that some lines of chickens have an inherent ability to regulate body temperature during heat stress. Mahmoud attributed this to the fact that the two lines of birds that were being used had differences in surface area to body weight ratio, and this was the key to their ability to lose heat at a greater rate. Mahmoud also noted that females from both lines had significantly higher expression levels of HSP 70 than did the males after heat shock

(Mahmoud, 2000). Genetic selection and domestication have resulted in commercial birds that are more susceptible to stress when compared to wild jungle fowl, which appear to be more resistant (Soleimani et al., 2011). Heat stressed commercial broilers showed a significant increase in HSP 70 production, which signaled a significant heat stress response, but when Red Jungle Fowl were faced with the same challenge, they showed no increase in HSP 70 production. This suggested that the Red Jungle Fowl is more tolerant to heat stress as compared to broilers (Soleimani et al., 2011). However, Red Jungle Fowl have higher constitutive HSP 70 levels as compared to typical broilers, and has been attributed to the fact that they are inherently more prepared to respond to unexpected change in their environmental temperature whereas commercial broilers have an unselected trait for increased susceptibility for heat stress due to a more controlled environment (Soleimani et al., 2011). There have been studies that show that even a 5°C increase in temperature can induce heat stress broilers and can decrease both body weight gain and food intake, and ultimately feed efficiency (Quinteiro-Filho et al., 2010). The impact of heat stress varies greatly depending on the duration of the stress and upon the severity of the stressor. There is a negative correlation with both the severity and duration of the heat stress on the productivity of broilers. Chickens have been shown to adapt to heat stress after chronic exposure, but they do not acclimate such that they perform equally with non-heat-stressed birds, but they reach a point that prevents further loss in performance (Gonzalez- Esquerra et al., 2005, Wang and Edens, 1998). There are studies showing that repeated daily exposure to acute heat stress improved the HSP response, and that improved HSP responses could possibly be involved in acquiring thermotolerance (Wang and Edens, 1994).

Thermotolerance

There has been much research aimed at developing methods to make poultry more thermotolerant. Since poultry are grown on such a large scale, even increasing feed conversion slightly could have a major impact on the profitability of a flock. If an effective method of thermotolerance is found, it could have a tremendous impact on commercial poultry production. There are commercial operations in North Carolina where a one point improvement in feed conversion ratio would yield over a million dollars monthly in increased revenue (Mahmoud and Edens, 2003). There have been many different methods developed to induce thermotolerance including the use of electrolytes (Ferket, 1992) heat acclimation (Davis et al., 1991) and changing feed and specific nutrient intake (McCormick et al., 1979). Most of these methods to induce thermotolerance have been based upon feed conversion and body weight. However, Wang and Edens (1998) determined the heat responses of the bird at the molecular level by involving HSPs in development of thermotolerance. The level of thermotolerance that an organism develops is related to HSP production, more specifically HSP70, and Wang and Edens (1998) determined that heat conditioned broilers and turkeys increased HSP production initially. However, after continued heat conditioning the HSP production plateaued indicating some thermotolerance had developed, which signaled a decreased need for the HSP 70 protection (Wang and Edens, 1998). The addition of organic selenium to broiler diets was shown to effectively improve thermotolerance in broilers. Broilers fed organic selenium-supplemented diets produced less HSP 70 when heat-shocked compared to broilers that had not been supplemented (Mahmoud and Edens, 2003 and 2005). Wang and Edens (2008) found that steroid hormones,

specifically testosterone, play an important role in controlling the amounts of HSP 70 produced in broiler males. Male broilers were selected based upon comb size, large combs (LC) and small combs (SC). After severe acute heat exposure, the mortality rates were much higher on the SC birds compared to the LC birds. This indicated that the LC males were more resistant to heat stress. It was found that the LC males have higher plasma testosterone and corticosterone levels. In a second supporting experiment, caponized males, normal males, and testosterone-supplemented caponized males were exposed acutely to high temperatures. The males with the highest amounts of testosterone produced the largest amount of HSP 70 and survived. This is believed to be due to the protection that the HSP 70 elicits to the cells during times of heat stress by their molecular chaperone function of prevention, elimination, and degradation of damaged proteins during times of heat stress (Wang and Edens, 2008).

Based upon a review of the literature, it is evident that Actigen plays some role in thermotolerance of organisms, but the actual mechanism by which this occurs is still unknown. This study focusing at the expression levels of HSP production under a mild heat stress is very important in understanding the role that Actigen plays in the gastrointestinal tract. There is still much research needed to better understand the effects Actigen has upon the gene expression of HSPs, as well as the role it plays in making chickens more thermotolerant.

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Materials and Methods

The experimental procedure used in this investigation was approved by the North Carolina State University Animal Care and Use Committee. One hundred sixty day old Ross 708 broiler chicks were obtained from a commercial hatchery (Mountaire Hatchery, Siler City, NC). The day old chicks were sorted by gender by feather sexing. The chicks were all weighed neck banded and placed into separate into pens by treatment group. There were 8 pens with 10 chicks per pen in each of the two identical environmentally controlled rooms. There were two replicates in each room consisting of two male control diet pens, two female control diet pens, two male Actigen diet pens, and two female Actigen diet pens. This provided 80 females and 80 males for this investigation for a total of 160 chicks to adjust for mortality, although only 144 were used for sampling. Each treatment pen was assigned randomly within the two rooms.

Brooding

The chicks were reared in two environmentally controlled rooms at the Dearstyne Avian Research Unit at North Carolina State University. Each room contained 8 (4'X3') pens with a 250 watt heat lamp shared between 2 pens and had approximately 5 inches of pine wood shavings as bedding. The rooms were kept at a constant 30°C for the first week, reduced to 28°C for the second week, reduced to 26°C for the third week, and reduced to 24°C for the remainder of the trial. The chicks were kept on 24 hours of incandescent light for the first 3 weeks and then were placed on 23 hours of light for the last 3 weeks of grow out. The feed and water were checked twice daily, feed was dispensed from hanging tube feeders and water

was provided in plastic waterers. The waterers were washed once daily to prevent litter build up. Both waterers and feeders were placed on concrete stands to prevent litter contamination.

Diets

The birds were fed 3 different diets. A Starter, Grower, and Finisher the starter was 3035 kcal/kg, and 22.855% CP, the grower was 3100 kcal/kg, and 20 %CP, the finisher was 3150 kcal/kg, and 18 %CP. All feeds were produced by the North Carolina Agriculture Research Service Feed Mill. To the experimental groups, 800g/ton of Actigen (Alltech Inc. Nicholasville, KY) was added to the starter, 400g/ton of Actigen was added to the grower, and 200g/ton of Actigen was added to the finisher. The birds were all given 2 pounds of starter per bird, 6 pounds of grower per bird, and 8 pounds of finisher per bird. The pens of birds were weighed weekly for six weeks. Mortalities were weighed and subtracted from the pen weights.

Sampling

At 3 and 6 weeks, 12 birds were taken randomly. Birds from each treatment were caught, and of those 12 birds, 6 were taken randomly to be heat-exposed, and the other 6 were sampled as controls. The heat-exposed birds were placed into a coop without feed or water then placed in the heat chamber that had a temperature of 41°C for a period of one hour. After the respective treatment, the birds were bled from the ulnar vein in the wing. The blood was collected in heparinized aqua cap tubes (Drummond Scientific Company, Broomall, PA) A 100 µL volume was then applied to an ISTAT EC8+ cartridge (Abbot Laboratories, Abbot Park, IL) for measurement of glucose, Na, K, Cl, pH, pCO₂, BUN, TCO₂, HCO₃, hematocrit, hemoglobin, base excess, and anion gap. Then, the birds were

ethanized by cervical dislocation. The breast was dissected and weighed, the liver and ileum were removed, approximately 10g samples were saved in RNAlater (Life Technologies, Grand Island, NY) for mRNA extraction, and 10g more were flash frozen in liquid nitrogen to be used for HSP quantification.

RNA extraction

RNA was extracted using the RNeasy mini kit (Qiagen, MA). Tissue 0.1g was placed into 350 μ l of RLT buffer (supplied from Qiagen), the sample was homogenized using a minibead beater (Biospec, Bartlesville, OK). The sample was then centrifuged at 16,000g for 3 minutes, the supernatant was removed and placed in to a tube containing 350 μ l of 70% ethanol, and was mixed by gently pipetting up and down. The sample was then placed over the RNA selective spin column (supplied with kit), and centrifuged for 15 seconds at 8,000g. The supernatant was discarded and 700 μ l of RW1 buffer (supplied from Qiagen) was added. The sample was centrifuged for 15 seconds at 8000g. The supernatant was discarded and 500 μ l of RPE buffer (supplied from Qiagen) was added. The sample was centrifuged at 8,000g for 15 seconds. The supernatant was discarded and the previous step was repeated, except that it was centrifuged for 8,000g for one minute. The collection tube was discarded and the spin column was placed into a new collection column. The sample was centrifuged for 1 minute at 16,000g to dry the column. The collection tube was discarded and the spin column was then placed into a clean pre-labeled 1.5ml tube. 50 μ l of nuclease-free water was added to the column to elute the RNA from the column. The sample was spun at 8000g for 1 minute, and immediately placed on ice. The sample was then nano-dropped (Nano-Drop 2000 Spectrophotometer, Thermo-Scientific) at 460 and 480 wavelengths to determine the

concentration and purity. Once all RNA samples had been extracted, they were diluted to concentrations around 500 μ L. Then 1 μ L of the RNA was placed into a 96-well plate, along with loading dye and Nuclease Free water. The plate was then run on a denaturing cycle on the 96 well Thermocycler (Applied Biosystems, Carlsbad, CA). The samples were then run on a 1% ethidium bromide gel. The gel was then visualized under the UV light box to make sure the RNA was not degraded. Once it had been determined there was no degradation of the RNA, cDNA was made.

cDNA

cDNA was made by using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Foster city, CA). RNA (1 μ g) from each sample was placed in a 96 well plate, and Nuclease Free water was added to make a final volume of 10 μ L. A master Mix was made and consisted of 2 μ L of 10x RT buffer, 0.8 μ l of 25XdNTP mix, 2 μ L 10 RT Random Primers, 1 μ L Multiscribe Reverse Transcriptase, and 4.2 μ L Nuclease Free water. 10 μ L of the Master Mix was added to all of the samples. The plate was mixed by gently vortexing, and briefly centrifuging. The plate was then placed in a Thermocycler and run with the manufacturers recommended program.

