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Associations of Single Nucleotide Polymorphisms in the Bovine Prolactin, Melatonin Receptor 1A, and Dopamine Receptor D2 Genes with Hair Coat Shedding Scores and Productivity Traits in Beef Cattle

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Associations of Single Nucleotide Polymorphisms in the Bovine Prolactin, Melatonin Receptor 1A, and Dopamine Receptor D2 Genes with Hair Coat Shedding Scores and Productivity Traits in Beef Cattle

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Abstract

Calving rate is a qualitative trait regulated by several genes and is strongly affected by the environment. With the development of biotechnology and gene identification, scientists are able to determine which genes affect these productivity traits to improve accurate selection decisions. Prolactin (PRL) has been associated with reproductive traits, melatonin receptor 1a (MTNR1A) has been associated with meat quality traits, and the dopamine receptor D2 (DRD2) gene has been associated with hair coat score (HCS) in cattle. Our objective was to determine associations between mutations in the PRL, MTNR1A, and DRD2 genes and cow-calf profitability traits. Genomic DNA was extracted from buffy coat samples of Angus-based crossbred cows (n = 170). Relationships were determined using mixed model ANOVA with genotype, year, and cow age group as main affects. When F-tests were significant, means were separated using multiple t-tests and Tukey's adjustment. We identified three single nucleotide polymorphism (SNP) sites in the PRL gene of cattle (C1286T, A1128T, G8398A), five SNP sites in MTNR1A (A541G, G575A, A583G, T679C, C721T) and one SNP site in DRD2 (A534G). Three years (2012 - 2014) of performance data were used to determine relationships to SNP genotypes. Mutation site C1286T, in the PRL gene, affected ($P = 0.038$) both calving rate and cow efficiency. Calf weaning weight and adjusted 205-d weight tended ($P < 0.10$) to be different for the C1286T SNP site. The SNP site A1134T affected ($P < 0.05$) calf birth weight, adjusted 205-day weight, and cow efficiency; and tended to affect ($P < 0.10$) calf weaning weight. Cows that were homozygous minor allele at MTNR1A SNP site, A583G, had increased hair coat scores (HCS) and calving rate. The DRD2 gene SNP site tended ($P = 0.10$) to affect HCS. These results suggest that PRL, MTNR1A, and DRD2 genes could be used as molecular markers for selection in cattle.

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Introduction

The beef cattle industry makes up a significant amount of the agricultural economy in the state of Arkansas. There are an estimated 1.64 million cows and calves in the state making Arkansas 20th in the nation (NASS, 2014). The value of cows and calves sold in 2012 totaled $766,476,000, making it the second largest livestock industry in the state behind poultry. The cattle industry is an essential asset to the subsequent advancement of the agricultural economy in Arkansas.

In 2012, Arkansas experienced a harsh drought where 97% of the state was categorized as severe. Livestock farmers suffered due to poor forage and scarce hay production. Cattle numbers were at their lowest level in the last 40 years, and farmers were reducing the size of their herds because of the limited supply of water and low quality forages (Kemper et al., 2012). The exposure to extremely high ambient temperatures and humidity can be detrimental to the animal causing decreases in milk production and quality, fertility, growth, and even death. High humidity will cause sweat to get trapped between the hair follicles causing an increased respiration rate. This causes the animal to spend more energy in thermoregulation (Finch, 1985).

Cattle that shed their winter coats in the early summer show less signs of heat stress than those who fail to shed their winter coats. A study conducted by Gray et al. (2011) concluded cows who shed their winter coats early weaned heavier calves. In that same study, a broad sense heritably was calculated and determined that early winter coat shedding was 0.35, making this a potential trait of selection for producers.

