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Introduction

Production of beef cattle represents a $60 billion industry in the United States (USDA, 2015). The American beef cattle industry loses an estimated $370 million annually due to heat stress (St-Pierre, 2003). As of 2003, this was equal to nearly 99 million pounds of beef lost (USDA, 2015). The average American consumed roughly 65 pounds of beef in 2003; this means that the 99 million pounds of beef lost to heat stress would have been enough to feed approximately 1.5 million Americans for an entire year (Barclay, 2012).

The stress response axis adjusts the physiology of the body in order to maintain homeostasis in the presence of stressors of any kind (Tsigos, 2002). One of the main components of the stress response axis is corticotropin-releasing hormone (CRH). CRH increases thermogenesis and inhibits appetite as well as inhibiting growth hormone (GH), thyrotropin-releasing hormone (TRH), and thyroid stimulating hormone (TSH) via somatostatin. Glucocorticoids also play an important role in stress response. Increases in glucocorticoids such as cortisol decrease the productivity of beef cattle by decreasing growth hormone release,

decreasing gonadotropin-releasing hormone (GnRH) secretion, decreasing reproductive hormones such as luteinizing hormone (LH) and follicle stimulating hormone (FSH), and decreasing sex steroids.

All animals respond to heat stress stimuli by increasing production of a large family of proteins called heat-shock proteins (HSPs) (Lindquist, 1988). HSPs help protect cells from the negative effects of heat and other stresses. They are typically classified by molecular weight: HSP10, HSP60, HSP90, etc. Many HSPs act as molecular chaperones and play vital roles in transporting proteins, protecting cells from apoptosis due to stress, and folding and unfolding of proteins. HSP 90 is highly conserved and has been found and sequenced from many diverse species including insects, mammals, birds, and bacteria.

Problem Statement

HSP90 has been discovered in beef cattle, but a gap in the research exists with this particular protein. This leads to the problem statement of this research project: Environmental stressors, heat stress in particular, can reduce livestock productivity by millions of dollars annually. Research is needed to help understand the relationship between polymorphisms of the genetic sequence that codes for HSP90, and the productivity of these animals.

Limitations

Possible limitations to this study include the fact that results determined for HSP90 may not be generalizable to other members of the HSP family. Uncontrollable environmental changes may also present threats to this study.

Literature Review

American Beef Cattle Industry

The United States is home to the largest fed-cattle industry in the world (USDA, n.d.a). Feed lots and cow-calf operations in the U.S. contribute over 23 billion pounds of beef annually to the production industry, making the U.S. the leading beef producer (USDA, n.d.a; USDA, 2015).

Profitability

As with any industry, a great concern of beef producers is profitability (Archer *et al.*, 1999). Profitability in the beef cattle industry relies heavily on the efficient provision of feed (Nkrumah *et al*., 2006). Because the cost of feed is so great, cattle farmers are concerned with ensuring that their cattle receive the most productive rations of feed possible so that no unnecessary excess is given. However, cattle farmers still need to simultaneously provide cattle with the necessary nutrition needed to develop.

Cow-Calf Enterprise

The cow-calf industry consists of both seedstock operations and commercial operations (Kahn, 2014). Seedstock operations are primarily concerned with producing breeding stock, whereas commercial operations produce cattle for slaughter, although seedstock operations occasionally raise additional commercial cattle. The majority of the beef produced in the United States comes from commercial cow-calf operations. These operations raise cross-bred cattle, and greatly outnumber purebred seedstock enterprises. Commercial cow-calf operations usually run on land appropriate for foraging, making it possible to raise cattle with lower feed costs (USDA, n.d.a). If environmental and weather conditions support the growth of forages, the cattle can be fed on forages all year. American cow-calf operations average 40 head of cattle per operation.

These operations are usually an extension of larger farms, or are small-scale operations used to supplement other income. However, 51% of U.S. beef cattle inventory consists of cattle raised on cow-calf operations of 100+ head. These farms account for 9% of U.S. beef operations. Because cattle on cow-calf operations may remain part of the stock for generations, it is possible for the herd to develop and produce cattle who have genetically adapted to environmental changes (Siegel & Gross, 2000).