Real Time PCR

The cDNA was pooled in two pools of 3 samples per pool. The first pool consisted of samples 1,2,3, and the second pool consisted of samples 4,5,6, from each treatment. All cDNA was diluted to a 1:20 ratio. 5 primer sets were designed (Table 1).

Table 1. Primers used for Real Time PCR and Gene expression	
Primer	Sequence
HSP70 Forward	GCGGAGCGAGTGGCTGACTG
HSP70 Reverse	CGGTTCCCCTGGTCGTTGGC
HSP90A Forward	CCTCCTCCATACGTGATGTGTCA
HSP90A Reverse	GCCTGGGCATTGATGAAGATG
HSP90B Forward	GGCTCTGGCATGCACGCTTC
HSP90B Reverse	TCTTCCACGGTCGCATCCACA
HSP90AA Forward	CCCCAGACACATGCCAACCGC
HSP90AA Reverse	AGGACTGGCCTCCTCAGCAGC
HSP60 Forward	GCAGATGCCGTAGCTGTCACCA
HSP60 Reverse	TGGGACTCCCCCAGCTTTGTT

All Real Time Reactions consisted of 1 μ L of cDNA, 1 μ L of primer, 10 μ L SYBER green, and 8 μ L nuclease free water. The Thermocycler parameters consisted of: 94°C for 7 minutes; 50 cycles at 95°C for 30 seconds, appropriate melting temperature (T_m) for 30 seconds, 72°C for 30 seconds; 72°C for 5 minutes; 95°C for 1 minute; 55°C for 1 minute, 80 cycles at 55°C for 30 minutes.

HSP evaluations

The flash frozen liver and intestine samples were pulverized, and 1 gram samples were homogenized in 4mL of protein buffer solution (0.05M Tris-HCL, pH=7.5, 0.15 M NaCl, 2mM DTT, 5mM EDTA, and 0.2% Tween-20). The samples are then centrifuged for 15 minutes at 10,000g. The supernatant was removed and centrifuged at 25,000g for 1 hour followed by transfer of the supernatant into a new tube. The total protein was then measured by using the Protein Assay Kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions, In brief, the sample was diluted 20x with distilled water and 10 μ L of the diluted sample was placed in a 96 well immunoassay plates. The working reagent (supplied with kit; 200 μ L) was placed into each well. The plate was shaken for 30 seconds on a plate shaker, then covered in parafilm and incubated for 30 minutes at 37°C. The plate was then placed in a micro plate reader and absorbencies read at 562 nm. Enzyme linked immunosorbent assay (ELISA) for liver and Ileum tissues were performed following the protocol described by Mahmoud and Edens (2003).

Statistics

Expression values were analyzed for treatment effects by ANOVA including Age, Diet, Heat exposure, and Sex and their interactions in the model (JMP 10, SAS, Cary, NC). Individual contrasts were made using a comparison of means using Tukey-Kramer (JMP 10). Significance levels were set at $P < 0.05$.

Results

There was a significant increase ($P < .0001$) in the expression of HSP 90 B in the Ileum of birds that were heated when compared to un-heated birds (Figure 1). The sex of the bird also played a significant role in the expression of HSP 90 B in the Ileum. Male birds had a significantly higher levels of expression of HSP 90 B ($P = .0002$) than the female broilers did (Figure 2). There were no significant differences in expression levels due to diet or age.

There was a significant increase ($P = .001$) in the expression of HSP 90 AA in the ileum of birds due to age with the 3 week birds having a higher level of expression (Figure 3). There was also a significant increase ($P < .0001$) in the expression of HSP 90 AA in the Ileum of birds that were heated when compared to non-heated birds (Figure 4). The sex of the bird also played a significant role in the expression of HSP 90 AA in the Ileum. Male birds had a significantly higher levels of expression of HSP 90 AA ($P = .0014$) than the female broilers did (Figure 5). There were no significant differences in expression levels due to diet.

There was a significant increase ($P = .0259$) in the expression levels of HSP 60 in the Ileum of the birds due to age with the 3 week birds having higher levels of expression (Figure 6). There was also a significant increase ($P < .0001$) in expression of HSP 60 in the Ileums of heated birds when compared to non-heated birds (Figure 7). There were no significant differences attributed to diet or sex.

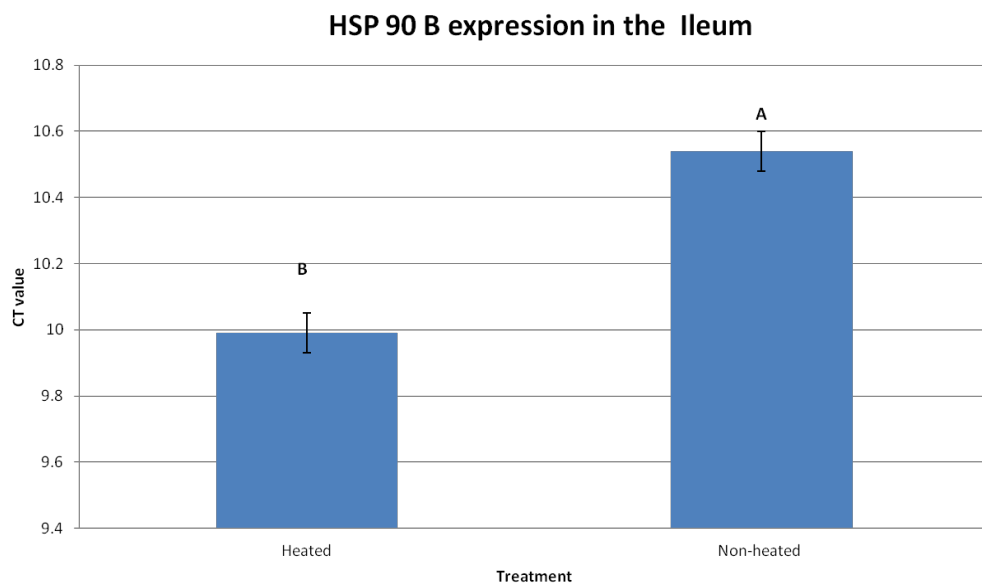


Figure 1. Mean CT values for gene expression of HSP 90 B in the Ileum of 3 and 6 week broilers based upon heat treatment. ^{A,B} indicates significant difference, $P \leq 0.05$

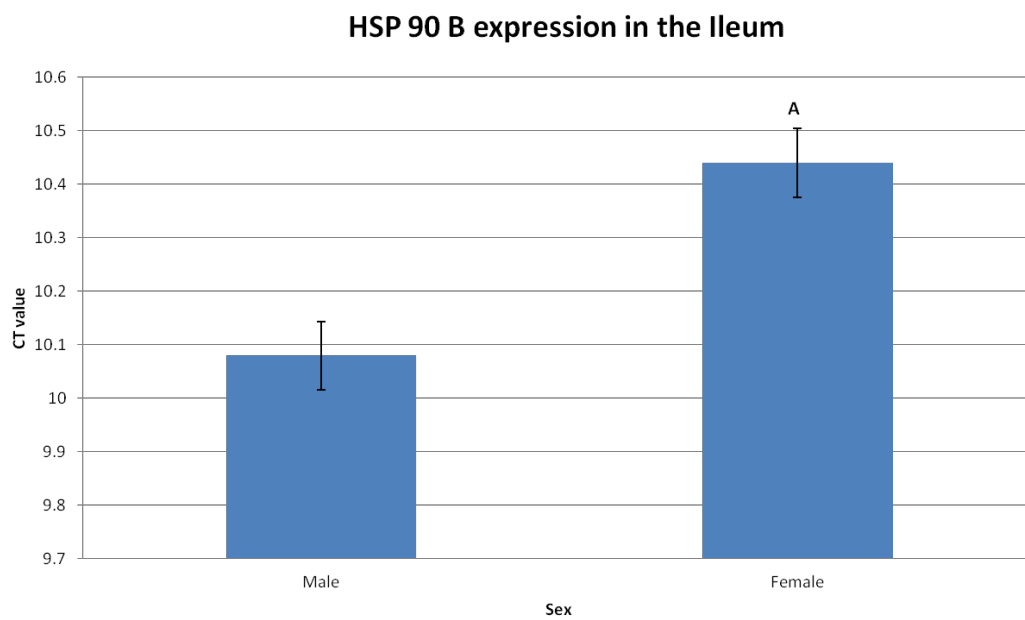


Figure 2. Mean CT values for gene expression of HSP 90 B in the Ileum of 3 and 6 week broilers based upon sex. ^{A,B} indicates significant difference, $P \leq 0.05$