Review of Literature
Characteristics of Hair

Hair Growth Cycle

The hair growth cycle in mammals consists of four main stages: anagen, catagen, telogen, and exogen. The anagen period is the active growth of the hair shaft from the base of the follicle. Catagen is the transitional period at which the bulb separates from the matrix cells. The resting phase is known as the telogen period in which the hair is loosely attached to the follicle. The fourth stage, the exogen period, occurs when the hair is shed (Paus and Cotsarelis, 1999; Stenn and Paus, 2001). It was previously suggested that cattle exhibited only one shedding period each year; however, two hair growth cycles were determined by Dowling and Nay (1960) and each follicle produces two hairs a year. When either the winter or summer hair coat is shed, it is closely related to when the subsequent cycle begins (Aiken et al., 2011).

Physiology of Hair Growth

Seasonal hair growth patterns occur regularly in many species such as cattle, mink, and sheep. The increase in day length in the spring begins the growth of the summer coat while the decrease in day length in the fall initiates the winter fur growth (Duby, 1972; Martinet, 1959, 1984; Rose, 1984, 1987). It was determined in Shorthorn cattle that altering the photoperiod caused an alteration in hair growth cycle (Yeates, 1955). The change in photoperiod throughout the year also impacts hormone secretion (Martinet, 1984; Rose, 1985). Circulating melatonin and prolactin concentrations are inversely related. Prolactin is correlated with day length in that it is increased during the summer and is decreased during the winter months. Melatonin, on the other hand, is secreted when the day length is shorter; increases during the winter

months and decreases during the summer months. In mink (*Mustela vison)*, Rose (1987) demonstrated that the density of hair may be affected by prolactin, but it may not be essential for the initiation of summer or winter hair growth. However, Craven et al. (2001) reported the seasonal ques of prolactin influence the hair growth cycle. A study with mares found that when administered prolactin in the winter the mares began to shed their winter coats (Thompson et al., 1997).

Nutritional Effects on Hair Coat

Endophyte-infected tall fescue (*Neotyphodium coendophialum loium arundinaceum)* is a main forage grass grown in many areas of the United States (Jackson et al., 1984). Rough hair coats during summer months are a common symptom of fescue toxicosis. The endophytic fungus produces ergot alkaloids which cause a restriction of blood flow and decreases the ability to dissipate body heat (Oliver, 2005). Circulating serum concentrations of prolactin are used as a biomarker for animals suffering from fescue toxicosis. The ergot alkaloids bind the dopamine receptors causing a decrease in prolactin concentrations. Nihsen et al. (2004) demonstrated that cattle grazing the forage with the endophyte had rough hair coats and decreased prolactin serum concentrations; whereas, animals grazing endophyte free tall fescue had normal hair coats and normal prolactin concentrations. In order to reduce the toxic effects, it has been demonstrated that feeding pelleted soybean hulls and treating animals with steroidal implants can induce some hair shedding as well as increase prolactin serum concentrations (Aiken et al., 2006). In order to determine the distribution of hair that is from new growth or improper shedding, Aiken et al. (2011) used a bleaching technique. They demonstrated that cattle with rough hair coats had increased rectal temperatures as the

ambient temperature and humidity increased. When animals where categorized as having shed their winter coats, their rectal temperatures were not elevated. The increase in rectal temperatures in animals expressing rough hair coats could be due to the inability to properly dissipate body heat or endocrine disruptors such as the altered prolactin concentrations.

The hair coat score (HCS) scale was first introduced by Turner and Schelgar in 1960. It was later adapted by Williams et al. (2006).

Table 1. Description of hair coat scores

Hair Coat Score	Description
5	Full winter coat, 0% shed
4	Initial shedding, 25% shed
3	Half way shed, 50% shed
2	Almost shed, 75% shed
1	Slick summer coat, 100% shed

First introduced by Turner and Schelgar in 1960. Adapted by Williams et al. (2006).

Genomic Selection

Over the last decade, beef cattle traits have been selected and studied through DNA markers. Single nucleotide polymorphism arrays have been made commercially available to cover a large portion of the genome and can be used as a tool to help make genetic selection decisions (Meuwissen et al., 2013). Quantitative traits are regulated by several genes and are strongly affected by the environment. With the development of biotechnology and gene identification, scientist are able to determine which genes affect productivity traits. Advancements increase the opportunity to make accurate selection decisions and quick changes within the herd. Determining the polymorphisms effects on productivity traits, such as

reproduction, will benefit producers and inform them of the genetic value of an animal early in its life.