Efficiency

Reproduction, a vital component of cow-calf operations, can be greatly affected by heat stress (Hansen, 2009). Heat stress can have negative effects on oocyte development and maturation, spermatogenesis, embryonic development, and foetal and placental growth, as well as lactation. There are two basic ways that heat stress can affect reproduction. Reproductive functions can be effected by changes in body temperature due to homeokinetic functions (i.e. blood is sent outward from the body core in order to dissipate more heat.) When cattle are heat stressed they consume less feed, lessening their production of metabolic heat. The decreased feed intake can also cause a disruption in the animal's energy balance and nutritional stability, which in turn can negatively affect fetal development, cyclicity, and conception rates. Heat stress can also cause homeokinetic systems to malfunction in the regulation of reproduction. The functions of germ cells, the developing embryo, and possibly other reproductive cells can be disrupted when the temperature of the animal increases above its regulated set point. Fetuses may experience reduced growth when the gestating animal is experiencing heat stress. Heat stress can also reduce the necessary nutritional intake of a fetus due to reduced nutritive intake of the gestating animal.

Physiology

In vertebrates, external stressors ignite a chain reaction of responses to deal with the stressor and to reestablish homeostasis (Boonstra, 2004; Sapolsky, *et al*., 2000). The sympathetic nervous system (SNS) prompts the adrenal medulla to release catecholamines (norepinephrine and epinephrine) within seconds of a stressor. These hormones enter into the general circulation, and the paraventricular nucleus (PVN) of the hypothalamus releases corticotropic releasing hormone (CRH) and arginine vasopressin (AVP) (Boonstra, 2004). This causes the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) into the bloodstream. The adrenal cortex then secretes glucocorticoids (GCs) in response to the release of ACTH within minutes of ACTH entering the bloodstream. The primary GC in birds and small mammals such as mice and bats is corticosterone. In mammals such as primates and ungulates, the primary GC is cortisol. This response causes the mobilization of ATP to muscle cells by stimulating hepatic gluconeogenesis. This response also results in the shunting of energy away from the peripheral tissues that are unnecessary for short-term survival. Appetite and feeding decreases, cognition sharpens, reproductive physiology is suppressed, inflammatory response decreases, immune function increases, and cardiovascular tone is enhanced.

Chronic Stressors

When environmental stressors become chronic (time frame of days to months), the resulting impacts of inhibition can impact long-term survival by causing an inability of the immune system to fight off disease, reductions in growth, and infertility (Boonstra, 2004). In response to chronic stressors, animals can have long-term effects that cause them to produce abnormally high levels of GC as a response to the presence of ACTH. Through harmful feedback regulation the hippocampus disturbs the response of the hypothalamic-pituitary-adrenal axis (HPA). However, this regulation can be reduced by chronic stress, which causes a down

regulation of GC receptors in the hippocampus thereby increasing GC concentrations. Chronic stressors can also cause an increase in the production of GC by causing adrenal hypertrophy, which enlarges the cells and increases the capacity to produce GC by the adrenocortical tissue.

HSP Family

An increase in the synthesis of certain proteins after cells were exposed to stressors such as heat shock was first discovered in drosophila cells in 1974, and the wide range of organisms that share this response was discovered soon after (Schlesinger, 1990). In more recent years scientists in this field have focused on the role of heat shock proteins as "molecular chaperones" (Schlesinger, 1990). Molecular chaperones are required for the folding of many proteins. They prevent protein structures that are malformed from occurring both in homeostatic conditions as well as when cells are exposed to stressors such as heat shock (Hartl, 1996). "Protein folding is the process by which the linear information contained in the amino-acid sequence of a polypeptide gives rise to the well-defined three-dimensional conformation of the functional protein." Many HSPs are part of protein families that are typically present and essential for normal cellular function (Schlesinger, 1990). Some of the most studied HSPs that increase in expression under stressful conditions are HSP27, HSP70 and HSP90. These three HSPs in particular are found in unusually high quantities in cancer cells and are increased even more after cells are exposed to a variety of death stimuli (Jego *et al*., 2013; Garrido *et al*., 2006; Wang, 2014). These proteins are very efficient at preventing apoptosis and thus blocking the cell death process (Joly *et al*., 2010; Wang, 2014). Research has shown that abnormally high expression of these HSPs escalates tumor growth, increases the chance that tumors will metastasize, and increases resistance to chemotherapy in tests on rodent specimens. As a result, inhibition of HSP27, HSP70 and HSP90 is being studied as a potential approach to cancer treatment.