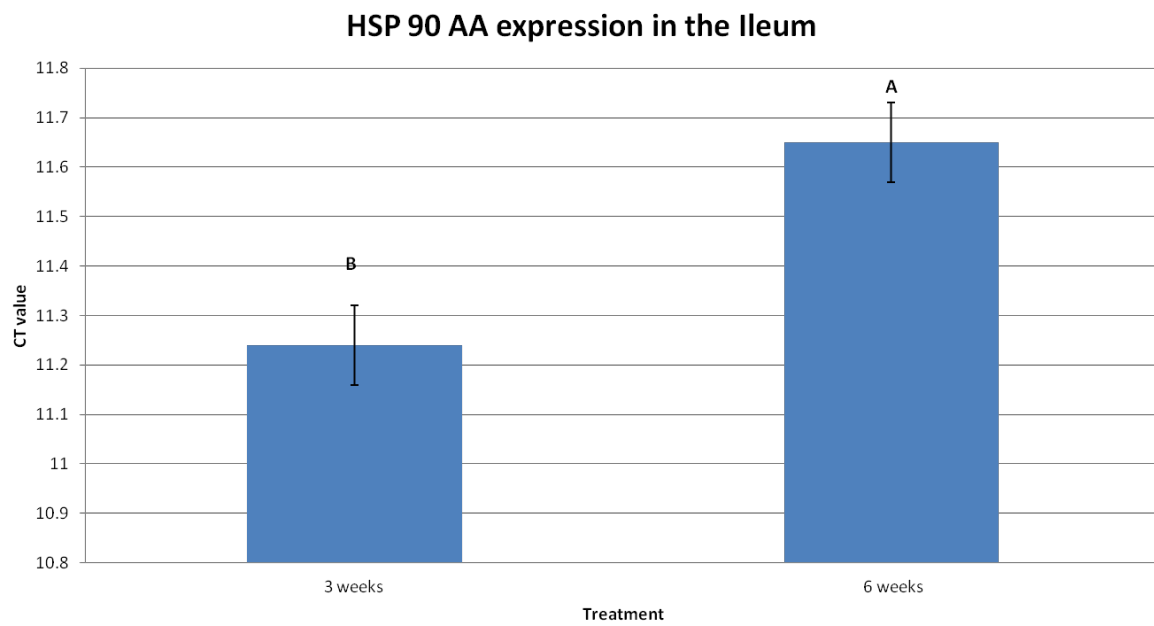


Figure 3. Mean CT values for gene expression of HSP 90 AA in the Ileum of 3 and 6 week broilers. ^{A,B} indicates significant difference, $P \leq 0.05$

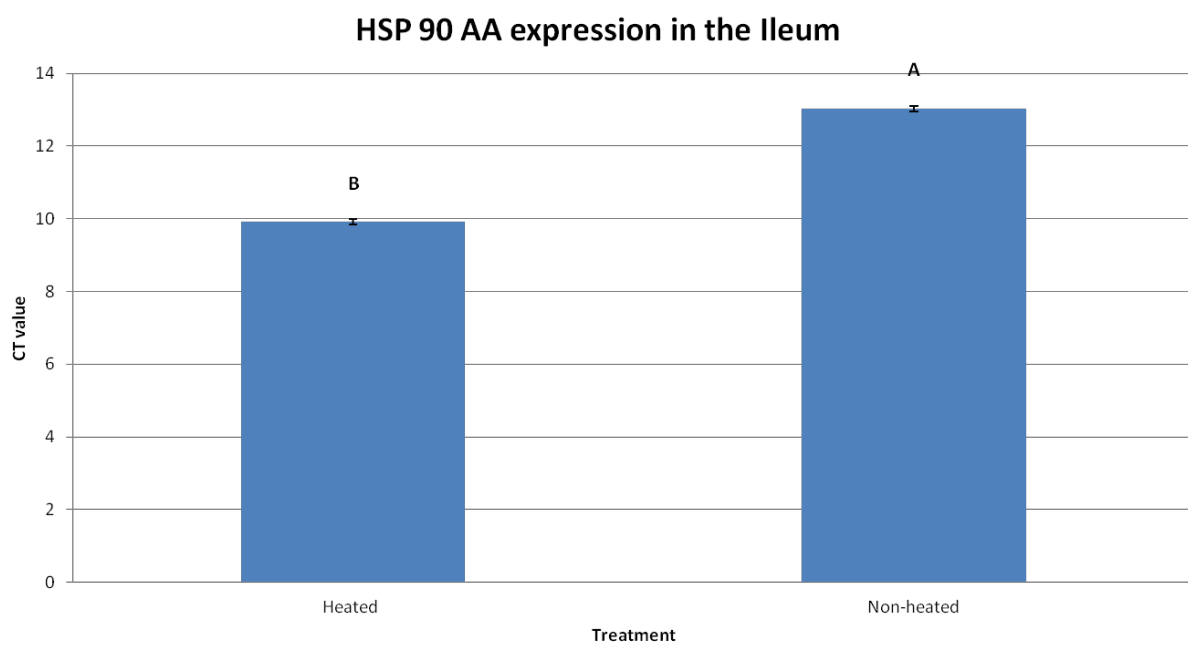


Figure 4. Mean CT values for gene expression of HSP 90 AA in the Ileum of 3 and 6 week broilers based upon heat treatment. ^{A,B} indicates significant difference, $P \leq 0.05$

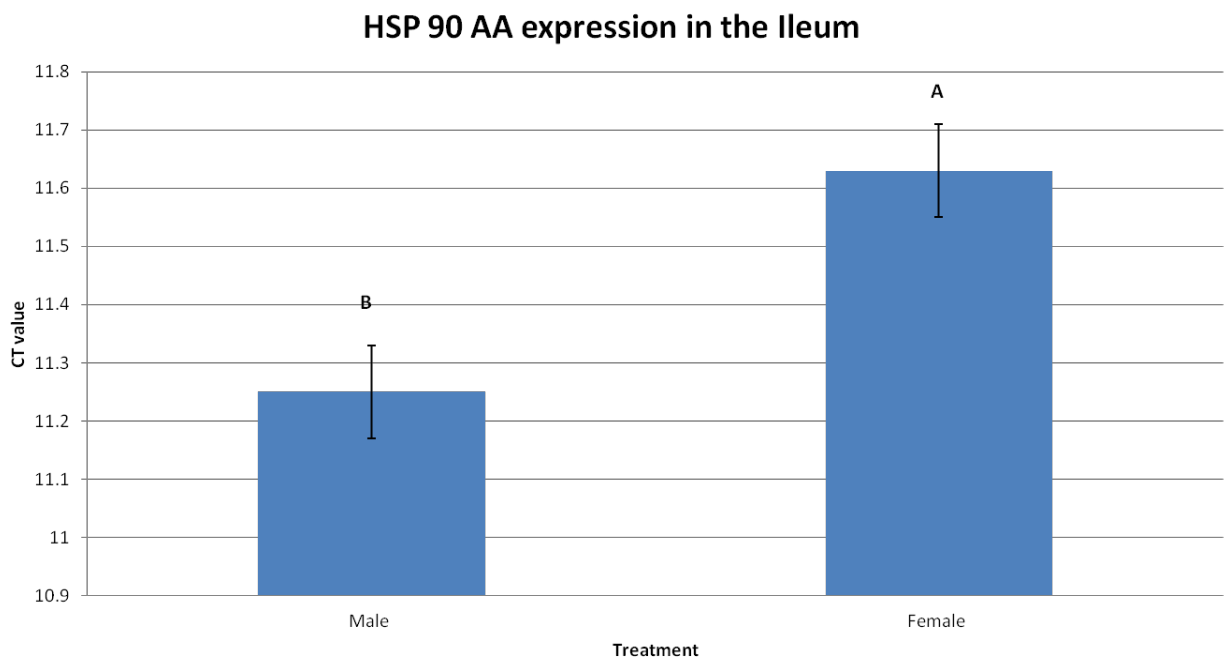


Figure 5. Mean CT values for gene expression of HSP 90 AA in the Ileum of 3 and 6 week broilers based upon sex. ^{A,B} indicates significant difference, $P \leq 0.05$

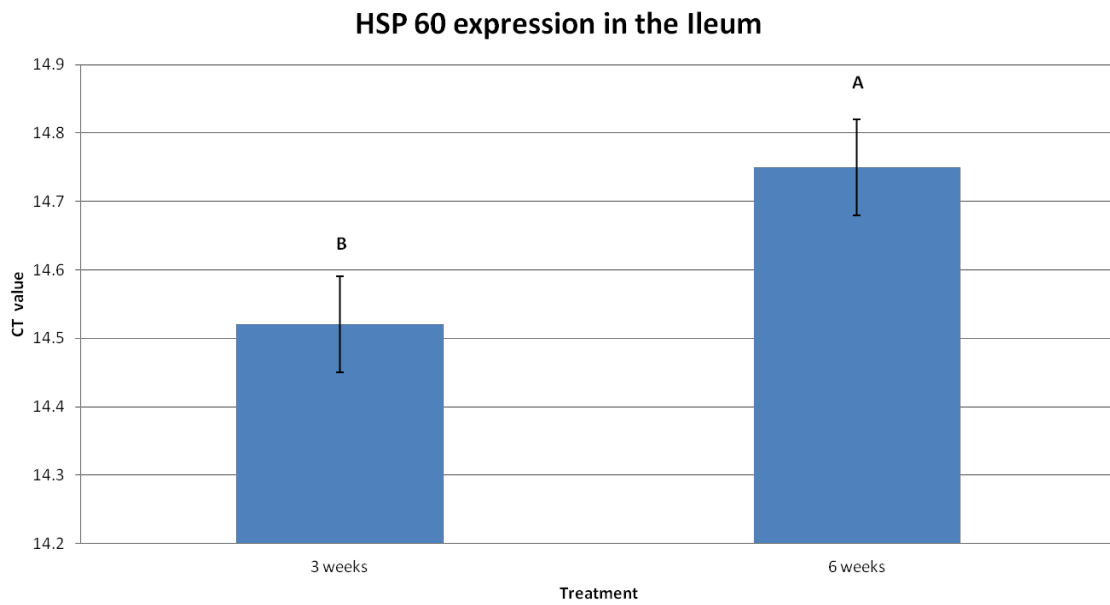


Figure 6. Mean CT values for gene expression of HSP 60 in the Ileum of 3 and 6 week broiler. ^{A,B} indicates significant difference, $P \leq 0.05$