Prolactin

Molecular mechanisms of prolactin

Prolactin is a protein hormone that is produced by the anterior pituitary and has been estimated to be involved in more than 300 functions (Bole-Feysot et al., 1998). Because of its biological function, the 198 amino acid hormone is a part of the cytokine receptor superfamily. The prolactin gene is located on chromosome 23 and is made up of five exons and four introns (Dietz et al., 1992).

Prolactin's structure is made up of three levels: primary, secondary, and tertiary. The primary structure contains a single chain of amino acids with three intramolecular disulfide bonds between six cysteine residues. The protein sequence and length can vary between species. In cattle, sheep, pigs, and humans the pituitary prolactin consists of 199 amino acids whereas in mice and rats is consists of 197 amino acids. The structure of the secondary level consists of a 50-50 split between loops and alpha helices (Freeman et al., 2000). Other types of secondary structures are beta pleated sheets and beta helix which are derived from variations in the right-handed alpha helices. This organization gives the structure thermodynamic stability and flexibility, and leads to the biologically active form of prolactin. The tertiary structure consists of four alpha helices arranged two up and two down and is thought to be similar to growth hormone. The nuclear magnetic resonance determines the attachment of the helices which are arranged in a Greek Key (Keeler et al., 2003).

The active form of prolactin is primarily known for initiating lactation and mammary development but has been further determined to have multiple roles within species. Prolactin receptors have been identified in the bovine granulosa cells (Lebedeva et al., 2001, 2004) and corpus luteum (Poindexter et al., 1979). Bole-Feysot et al. (1998) demonstrated that mice were unable to conceive when the prolactin receptors were non-functioning.

Problems can arise within the body from abnormal concentrations of circulating prolactin. Halbreich and Kahn (2003) used the human model to demonstrate that patients suffering from osteoporosis and depression have greater serum concentrations of prolactin. Treatments for abnormal prolactin concentrations are often difficult to control. Side effects include an imbalance of physical and hormonal responses such as weakened growth hormone release or insulin resistance; however, the severity varied from patient to patient (Melkersson et al., 1999). Due to this inconsistency between individuals, it is reasonable to conclude the genetics and environment affect prolactin.

Prolactin Genotypes

Several SNP in livestock have been identified and associated with different traits. Milk fat and milk yield are associated with a SNP at bp 8398 (guanine to adenine transversion) where heterozygous animals had the greatest milk yield and the homozygous animals displayed the greatest fat content (Brym et al., 2005).

More recently, polymorphisms in the promotor region of the PRL gene have been associated with cattle productivity traits (Looper et al., 2010). Mutations at bp 1286 (cytosine to thymine transversion) and bp 1167 (adenine to guanine transversion) had an impact on calving

percentages when animals were grazing either endophyte-infected tall fescue (*Lolium arundinaceum* (Schreb.) Darbish) or common bermudagrass (*Cynodon dactylon* (L.) Pers). It was also determined that calves from AG cows were both heavier and taller at weaning than those from AA cows at the A1167T SNP site. Within the same study, the allelic frequency distributions were influenced by breed. Purebred Brahman had thymine as the primary allele while purebred Angus had cytosine as the primary allele (Looper et al., 2010).

Dopamine

Molecular Mechanisms of Dopamine

Dopamine is a catecholamine neurotransmitter in the mammalian brain and controls multiple functions. It plays many roles within the body such as hormone secretion, renal function, and cognitive behaviors. Dopamine is produced in several areas in the brain including the striatum, substantia nigra, and the ventral tegmental area. These areas define three principal neuronal pathways, the mesocorticolimbic, the nigrostriatal, and the tuberoinfundibular (Civelli et al., 1993). The mesocorticolimbic pathway connects the ventral tegmental area with the limbic forebrain and is thought to be a part of emotional balance. The nigrostriatal pathway runs from the substantia nigra to the striatum and is involved in the production of movement. The tuberoinfundibular pathway begins in the hypothalamus where the dopamine is transported to the pituitary gland through the portal blood. The dopamine that is transported to the pituitary regulates the production of prolactin, which influences fertility and lactation in humans (Civelli et al., 1993).