In another research study conducted by Florence Favatier, Liza Bornman, Lawrence E. Hightower, Eberhard Gunther, and Barbara S. Polla, polymorphisms of HSP70-1 in humans was analyzed to determine if there were differences in expression when comparing cell lines with C or A alleles (Favatier, 1997). In this study HSP70-1 expression was analyzed after heat shock in peripheral blood cells that were mononuclear from people with different HSP70-1 genotypes. No difference in expression was found. However, whether or not different polymorphisms of HSP70 make certain people less susceptible to disease remains an open issue. According to these researchers, only a limited amount of these HSP polymorphisms have yet to be discovered, and single nucleotide or amino acid changes might only be detected by sequencing, which was not utilized in this study. They also agree that further studies of polymorphisms (HSP90 in particular) should prove to show significant differences. In our study, we analyzed HSP90 through the use of genetic sequencing, as was suggested by this team of researchers.

Transcription Factors

When abundant stress is experienced, HSPs are promptly induced (Shamovsky & Nudler, 2008; Wang, 2014). This is a phenomenon known as the heat shock response (HSR). Heat shock transcription factors (HSFs), the upstream transcriptional controllers of HSPs, mediate the HSR at the transcriptional level. HSF1-4 and HSFY have been identified as vertebrate HSFs, and each of these "exhibit a similar structure with a highly conserved amino-terminal helix-turn-helix DNA-binding domain and a carboxy-terminal transactivation domain" (Green *et al*., 1995; Nakai *et al*., 1997; Sistonen *et al*., 1994; Wang, 2014). The chief transcription factor for the HSR is HSF1 (Åkerfelt *et al*., 2010; Wang, 2014; Whitesell & Lindquist, 2009). HSF1 coordinates the continued existence of cells in response to different forms of cellular stress and normalizes the expression of HSPs (Åkerfelt *et al*., 2007; Whitesell & Lindquist, 2009).

HSF1 serves as a sedentary monomer in physiological conditions (Wang, 2014; Westerheide *et al*., 2006;). HSF1 can be bound to the HSP70 and HSP90 sequence when not under stress to cancel the transcription function. It can also separate itself from the HSP sequence during cellular stress to activate transcription of the HSP. HSF1 then is phosphorylated and moves to the nucleus of the cell. HSF1 attaches to DNA elements that are present in the genes that code for heat shock proteins. These cis-acting DNA elements are called heat shock elements (HSEs). They begin the transcription process of the genes that code for HSPs such as HSP27, HSP70 and HSP90 (Wang, 2014; Xia *et al*., 2012). Therefore, inhibiting the production of HSP90 will initiate HSF1 with the amplified expression of HSP27 and HSP90 (Wang, 2014; Westerheide *et al*., 2006).

HSP90

HSP90 is a ubiquitous protein chaperone produced by all eukaryotic cells (Krause *et al*., 2004). It has been repeatedly found to be highly conserved throughout evolution and makes up 1% of all cellular protein (Wang, 2014). HSP90 is a molecular chaperone that requires ATP. Several isoforms have been found in humans, the most studied being the constitutive form HSP90β, and the inducible form HSP90α (Subbarao *et al*., 2004; Wang, 2014). HSP90β is expressed at high levels and is slightly induced following heat stress. HSP90α, however, has been proven to be expressed at much lower levels than HSP90β under homeostatic conditions, but highly induced under conditions of heat stress.