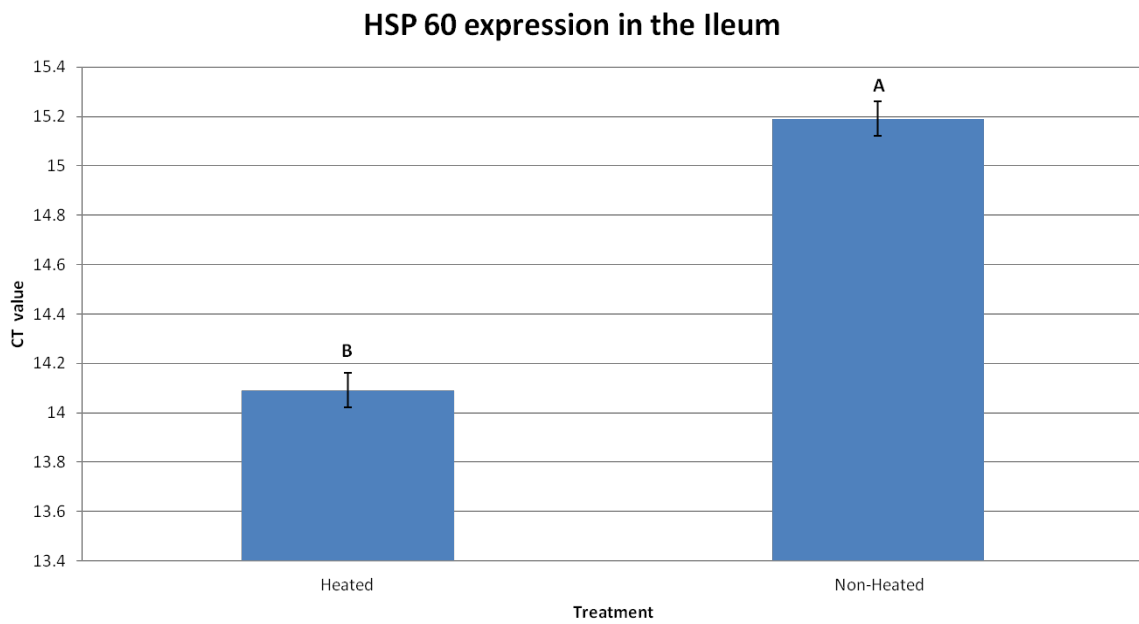


Figure 7. Mean CT values for gene expression of HSP 60 in the Ileum of 3 and 6 week broilers based upon heat treatment. ^{A,B} indicates significant difference, $P \leq 0.05$

There was a significant increase ($P=.0097$) in the expression levels of HSP 70 in the Ileum of the birds due to age with the 3 week birds having higher levels of expression (Figure 8). There was also a significant increase ($P=<.0001$) in expression of HSP 70 in the Ileums of heated birds when compared to non-heated birds (Figure 9). There were no significant differences attributed to diet or sex.

There was a significant increase ($P=<.0001$) in the expression of HSP 90 A in the Ileum of birds that were heated when compared to non-heated birds (Figure 10). The sex of the bird also played a significant role in the expression of HSP 90 A in the Ileum. Male birds had a significantly higher levels of expression of HSP 90 A ($P=<.0001$) than the female broilers did (Figure 11). There were no significant differences in expression levels due to diet.

There was a significant increase ($P=.004$) in the expression of HSP 90 AA in the liver of birds due to diet. Birds fed the Actigen diet had lower expression rates when compared to birds fed control diets (Figure 12). There were significant differences ($P=.0119$) in the expression of HSP 90 AA in the liver due to age, with the younger birds having the higher expression levels (Figure 13). Heating also caused a significant increase ($P<.0001$) in expression of HSP 90 AA in the livers (Figure 14). There were no significant differences attributed to a sex effect for HSP 90 AA.

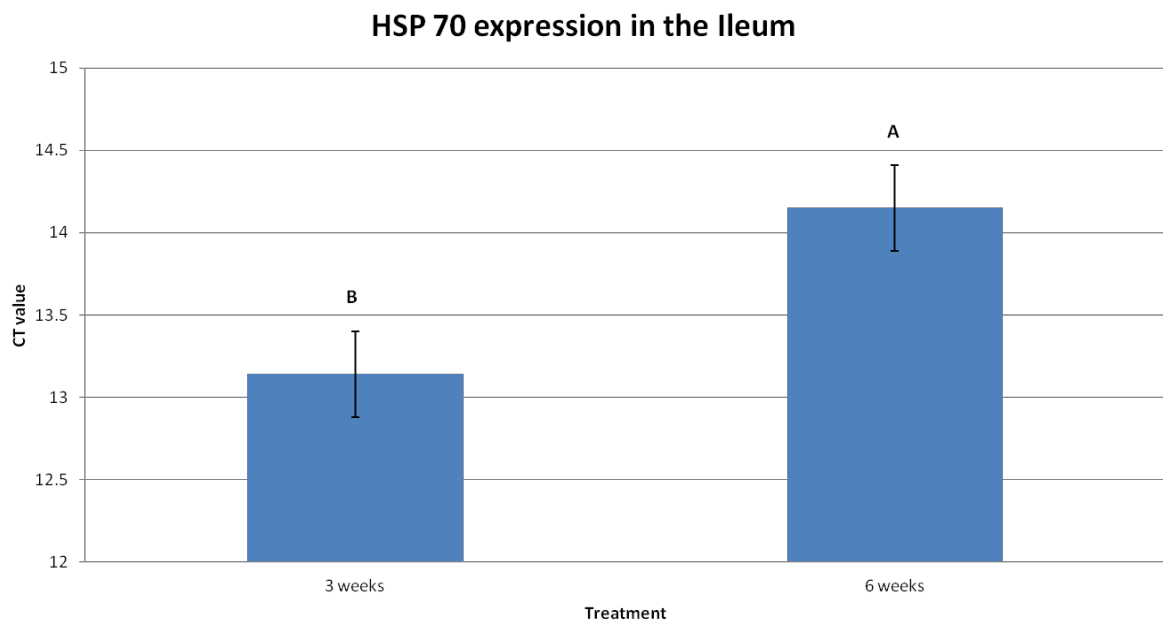


Figure 8. Mean CT values for gene expression of HSP 70 in the Ileum of 3 and 6 week broilers. ^{A,B} indicates significant difference, $P \leq 0.05$

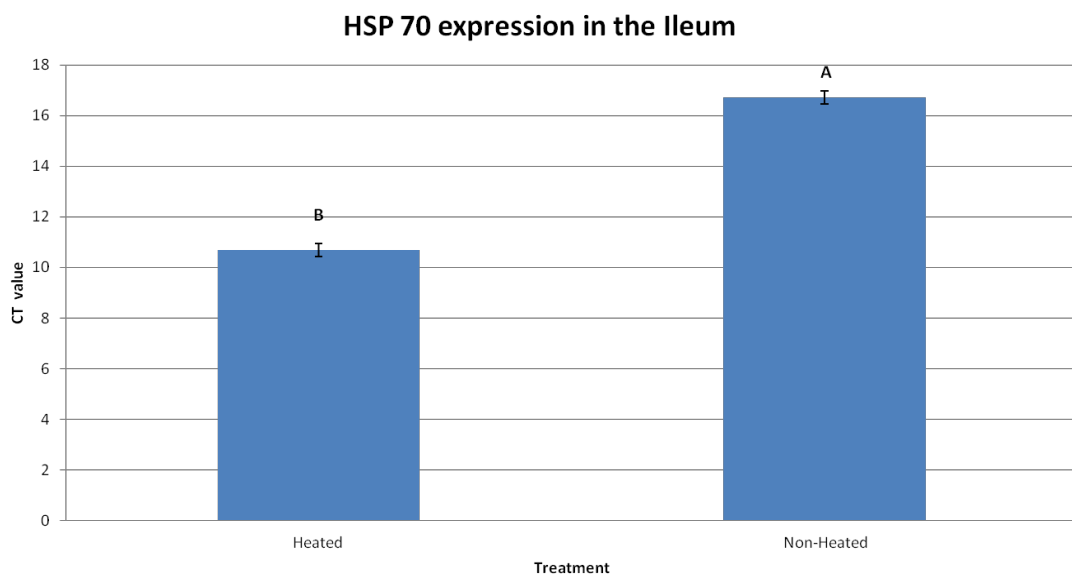


Figure 9. Mean CT values for gene expression of HSP 70 in the Ileum of 3 and 6 week broilers based upon heat treatment. ^{A,B} indicates significant difference, $P \leq 0.05$

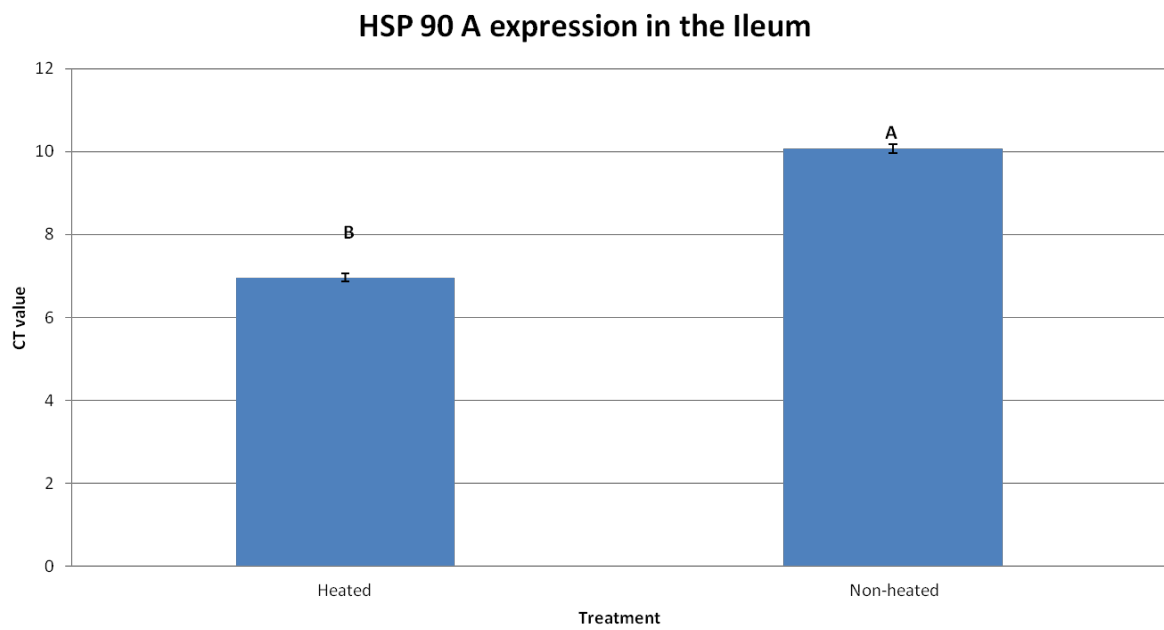


Figure 10. Mean CT values for gene expression of HSP 90 A in the Ileum of 3 and 6 week broilers based upon heat treatment. ^{A,B} indicates significant difference, $P \leq 0.05$