These dopaminergic actions are mediated by five receptor subtypes (D_{1-5}) which are further divided into two major subclasses, the D_1-like and D_2-like receptors. These receptors are members of the large G-protein coupled receptor superfamily. Within the central nervous system, the different subclasses perform different physiological roles. The D_1-like receptors are primarily post-synaptic and the D_2-like receptors are both pre- and post-synaptic. Receptors D_1 and D_5 are D_1-like because they stimulate adenylyl cyclase activity, have similar modes of action, share high amino acid sequence identity, and they do not contain introns in their protein coding region. The D_2, D_3, and D_4 receptors are D_2-like because of their pharmacology and identical amino acid sequences. However, D_2-like receptors do contain introns within their protein coding regions (Civelli et al., 1993; Jaber et al., 1996).

Dopamine Receptor D_2

The D_2 receptor is expressed in the pituitary where it controls prolactin secretion (Creese et al., 1997). Two isotopes of the D_2 receptor have been reported. The two forms are different by 29-amino acids in the third intracellular loop (Grandy et al., 1989; Civelli et al., 1993). During maturation of the D_2 receptor pre-mRNA, an alternative splicing event occurs. The isotopes are not tissue or species specific and exist in human, rat, bovine, mouse, and frogs. Little is known about their biological importance (Grandy et al., 1989; Civelli et al., 1993).

Dopamine Receptor D_2 (DRD2) Genotypes

Mutations within the dopamine D_2 receptor gene have been linked to temperament in sheep. A study was conducted to compare nervous tempered sheep to calm sheep. It was confirmed that a combination of polymorphisms within the CYP17 gene, and the dopamine

receptor D_2 and D_4 genes could be used as genetic markers for temperament (Qui et al., 2016). A study conducted on men found a mutation in exon 6 of the DRD2 gene. This polymorphism was linked to personality disorders and hypertension (Rosmond et al., 2001).

Animals grazing endophyte-infected tall fescue have decreased prolactin concentrations in serum (Nihsen et al., 2004). Dopamine is known to inhibit prolactin secretion. The ergot alkaloids that are produced by the endophyte bind dopamine receptors (Larson et al., 1991). It was demonstrated by Henson et al. (1987) that wethers grazing endophyte-infected tall fescue and treated with a dopamine antagonist had decreased dopamine concentrations and increased prolactin serum concentrations. Equivalent to Henson, Lipham et al. (1989) showed an increase in serum prolactin in steers grazing endophyte-infected tall fescue when treated with a dopamine antagonist.

Campbell et al. (2014) associated a mutation with hair coat score; dopamine receptor D_2 genotypes were tested among steers grazing either endophyte-infected tall fescue or a novel variety and the relationship between genotypes and hair coat shedding scores. It was determined that animals carrying either one or two copies of the primary allele had lower hair coat scores when compared with the homozygous minor allele.

Melatonin

Molecular Mechanisms of Melatonin

Melatonin is an endocrine hormone that is produced by the pineal gland and known to help regulate circadian rhythms, sleep, and cellular growth (Rose, 1987). The essential amino acid tryptophan is converted to serotonin by tryptophan hydroxylase. Melatonin is synthesized