HSP90 is a homodimer consisting of three distinguishable parts (Hartl *et al*., 2011; Taipale *et al*., 2010; Wang, 2014). First, the ATPase activity of HSP90 is controlled by the ATP-binding and hydrolyzing pocket of the amino (N)-terminal domain. Second is the middle region which is charged and is responsible for the recognition and binding of the protein target that is to

be chaperoned. Third is the carboxy (C)-terminal dimerization domain. This domain directs HSP90 dimerization (Wang, 2014).

Polymorphisms of HSP90

The presence of intrinsically expressed genetic polymorphisms, as well as acquired resistance to HSP90 inhibitors, has been recognized in humans (Duerfelt, 2010). DT-diaphorase and cytochrome P450 3A4 (CYP3A4) are included in these polymorphisms. There is concern over similar difficulties arising in the future with other HSP90 inhibitors, making it increasingly relevant to study these polymorphisms. Several polymorphisms of HSP90 have been shown to result in reduced HSP90 activity.

Objectives of this Study

To my knowledge, no data has been shown to prove the existence of polymorphisms in the genetic sequence that codes for HSP90 in cattle. This study intends to determine if polymorphisms of HSP90 exist in beef cattle, and to determine if these polymorphisms can be reliably used to determine beef cattle productivity, and thus, profitability.

Methods

Purpose and Objectives

The overall purpose of this experiment is to improve the beef cattle industry by developing a better understanding of beef cattle genetics. To meet this purpose, the following objectives were created:

1. Determine if polymorphisms exist in HSP90 genes of cattle.
2. Determine if these polymorphisms relate to beef cattle efficiency.

Design

The design of this experiment was a randomized incomplete block. It was random because the genetic sequence of the cattle is unique to each cow and cannot be selected by the experimenter, incomplete because every cow was not represented in every year, and blocked by year. The genotype was the independent variable of this experiment.

Participants and Sampling

For this experiment DNA was extracted from white blood cells of Angus crossbred beef cows (n=26; University of Arkansas IACUC approved protocol #13062) that were grazed on endophyte-infected tall fescue and common Bermuda grass. Cattle had unlimited access to water and trace mineral supplements. For this study, 26 cows were used because this was the number of cows available for research at the University of Arkansas farm. For each cow we have at least 3 years of production data such as Julian calving date, calf birth weight, and weaning weights of the cows and their calves. In addition, we have hair coat scores for those same cows during spring and summer months of those same years.

Blood from the cattle was collected with EDTA treated tubes by jugular venipuncture. Samples were cooled on ice once collected. The samples were then centrifuged at 2,500 g at 5℃ for 25 minutes. The plasma was decanted and the buffy coats were collected and placed in 1.5 mL tubes and stored at -80℃ until they were further analyzed.

DNeasy® Blood and Tissue Kit protocol was used to isolate DNA from the buffy coats. A C1000 Touch™ thermal cycler was used to amplify DNA segments via polymerase chain reaction (PCR).

Data Collection

The first denaturation process was administered at 94 ℃ for 2 minutes for each amplification protocol, followed by 35 cycles for 30 seconds at 94℃, 55℃ for 1 minute, and at 68℃ for 1 minute. The last step, which completed the process, lasted for 10 minutes at 68℃ and was cooled to 8℃. The amplification reactions each included 2.5µL of DNA (20ng/µL), 1.25 µL of forward primer, 1.25 µL of reverse primer, and 45 µL of Platinum PCR Supermix. 10µL of amplification products and 100 bp DNA ladder were inserted into individual 1.2% agarose wells. These samples were then separated using electrophoresis (TBE buffer; 30 minutes at 130 volts). The samples were then stained with ethidium bromide and visualized by using a UVP Epi Chemi II Darkroom. The DNA ladder was then used to compare the expected amplicon size. Samples were then purified with GenScript QuickClean II PCR Extraction Kit after being confirmed as the accurate amplicon size. After the purification process was complete, a Qubit® Flurometer was used to quantify the purified product.