Figure 11. Mean CT values for gene expression of HSP 90 A in the Ileum of 3 and 6 week broilers based upon sex. ^{A,B} indicates significant difference, $P \leq 0.05$



Figure 12. Mean CT values for gene expression of HSP 90 AA in the liver of 3 and 6 week broilers based upon diet. ^{A,B} indicates significant difference, $P \leq 0.05$

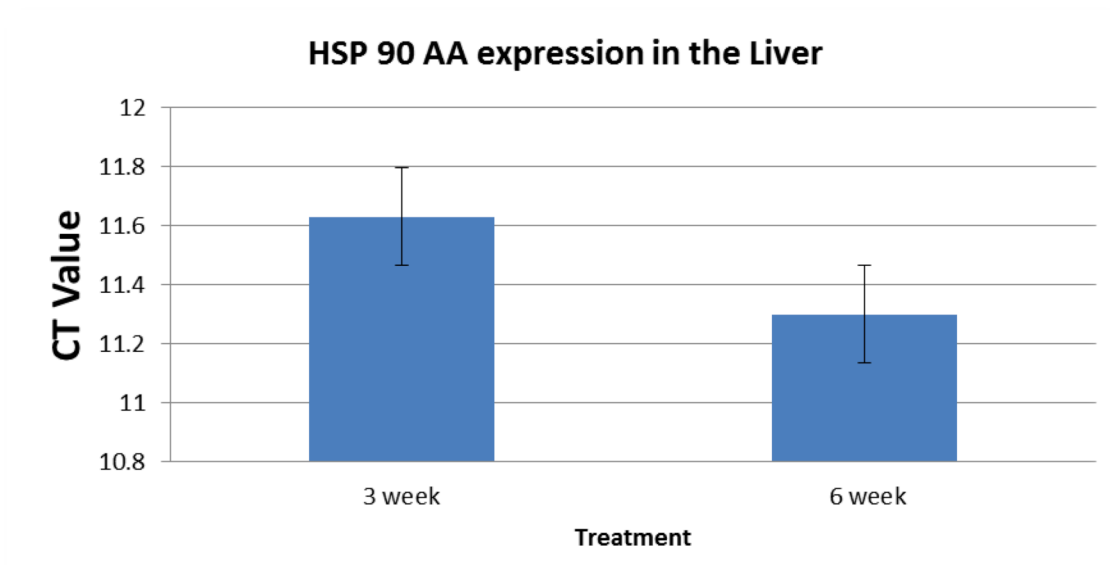


Figure 13. Mean CT values for gene expression of HSP 90 AA in the liver of 3 and 6 week broilers based upon age. ^{A,B} indicates significant difference, $P \leq 0.05$

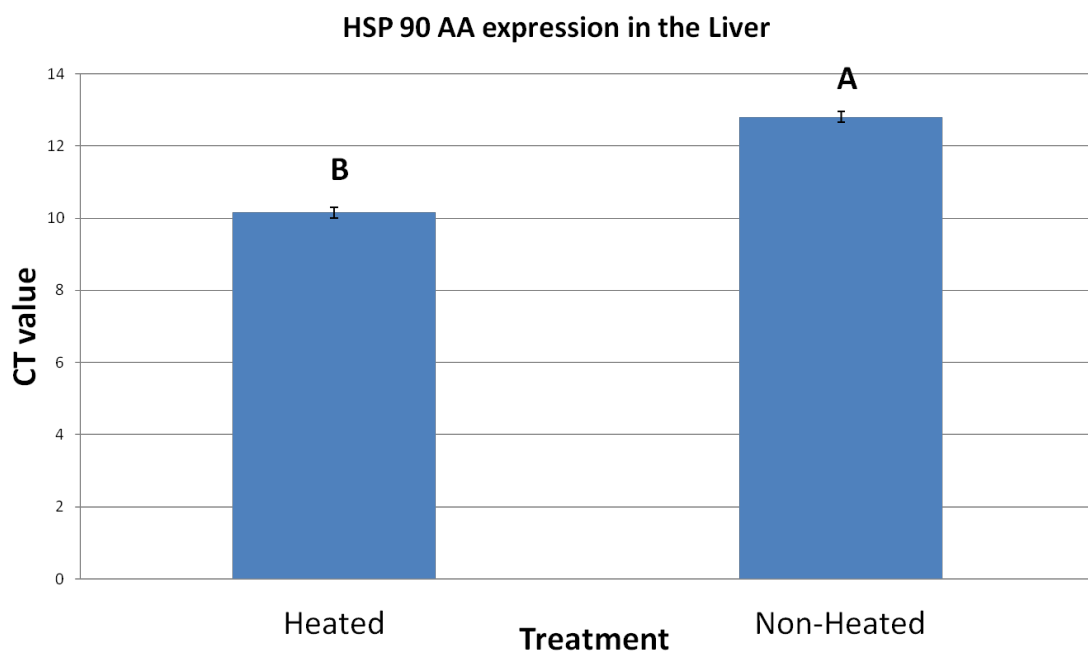


Figure 14. Mean CT values for gene expression of HSP 90 AA in the liver of 3 and 6 week broilers based upon heat treatment. ^{A,B} indicates significant difference, $P \leq 0.05$

There was a significant increase ($P=.0038$) in the expression of HSP 90 B in the liver of birds due to diet. Birds fed the Actigen diet had lower expression rates when compared to birds fed control diets (Figure 15). There was a significant increase ($P=<.0001$) in the expression of HSP 90 B in the liver of birds due to age with the 6 week birds having a higher level of expression (Figure 16). There were no significant differences attributed to heating or sex.

There was a significant increase ($P=.0002$) in expression of HSP 60 in the livers of heated birds when compared to non-heated birds (Figure 17). There were no significant differences observed due to age, diet, or sex effects.

There was a significant increase ($P=<.0001$) in expression of HSP 70 in the livers of heated birds when compared to non-heated birds (Figure 18). There were no significant differences observed due to age, diet, or sex effects.

There was also a significant increase ($P=<.0001$) in expression of HSP 90 A in the livers of heated birds when compared to non-heated birds (Figure 19). There were no significant differences observed due to age, sex, or diet.

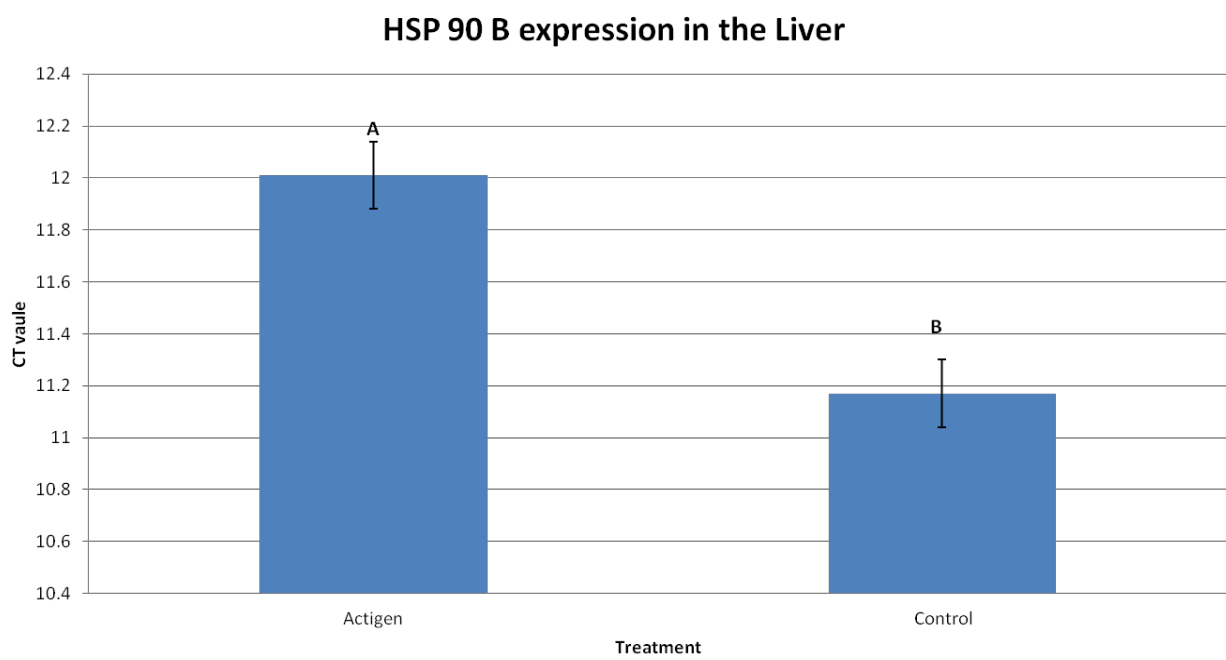


Figure 15. Mean CT values for gene expression of HSP 90 B in the liver of 3 and 6 week broilers based upon diet. ^{A,B} indicates significant difference, $P \leq 0.05$