from serotonin by N-acetylserotonin (Bernard et al., 1999). Deficiencies of tryptophan in the diet can influence melatonin synthesis. Melatonin secretion is rhythmic with peak concentrations occurring during the night (Zimmerman, 1993). The seasonal day length is reflected on the duration of the peak (Gall et al., 2002). This daily rhythm can affect many physiological functions such as reproduction (Chemineau et al., 1992) and hair growth (Santiago-Moreno et al., 2004). Melatonin is not stored at significant concentrations within the body and upon synthesis, it is immediately released into the blood (Ganguly et al., 2002). Melatonin affects are mediated by two melatonin receptor subtypes, melatonin receptor 1a (MTNR1A) and melatonin receptor 1b (MTNR1b). Both receptor subtypes are found in mammals, members of the seven-transmembrane G protein-coupled receptor family, and they are located on the cells of the pituitary parstuberalis and suprachiasmatic nucleus (Gall et al., 2002). The two receptor types are different in that MTNR1A is coupled to different G proteins that mediate adenylyl cyclase inhibition and phospholipase Cβ activation. Receptor MTNR1B is also coupled to inhibition of adenylyl cyclase, but it inhibits the soluble guanylyl cyclase pathway (Gall et al., 2002; Reppert et al., 1996). A third subtype (Mel_{1c}) was found in the chicken brain but has not been found mammals (Gall et al., 2002).

Melatonin Genotypes

Several recent studies have shown that melatonin is involved in the regulation of hair growth and reproduction. A study by Santiago-Moreno et al. (2004) used the sheep model to examine whether mouflons are able to exhibit normal hair growth and shedding cycles when exposed to constant long day and short day photoperiods. Mouflon sheep were either held under normal photoperiod conditions or implanted with melatonin or were exposed to a

constant long day photoperiod. It was determined that sheep exposed to constant long days began the hair growth cycle two months earlier but had slower hair growth rate compared to control and implanted animals. This suggests that melatonin and prolactin depend on the cycle of growth and shedding (Santiago-Moreno et al., 2004). Melatonin affects are regulated by specific melatonin receptors; therefore, SNPs within the MTNR1A gene could affect HCS and productivity traits. Mutations in the MTNR1A gene have been studied in chicken, pig, Sarda goat, Rasa Aragonesa sheep, and Sika deer, and have shown significant relationships with reproductive activity (Li et al., 2013; Ramírz et al., 2009; Chu et al., 2007; Martínez-Royo et al., 2012; Yang et al., 2014). Yang et. al., associated MTNR1A genotypes with meat quality traits in Qinchuan cattle.

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Chapter 2

Associations of Single Nucleotide Polymorphisms in the Bovine Prolactin Gene with Productivity Traits in Beef Cattle

Introduction

Cattle productivity can be affected by multiple factors including heat stress and the consumption of forages containing ergot alkaloids (i.e. endophyte-infected tall fescue; E+). Most tall fescue (E+; [*Lolium arundinaceum* (Schreb.) S. J. Darbyshire]) plants are infected by an endophytic fungus (*Neotyphoduym coenophialum*) that produce ergot alkaloids. The E+ fescue is one of the most prominent forage grasses grown across the southeastern United States (Jackson et al., 1984). Ergot alkaloids can be detrimental to cattle reproductive efficiency because they inhibit the animal's ability to tolerate heat (Brown et al., 2000; Burke et al., 2001), as well as cause a decrease in calving percentage and weaning weight (Brown et al., 1993, 1997; Burke et al., 2001; Looper et al., 2009). One approach to solving reproductive inefficiency is to establish breeding programs that select animals with quality phenotypic traits. The negative effects of ergot alkaloids can be improved through heterosis and breed of cattle, demonstrating the relationship between reproductive efficiency and genetic linkage (Brown et al., 1997, 2000). Making early selection decisions could result in decreased development cost for replacement heifers.

There are various genes known to be related to economic traits in different species, and they can be used for selection criteria. These economic traits are controlled by several genes and are strongly affected by the environment. With the development of gene identification technologies, scientists are able to make accurate selection decisions. The study of mutations and their effects on economic traits, such as reproduction, are beneficial to producers and

inform them of the genetic value of an animal early in its life. One of those genes is prolactin which has been thought to play a role in cattle reproduction (Flores et al., 2008). Prolactin is a protein hormone secreted by the anterior pituitary. The active form of prolactin is primarily known for initiating lactation and mammary development, but it has multiple physiological roles. Prolactin receptors have been identified in the bovine granulosa cells (Lebedeva et al., 2001) and corpus luteum (Poindexter et al., 1979) suggesting a mechanism for prolactin's influence on reproduction. Bole-Feysot et al. (1998) demonstrated that mice were unable to conceive when the prolactin receptors were non-functioning. Prolactin may be an indicator of nutritional status and act as a communication limb between the reproductive system and brain (Flores et al., 2008).