The amplification products were sequenced in forward and reverse directions for each animal. The PCR product (8 µL) and the Primer mix (4µL) were sent for sequencing at Eurofins SimpleSeq in Louisville, KY. The program Clustal Omega (HTTP://www.ebi.ac.uk/Tools/msa/clustalo/) was used to compare the genetic sequences. Each single nucleotide polymorphism (SNP) was identified after multiple alignments were conducted by Clustal Omega. The SNP site of each sample was named according to the distance from the first base in the forward primer of the SNP. The primary allele is indicated by the first letter in the name of the SNP site, and the second letter represents the minor allele. Homozygous and heterozygous genotypes were both identified using Mega and sequence chromatograms.

Data Analysis

MIXED MODEL procedures of SAS were used to analyze quantitative data. This study utilized repeated measures analysis using the maximum likelihood method. The animal was the experimental unit. Tukey's adjustment and multiple t-tests were performed when F-tests for main affects were significant ($P < 0.05$).

Results

A transition from adenine to guanine was detected at base 97 of the 283 base amplicon (Figure 1). In this population of 26 cows, 19 were homozygous for adenine, 7 were heterozygous, and 0 were homozygous for guanine, which resulted in a minor allele frequency of 13.46%. Calving traits and cow weaning weight were not ($P > 0.40$) associated with genotype at A97G (Table 1). However, 205-day adjusted calf weight was associated ($P = 0.0002$) with A97G genotype (188 vs. 208.1 ± 7.1 kg; respectively AA and AG). This increased weaning weight resulted in a trend ($P = 0.08$) for increased cow efficiency for AG cows (Table 1). However, hair coat score for AG cows (1.6 ± 0.17) was lower ($P < 0.03$) in June than AA cows (2.2 ± 0.11; Figure 2).

Conclusion

According to the results of this study, there is evidence to suggest a link between polymorphisms of HSP90 and 205-day adjusted calf weight, as well as cow efficiency. These factors directly impact beef cattle productivity, ultimately suggesting an association between polymorphisms of HSP90 and beef cattle productivity. This connection suggests that this gene could be used as a genetic marker for heat stress as well as productivity. Further study of SNPs of the HSP90 gene would allow beef cattle producers to be more informed and potentially allow for selection of cattle that are more tolerant to heat stress.

	A97G Genotype[a]			
Trait	AA	AG	SEM[b]	*P*-value[c]
Number of cows	19	7	-	-
Calving				
Rate, %	89.5	89.5	-	1.0
Julian, day	269.4	273.4	7.3	0.65
Adj. birth weight[d], kg	33.8	35.8	2.0	0.40
Weaning				
Cow weight, kg	521.7	521.8	24.5	0.99
Adj. 205-d calf weight[d], kg	188.0	208.1	7.1	0.0002
Cow efficiency[e], %	36.8	41.5	2.6	0.08

Table 1. Effects of heat shock protein 90 genotype at single nucleotide polymorphism site A97G on cattle productivity traits.

[a]Single nucleotide polymorphism occurred at the 97th base of the 283 base amplicon. Letters indicate the primary and minor alleles, respectively.

[b]Mean standard error of the least squares means.

[c]F-test probability of main effects for A97G genotype.

[d]Birth weight and 205-d weaning weights were adjusted as recommended by the Beef Federation (2010).

[e]Cow efficiency was calculated by dividing each calf's 205-day adjusted weaning weight by dam weight at weaning and expressed as a percent.

GTGACGATCTCCAACAGGCTTGTGTCGTCACCCTGCTGCATCGTGACCAGCACCTA
CGGCTGGACCGCCAACATGGAGCGCATCATGAAAGCCCAG**[A/G]**CGCTTCGGGACA
ACTCGACCATGGGCTACATGATGGCCAAAAAGCATCTGGAGATCAACCCTGACCAC
CCCATCGTGGAGACCCTGCGGCAGAAGGCAGAGGCGGACAAAAACGACAAGGCCG
TCAAGGACCTGGTGGTGCTGCTGTTCGAAACTGCACTGCTCTCC**TCTGGCTTCTCG**
CTTGAGG

Figure 1. Amplicon (283 bases) of bovine heat shock protein 90 (NCBI Reference Sequence: NM_001079637.1). Forward and reverse primer sequences are bold and in green, and single nucleotide polymorphism of interest (A97G) is bold in red.