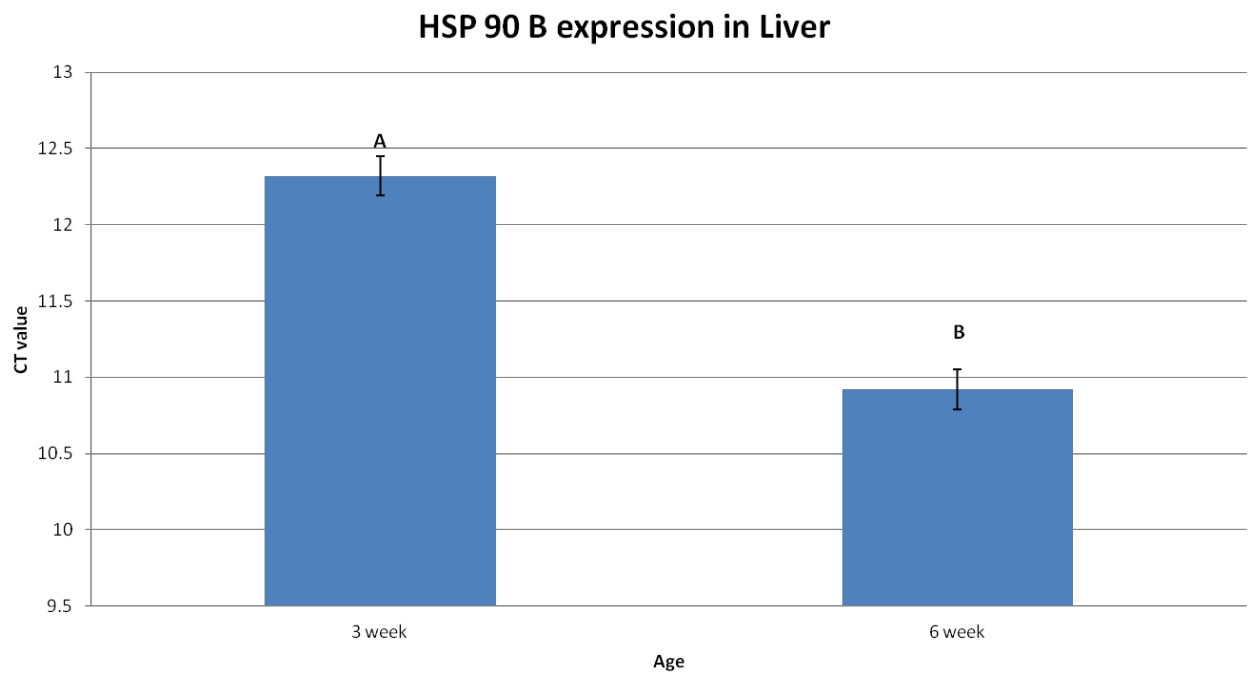


Figure 16. Mean CT values for gene expression of HSP 90 B in the liver of 3 and 6 week broilers. ^{A,B} indicates significant difference, $P \leq 0.05$

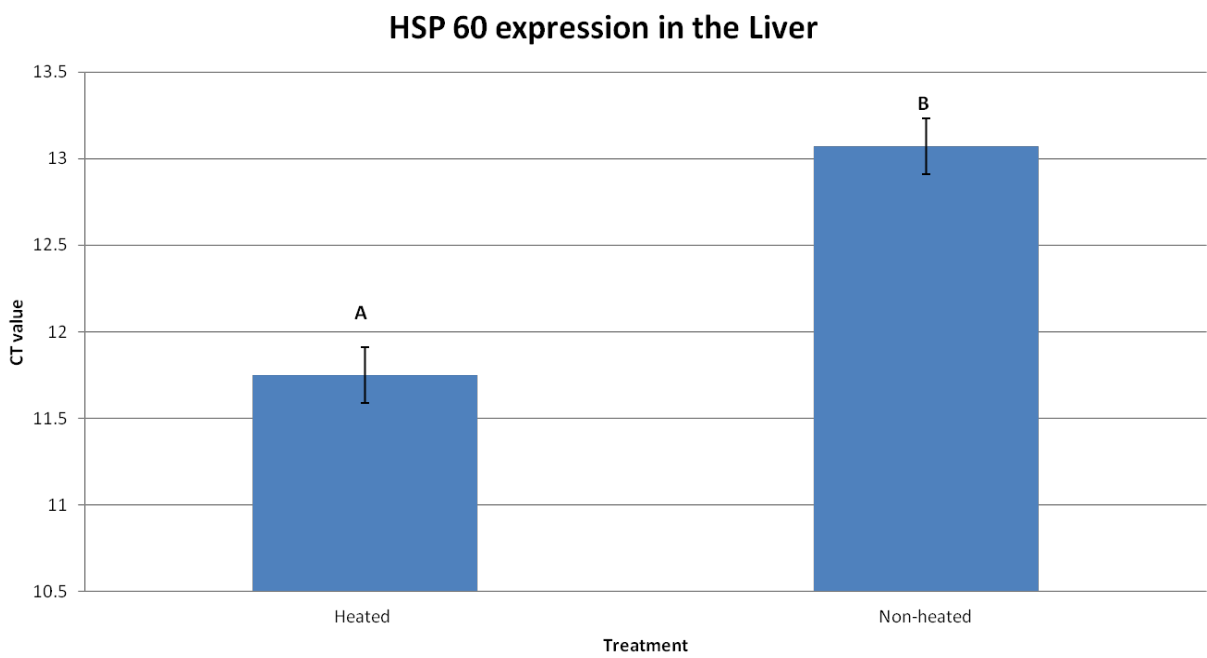


Figure 17. Mean CT values for gene expression of HSP 60 in the Ileum of 3 and 6 week broilers based upon heat treatment. ^{A,B} indicates significant difference, $P \leq 0.05$

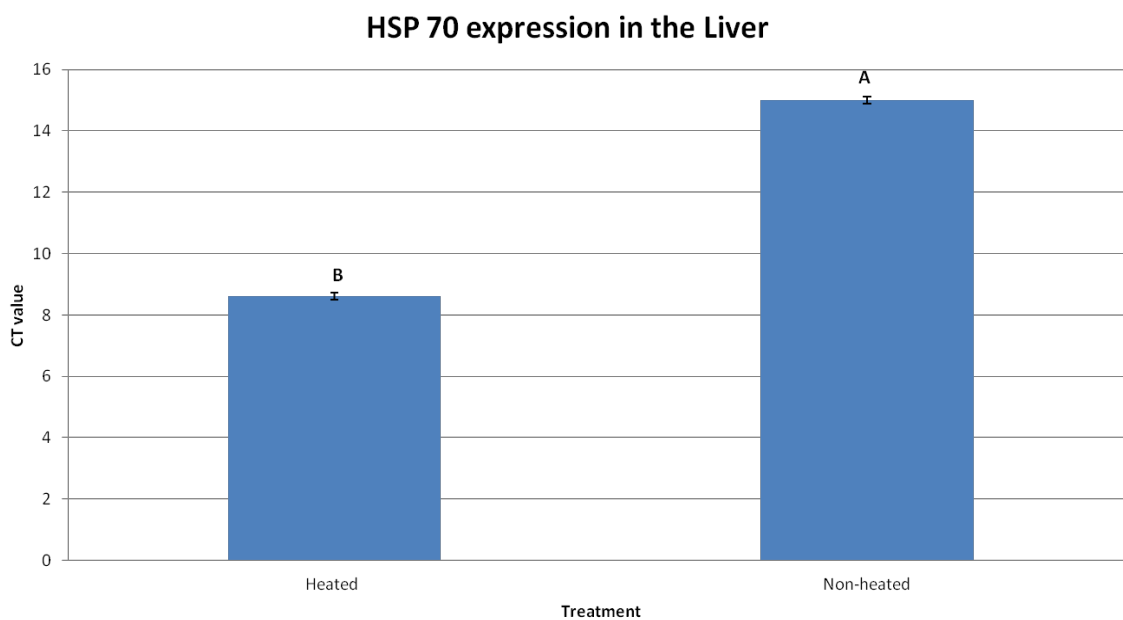


Figure 18. Mean CT values for gene expression of HSP 90 B in the Ileum of 3 and 6 week broilers based upon heat treatment. ^{A,B} indicates significant difference, $P \leq 0.05$



Figure 19. Mean CT values for gene expression of HSP 90 A in the Ileum of 3 and 6 week broilers based upon heat treatment. ^{A,B} indicates significant difference, $P \leq 0.05$

There were no significant differences in the 3 week blood salt compositions due to any variable (Table 2.) However there were many changes in the other blood parameters. There were significant differences in the total CO₂ at 3 weeks due to sex (P=.0457), as well as due to heat (P=.0001). There was a significant difference in glucose in 3 week broilers due to sex (P=.0036) in which the males had higher levels. PCO₂ levels were also affected. Birds fed Actigen had higher levels (P=.0391) than the birds fed the control diet, as well as the heated birds having higher lower (P<.0001) levels than the non-heated birds. There was a decrease in HCO₃ (P=.0493) in the males, as well as a decrease in the heated birds (P=.0001) (Tables 3 &4) There are also differences attributed to Feed*Sex in Na(P=.0255), and pCO₂ (P=0.0181). sex * heat interactions as well as feed *heat interactions caused changes in tCO₂ (P=.0038, and .0079) as well as HCO₃ (P=.0037, and .0079). Feed*sex*heat interactions were significantly different for tCO₂(P=.0079), Glucose (P=.0311), and HCO₃ (P=.0064). (Tables 3&4.)