Several mutations in the bovine prolactin gene (PRL) have been identified and associated with different traits. Milk fat and milk yield were associated with a SNP at bp 8398 (guanine to adenine tranversion) where heterozygous animals had the greatest milk yield, and the homozygous animals displayed the greatest fat content (Brym et al., 2005). More recently, polymorphisms in the promotor region have been associated with cattle productivity traits (Looper et al., 2010). Mutations at bp 1286 (cytosine to thymine transversion) and bp 1167 (adenine to guanine transversion) had an impact on calving percentages when animals were grazing either E+ or common bermudagrass. It was also determined that calves from AG cows were both heaver and taller at weaning than those from AA cows at the A1167T SNP site. The objective of this study is to evaluate two polymorphisms in the enhancer region and one mutation in the coding sequence of the PRL gene of cattle as potential biomarker of productivity traits in beef cows.

Materials and Methods

Animals and Management

Crossbred Angus-based cows (*n* = 170) grazed mixed grass pastures of common bermundagrass (*Cynodondactylon*(L.) Pers.) and endophyte-infected tall fescue (E+; *Lolium arundinaceum*(Schreb.) Darbysh). Animals had a*d libitum* access to trace mineral supplement and water. Cows were on a fall-calving schedule and averaged 5.4 ± 3 years of age.

Heifers (n = 136) and cows (n = 264) were estrous synchronized and artificially inseminated (AI) beginning in November then placed with a bull for approximately 60 d. Transrectal ultrasonography (Aloka 500v with 5-MHz transducer, Aloka Inc., Wallington, CT) was used approximately 70 d after initial AI to determine pregnancies resulting from AI or natural service, and overall pregnancy rates were determined by ultrasonography between d 100 and 115. Cows calved from August – December and calves were weaned at approximately 223 ± 39 d of age. At weaning, animals were vaccinated and dewormed.

Overall calving rate, cow pre-breeding weight, cow pre-breeding BCS, julian calving date, calf birth weight, cow BCS at weaning, cow weight at weaning and calf weight at weaning were recorded over three consecutive years (2012, 2013, and 2014). Calf weaning weights were adjusted using the 205-d adjusted weaning weight formula (BIF, 2010). Cow efficiency was calculated by dividing the 205-d adjusted weaning weight by the cow weight at weaning.

Blood from all cows were collected via jugular venipuncture in EDTA treated tubes and samples were placed on ice immediately after collection. Samples were centrifuged (2500 *g* for 25 min) and buffy coats harvested and stored at -20°C until DNA extraction.

DNA isolation and genotyping

Genomic DNA (gDNA) was extracted from buffy coats using the DNeasy blood and tissue kit (QIA-GEN, Valencia, CA). A Qubit® Fluorometer (Invitrogen, Carlsbad, California) was used to quantify DNA following purification. Genomic DNA (600ng) was pipetted into 96-well plates and placed in a drying oven (~ 50°C) overnight. Dried plates were covered with adhesive sealing film, mailed to Geneseek (Geneseek, Lincoln, NE) and genotypes determined using the sequenom technique.

Statistical Analysis

Data were analyzed using mixed model ANOVA (SAS inst. Inc., Cary, NC) with the main effects of genotype, year, and parity. A repeated measures analysis using the maximum likelihood method was utilized (Jennrich and Schluchter, 1986). The dependent variables were pre-breeding BCS, pre-breeding cow weight, Julian calving date, calf birth weight, calf weaning weight, 205-d adjusted weaning weight, cow BCS at weaning, cow weight at weaning, and cow efficiency. Each SNP was analyzed in separate models. Cows were divided into groups by age; young (≤ 3 years), adult (4 – 10 years), and mature adult (≥ 11 years). The experimental unit was animal. When *F*-tests for main affects were significant ($P < 0.05$), multiple t-tests and the Tukey's adjustment were performed to separate means.