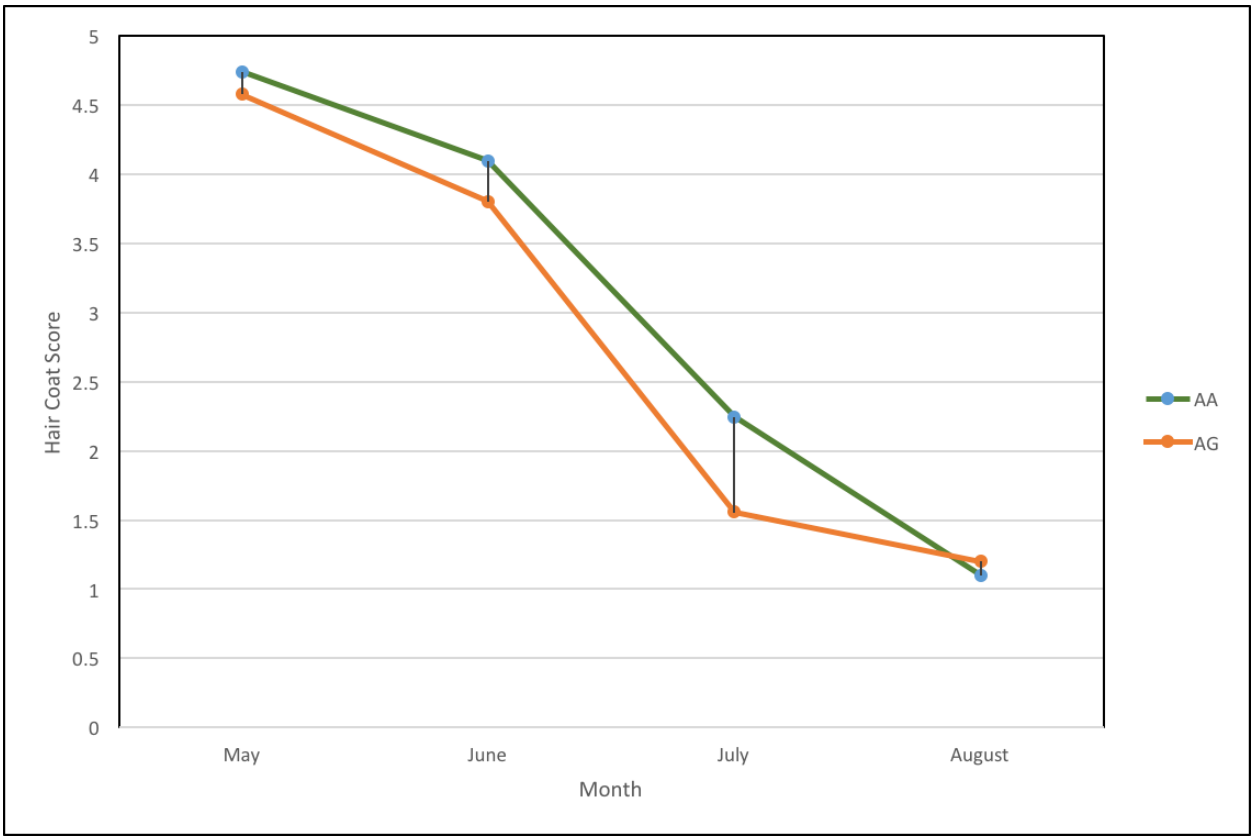

Figure 2. Resulting hair coat scores by month of AA and AG single nucleotide polymorphisms at base 97 of the 283 nucleotide amplicon.

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