Table 2. ANOVA analysis of blood salt composition from ISTAT for week 3 necropsy

	MCN	MCH	MAN	MAH	FCN	FCH	FAN	FAH
Na	144.17	148.33	140.33	145.33	143.5	146.33	143.83	145
K	5.38	5.57	4.78	5.13	4.92	5.2	5.02	5.13
Cl	109.5	120.83	111	113.5	109.83	113.5	109.17	95.17

Means of blood salt composition for 3 week bleed, ^{ab} Denotes significance at P≤.05 level

Table 3. MANOVA analysis of blood parameters from ISTAT for week 3 necropsy

	MCN	MCH	MAN	MAH	FCN	FCH	FAN	FAH
tCO ₂	32.83 ^a	18.83 ^c	30.83 ^{ab}	27.00 ^b	30.67 ^{ab}	27.33 ^{ab}	31.17 ^{ab}	27.83 ^{ab}
Glucose	260.17	248.17	237.33	265.83	230.67	234.83	239.33	225.17
pH	7.55	7.52	7.49	7.51	7.49	7.56	7.51	7.56
pCO ₂	36.23 ^{ab}	21.98 ^c	38.97 ^a	32.68 ^{ab}	39.08 ^a	29.3 ^{bc}	37.11 ^{ab}	30.30 ^{abc}
HCO ₃	31.65 ^a	18.13 ^b	29.53 ^a	26.12 ^a	29.35 ^a	26.45 ^a	29.95 ^a	26.90 ^a
An Gap	8.67 ^b	15.00 ^a	9.50 ^b	10.83 ^b	8.67 ^b	11.67 ^{ab}	10.00 ^b	11.17 ^{ab}

Means of blood parameters for 3 week bleed, ^{abc} Denotes significant at P≤.05 level

Table 4. MANOVA analysis of blood composition from ISTAT for week 3 necropsy

	Feed	Sex	Heat	Feed*Sex	Sex*Heat	Feed*Heat	Feed*Sex*Heat
Na	-	-	-	0.0255	-	-	-
K	-	-	-	-	-	-	-
Cl	-	-	-	-	-	-	-
tCO ₂	-	0.0457	<.0001	-	0.0038	0.0079	0.0079
Glucose	-	0.0036	-	-	-	-	0.0311
pH	-	-	-	-	-	-	-
PCO ₂	0.0391	-	<.0001*	0.0181	-	-	-
HCO ₃	-	0.0493	<.0001*	-	0.0037	0.0079	0.0064

P values from MANOVA analysis of blood composition from ISTAT for 3 week necropsy, lack of P value denotes not significantly different.

There was a significant increases ($P=0.0083$) in the amount of sodium in the blood of males birds on the Actigen diet when compared to the other treatments (Table 5.) There were significantly lower levels ($P<0.0001$) of potassium in the male birds than the female birds (Table 5.) The male birds on the Actigen diet also had higher levels ($P<0.019$) of chloride than did the other treatments.

MANOVA analysis of blood salt composition from ISTAT for week 6

Table 5. necropsy

	Male	Female		Actigen	Control		Heated	Non heated	
	Mean	SEM		Mean	SEM		Mean	SEM	
Na	147.57	150.3	1.14	150.22	147.65	1.14	149.64	148.29	1.16
K	5.13*	5.89*	0.11	5.33	5.69	0.12	5.4	5.61	0.11
Cl	112.65	114.78	1.17	114.57	112.87	1.17	114.54	112.96	1.14

Means from MANOVA for blood salt composition for 6 week bleed, * denotes significance at $P\leq 0.05$

There was a decrease in pH and pCO_2 due to sex, with the females having the highest pH and the males having the lowest pCO_2 (Table 6.) There were several differences due to heating the birds, with the heated birds having significantly higher pH ($P=0.0225$) and AnionGap ($P=0.0033$) and having significantly lower total CO_2 levels ($P=0.0010$), lower pCO_2 levels ($P=0.006$) and lower HCO_3 levels ($P=0.0021$) than the non-heated birds. There

were interactions from sex*heat and feed*sex*heat for Anion gap (P=.0158) (Tables 6 and 7.)

There was a significant difference (P=0.017) in the breast weight of the birds on the Actigen diet in the males at 3 weeks of age, but no other significant differences were observed (Table 8).

MANOVA analysis of blood salt composition from ISTAT for week 6 necropsy

Table 6.

	Male			Female			Actigen		Control		Heated		Non heated	
	Mean	SEM		Mean	SEM		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
TCo2	3.78	32.08	0.56	31.3	31.56	0.56	30.04	32.71	0.56					
Glucose	254.57	264.17	3.74	256.91	261.82	3.74	262.86	256.17	3.8					
pH	7.5*	7.45*	0.015	7.48	7.46	0.015	7.5*	7.44*	0.015					
PCO2	38.5*	45.44*	1.53	40.73	43.21	1.58	37.66*	45.92*	1.56					
HCO3	29.68	30.7	0.54	30.07	30.13	0.54	28.9	31.38	0.52					
An gap	10.39	10.65	0.48	10.86	10.17	0.49	11.6	9.54	0.5					

Means from MANOVA for blood composition for 6 week bleed, * denotes significance at P≤.05

MANOVA analysis of blood parameters from ISTAT for week 6

Table 7.

necropsy

	Sex	Feed	Heat	Feed*Sex	Sex*Heat	Feed*Sex*Heat
Na	-	-	-	0.0083*	-	-
	<.0001	-	-	-	-	-
K	*					
Cl	-	-	-	0.0196*	-	-
tCO ₂	-	-	0.0019*	-	-	-
Glucose	-	-	-	-	-	-
pH	0.0192*	-	0.0225*	-	-	-
PCO ₂	0.0029*	-	0.0006*	-	-	-
HCO ₃	-	-	0.0021*	-	-	-
An gap	-	-	0.0033*	-	0.0158	0.0158

P values from MANOVA analysis of blood composition from ISTAT for 6 week necropsy, lack of P value denotes not significantly different.

Table 8. Breast weight in grams for broilers at 3 and 6 weeks.

	Actigen	Control	P-Value	N
Male 3 week	182.75	165.08	0.017*	12
Male 6 week	721.67	736.92	0.0612	12
Female 3 week	168.74	167.97	0.9301	12
Female 6 week	615.48	636.692	0.4777	12

Analysis of Breast weight in grams at both 3 and 6 week necropsies due to diet and sex,

* denotes significance at $P \leq .05$ level

There were significant changes in the protein levels of HSP 70 in the Ileum of the 3 week birds, Diet (P=.0257) with birds on the Actigen diet having the highest levels of HSP 70 present and heat (P=.009) with the heated birds having the highest levels. There were several changes in the liver of the 3 week birds the heated birds had higher HSP 70 concentrations (P=0.009), the females had higher concentrations of HSP 70 (P=0.0014) and there were effects due to diet*heat (P=0.0005), as well as diet*sex (P=0.0124). There were no differences in concentration in the ileum of week 6 birds, and there was only a difference due to heat (P=0.0307) in the livers of week 6 birds (Tables 9, 10).

Table 9.		HSP 70 Protein in 3 & 6 week broilers for liver and Ileum				
	Diet	Heat	Sex	Diet*Heat	Diet* Sex	Diet*Heat*Sex
Week 3 Ileum	0.0257	0.009	-	-	-	0.034
week 3 Liver	-	0.009	0.001	0.0005	0.0124	-
Week 6 Ileum	-	-	-	-	-	-
Week 6 Liver	-	0.0307	-	-	-	-

P Values from MANOVA of HSP 70 in 3 and 6 week broilers for the liver and Ileum, lack of number indicates no significance

ANOVA Mean HSP 70 Proteins present in 3&6 week broilers for liver and Ileum								
Table 10.	MCN	MCH	MAN	MAH	FCN	FCH	FAN	FAH
Wk 3 Ileum	.0129 ^b	.0263 ^a	.0256 ^a	.0257 ^a	.0230 ^{ab}	.0254 ^a	.0242 ^{ab}	.0325 ^a
wk 3 Liver	.0355 ^{bc}	.0371 ^{bc}	.0255 ^c	.0401 ^b	.0395 ^{bc}	.0368 ^{bc}	.0379 ^{bc}	.0573 ^a
Wk 6 Ileum	.0404 ^a	.050 ^a	.0450 ^a	.0338 ^a	.0499 ^a	.0355 ^a	.0393 ^a	.0426 ^a
Wk 6 Liver	.0234 ^a	.039 ^a	.0251 ^a	.0289 ^a	.0284 ^a	.0295 ^a	.0237 ^a	.0410 ^a

ANOVA means of HSP 70 concentration in 3 & 6 week liver and Ileum samples, Means with differing ^{abc} letter are significantly different at the $P \leq .05$ level

Discussion and Conclusions

In this study, broilers were given either a control diet or a diet supplemented with Actigen. The birds were then mildly heat stressed at 41° C for one hour. The ileums and livers were collected for gene expression using real time PCR and protein analysis, blood was collected for blood metabolite profiles. It is well documented that a thermal stressor induces in an animal rapid synthesis of HSPs in all tissues (Liew et al. 2003, Yu et al, 2008, Wang and Edens, 1998, 2008). Since heat stress can be such a common unavoidable problem in commercial poultry production, it is very important that knowledge be gathered on this issue. The data from this investigation followed in the same patterns as those which preceded this work, showing significant alteration in many physiological parameters as well as HSP induction. There were significant increases in the expression levels of all HSP genes in the ileum due to heating, HSP 90 B ($P = <.0001$), HSP 90 A ($P = <.0001$), HSP 60 ($P = <.0001$), HSP70($P = <.0001$). This is in agreement with past publications of HSPs, that when an animal is acutely heat stressed the expression of HSPs significantly increases in an attempt to protect the organism and its genome from the stressor (Pauli and Tissieres, 1990, and Fayet et. al., 1989, Wang and Edens, 1998, 1994, 2008). Similar results were also observed in the gene expression levels. In the livers there were significant increases in all HSP mRNAs except for HSP 90B. Mahumoud (2000) reported that HSP 90 B is normally found at higher levels than most HSPs at normal temperature and only slightly induced by heat stressing. These data help to establish that heat stressed the birds evoked a response that was similar to previous

work with HSP and that it was enough to elicit a response, which showed that other comparisons such as blood chemistries were accurate.

There is research suggesting that gender plays a role in many aspects of organism's response to stressors. This difference has been attributed to glucocorticoid and steroid hormones. HSPs have been found to interact with the hormone binding domain (HBD) of the nuclear receptors. The HSPs aid in the folding of the HBD to form a conformation that has a very high binding affinity for steroid hormones of the nuclear receptors (Pratt and Toft, 1997). Since hormones play such diverse roles within animals, the sexually dimorphic response to the heat stressor was not surprising. Male broilers showed a significant increase in the expression levels of both HSP 90 A ($P < .0001$), HSP 90 B, ($P = .0002$), as well as HSP 90 AA, ($P = .0014$) in the ileum differing from females, which further supports that the HSP 90 family is involved in the steroid receptor pathway. Wang (1992) demonstrated that there were significant interactions of hormones on HSPs and that both testosterone and estrogen levels played significant roles in HSP production in male and female broilers, respectively, when they were subjected to a heat stressor. A number of studies have shown that the HSP 90 family is intimately involved with steroid hormone receptors in animals (Wang and Edens, 2008, Lin, 2007, Barral et al. 2004, Schlesinger, 1990, and Hightower, 1991).

Actigen is a second generation yeast outer cell wall derivative from *Saccharomyces cerevisiae* (Hooge, and Connolly, 2001). BioMos has been shown to improve gut health in broilers, illustrated by a significant increase in poult bodyweight associated with increased villi length and area, decreased number of goblet cells (Bradley et al., 1994) BioMos has also been shown to help combat some of the oxidative damage that results from heat stress in

broiler chicks (Sohail et al., 2011). The mechanism of this beneficial effect is not yet clear, but it is believed to be associated with increased numbers of gut microbes, which potentially release a bioactive substance that could prevent oxidative damage (Sohail et al., 2011). BioMos is believed to interact with carbohydrate and mannose receptors on the intestinal epithelial cells (Seifert and Watzl, 2007). However, in this current research, a biological challenge was not imposed on the broilers, but we still observed a significant influence of Actigen on the induction or suppression of various HSPs. There were significant decreases in HSP90 B (P=.0038) HSP 90 AA (P=.0040) HSP 70 (P=.0211) expression due to Actigen in the livers. There were no significant decreases in expression levels for the ileum. However, the expression levels in the ileum tend to be lower for all of the HSP genes except HSP 90 B but were not significantly different due to heat. These data suggest that Actigen may be able to lower the expression levels of HSPs in poultry that undergo exposure to a heat stressor while still developing thermotolerance. An explanation for this phenomenon could be that Actigen caused a primary effect in the ileum that may or may not be due to HSPs directly, and it has been reported that MOS affects many pathways where multiple heat-related changes could occur (Xiao et al. 2012). This change might be related to secondary effects in the liver which allows for decreased HSP production in the livers and not in the ileum.

The data for the quantification measure of HSP 70 are similar to the gene expression data in that there were more significant increases in HSP 70 production in the younger birds, and that there was a general increase in HSP 70 production in most tissues. There was not a significant heat induced increase in the ileum HSP 70 at 6 weeks of age. However it is believed that gene expression levels do not always correlate to the actual production of the

specified proteins (Celi et al., 2011). There are many factors that can affect mRNA translation to proteins. In this investigation, there were some contradictions between the real-time data and the protein data where the diet caused a significant change in the ileum of 3 week birds in actual protein concentration and no increases in the liver, which were in reverse of the real-time data. Females were found to have higher expression levels of HSP 70 at 3 weeks in the liver ($P=.0014$) and, though not significantly different, also in the ileum. This is supported by Wang (1992) in that females synthesize more HSPs than male birds.

There were significant differences in gene expression of HSP 90 AA ($P=.001$), HSP 60 ($P=.0259$), HSP 70 ($P=.0097$) in the ileum and HSP 90 B ($P=.0001$) and HSP 90 AA ($P=.0119$) in the liver due to an age difference with the 3 week birds having the highest level of expression. This could be due to 3 week old birds being less developed and have less established pathways and are more able to reallocate resources in response to the stressor. Older birds may mount less of a response. Mahumoud (2000) stated that an animal's response is based upon several factors, one being the age at which the animal is exposed to the stressor. Early exposure to a stress may provide enhanced stress response.

Heat stressors are classical inducers of non-specific physiological responses in birds and other animals causing depression or inhibition of growth, production, and livability of animals. HSP expression is one way assessing whether a bird has been exposed to a heat stressor, but HSPs are not the only sign of heat stress response. There are many physiological changes that occur in a bird that has been heat stressed (Edens, 1977). One sign that a bird has been subjected to heat stress is the change in blood chemistry. When birds are heated, they begin to pant heavily. This panting severely decreases the amount of

CO₂ in the birds blood (Edens, 1977). This panting or “blowing off CO₂” causes a condition known as respiratory alkalosis as indicated by a more alkaline blood pH. The pH of the blood is buffered by HCO₃, and HCO₃ is dependent on blood CO₂, which in the long term can cause blood CO₂ and blood HCO₃ to become too low causing an interruption in the respiratory process (Edens, 1976 and 1977). However, with decreased respiratory rate CO₂ and HCO₃ rapidly elevates, causing blood pH to rapidly return to a more acidic state, which now is very difficult to arrest thereby pushing the bird to a point of heat prostration. The developing heat prostration acts to promote a response similar to positive feedback in many systems allowing for eventual heat-related mortality. There are many factors that can affect poultry’s response to heat. Gender plays a role in this response, perhaps due to surface area to body weight ratio, or perhaps due to differences relative to the difference in sex hormones (Wang, 1992). The blood composition in response to heat stress in this study shows several significant differences that agree with previous heat stress work (Edens, 1997). There were significant differences in the total CO₂ at 3 weeks due to sex (P=.0457), as well as due to heat (P=.0001). These differences correspond to previous work by Edens (1997) who reported that birds under heat stress have decreased levels of CO₂ in the blood, the females had higher TCO₂, which can also be explained by Wang (1992) who showed that females tolerate heat stress better than males. Wang (1992) suggested that these differences may be influenced by sex hormones or by the difference in HSP production.

There was a significant difference in glucose in 3 week broilers due to sex (P=.0036) in which the males had higher levels. Although not significant, there were slight numerical increases in both the control birds and the heated birds. These data are supported by Edens

(1976 and 1977) who demonstrated that heat stress increases glucose levels in the birds. Since the males had higher blood glucose levels it again supports the claims by Wang (1992) that males are more susceptible to heat stress than females, in birds fed Actigen, blood glucose levels were higher ($P=.0391$) than the birds fed the control diet. Actigen fed heated birds had higher ($P<.0001$) blood glucose levels than the non-heated birds. Since HCO_3 and pCO_2 respond in parallel, it makes sense that a decreased HCO_3 ($P=.0493$) and CO_2 in non-heated males, might suggest CO_2 and HCO_3 in the heated birds ($P=.0001$). However CO_2 and HCO_3 were not significantly decreased, but might have shown a slight increase in control and heat stressed birds fed Actigen. Edens (1977) showed that birds under heat stress have decreasing pCO_2 and HCO_3 so these higher pCO_2 and HCO_3 in the males suggest that the birds are not as stressed as the birds with the lower levels. Thus, Actigen has some effects on the heated birds that gives them an enhanced capacity them more capable of dealing with that heat stress.

The gender dependent differences in pH ($P=.0192$), pCO_2 ($P=.0029$) and gender dependent differences in pH ($P=.0225$), pCO_2 ($P=.0006$) due to heating in 6 week old birds follow the same pattern as in the 3 week birds showing that the birds were in fact under heat stress and were exhibiting the classical responses reported by Edens (1977). In summary, the decrease in CO_2 , HCO_3 , and pCO_2 as well as the increase in pH and potassium were indicative of the birds being under heat stress. The fact that the heated birds on the Actigen diet had less of a change in blood chemistry is evidence of the role that Actigen plays in allowing the bird to tolerate heat stressors better than birds that were not on the Actigen diet.

There was a significant increase in breast weight for males given the Actigen diet at 3 weeks of age, but there were no other significant treatment associated changes in breast weight at 3 weeks or 6 weeks of age. These data are similar to Zhang et al. (2005) who reported BioMos feeding improved weight gain but not significantly different for all groups of birds in the trial.

These data do support totally the claim that Actigen plays role in thermotolerance in heat stressed poultry. The actual mechanism by which Actigen works in heat stressed birds is still somewhat unknown. There is researched focused at investigating the actual pathways by which MOS exerts its influence in birds by Xiao et al. (2012). However the pathways, which are affected, are very numerous and encompass many different physiological functions of the bird. Thus, trying to pin point the actual mechanism by which Actigen works could prove to be an extremely difficult task to accomplish. It is known that Bio MOS improves several functions within the birds, such as improved gut function and increase villi length (Bradely et al. 1994) as well as increase beneficial bacteria in the gut and decrease detrimental bacteria (Baurhoo et al. 2007, Sims et al. 2004). From the results of this study, it is evident that there is some interaction between Actigen and HSP production. More research is needed to determine the functional role of Actigen in the bird that allows for improved resistance to heat stressors.

These findings should be useful to the commercial poultry industry to help combat heat stress via the incorporation of feed additives. Since birds on Actigen are more tolerant to heat, they should survive sudden acute spikes in temperature, possibly by being

preconditioned to respond nonspecifically. Thus, under chronic heat stress, the birds should fare better than birds on an Actigen free diet.

Future studies

There is still much to be learned about Actigen and the pathways by which it works. Actigen has been shown to be involved in many of the physiological responsiveness of broilers under heat stress as indicated by body weight gain, egg quality factors, probiotics, and immune response. Actigen's influence on physiological responsiveness of poultry, swine, and cattle need to be assessed. Research should be done, covering a broad range of topics, to elucidate the actual mechanisms by which Actigen exerts its beneficial effects. There are lines of poultry that are more tolerant of heat than other lines, and it would be interesting to see the effects of Actigen on heat stressed heat tolerant birds. In order to improve the evaluation of Actigen during heating episodes, it would be important to utilize RNAseq which would provide a greatly expanded database from which a better understanding of Actigen's influence could be obtained. Additionally the role of parental nutritional status as it influences the predisposition of their offspring in relationship to development of obesity and other problems would be very valuable, but the role of Actigen feeding on progeny responses to heat stress also holds much promise. Furthermore, since there is an interaction among gender, sex steroids, and heat tolerance it would be of interest to address this issue similarly using the approach of Wang and Edens (2008) but only use females and estrogen treatments.

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