Results and Discussion

Identification of polymorphisms

Two SNP sites within the promoter region and one SNP site within the coding sequence of the PRL gene were identified (Table 1).

Base position 1286

A transition from cytosine to thymine was detected at base 1286. One-hundred eleven cows were either heterozygous or homozygous with the minor allele (Table 2). Calving rate was affected ($P = 0.038$) by genotype (Table 3). Calf weaning weight and adjusted 205– d weight tended ($P \leq 0.10$) to be affected by genotype. Cow efficiency was affected ($P = 0.03$) by genotype (Table 4). An interaction between genotype and year was detected, however, there were no genotype differences within year.

Base position 1134

A transversion from adenine to thymine was detected at base 1134. Forty-one animals were heterozygous (Table 2). Calving rate tended ($P = 0.071$) to be affected by genotype (Table 3). Calf birth weight and calf weaning weight tended ($P \leq 0.10$) to be affected by genotype (Table 4). Calf weaning weight tended ($P = 0.07$) to be altered by cow age group, where the young homozygous A animals had the lowest weaning weights (Table 5).

Base position 8398

A transition from guanine to adenine was detected at base 8398. Eighty-six cows were either heterozygous or homozygous with the minor allele (Table 2). There were no genotype effects on any of the cattle productivity traits (Table 4).

Main Effects of Cow Age and Year

Main effects of year and cow age group affected ($P < 0.05$) cow pre-breeding BCS and weight, calf birth weight, weaning BCS, cow weight at weaning, calf weaning weight, adj. 205-d

weight, and cow efficiency (Table 5). Year affected ($P < 0.05$) pre-breeding BCS and weight, Julian calving date, calf birth weight, cow weight at weaning, calf weaning weight, adj. 205-d weight, and cow efficiency (Table 5).

Discussion

Prolactin consists of 198 amino acids and is part of the cytokine receptor superfamily. It is produced by the anterior pituitary and has more than 300 physiological functions (Bole-Feysot et al., 1998). The active form of prolactin is primarily known for initiating lactation and mammary development but has been associated with fecundity in cattle (Flores et al., 2008). A previous study by our lab demonstrated that C1286T had an impact on calving percentages when animals were grazing either endophyte-infected tall fescue or common bermudagrass (Looper et al., 2010). In the current study, polymorphisms associated with the prolactin gene were associated with calving rates. The precise mechanism of how the prolactin SNP altered calving rate was not studied; however, prolactin receptors have been identified in the bovine granulosa cells (Lebedeva et al., 2001) and corpora lutea (Poindexter et al., 1979)

Abnormal concentrations of circulating prolactin result in physiological dysfunction. In humans, patients suffering from osteoporosis and depression have greater serum concentrations of prolactin (Halbreich and Kahn, 2003). Treatments for abnormal prolactin concentrations are often difficult to control. Side effects include an imbalance of physical and hormonal responses such as weakened growth hormone release or insulin resistance (Melkersson et al., 1999). The prolactin SNP G8398A was associated with milk fat and milk yield in dairy cattle; specifically, heterozygous animals had the greatest milk yield and homozygous

cows displayed the greatest fat content (Brym et al., 2005). We did not evaluate growth hormone or insulin in this study; however, we did find SNP effects on calf weaning weights and cow efficiency which suggests the growth axis may have been altered. Additional studies will be conducted to determine the precise mechanism linking polymorphisms and productivity traits.

Our results confirm that cow age at calving affects weaning weights of cows and calves (Doornbos et al., 1984, Triplett et al., 1995, Singh et al., 1970). In our study, we categorized cows based on their age (≤ 3 years old, 4-10 years old, ≥ 11 years old) rather than parity. However, our results are consistent with the parity adjustments for 205-d weaning weights recommended by the Beef Improvement Federation (2010).

The relationship between BCS and reproductive performance has been well established. A BCS of approximately six for a 2 year old cow is considered ideal for reproductive success (Whittier et al., 2005; Spitzer et al., 1995). Both younger and older cows in this study had BCS that were less than ideal which may have led to compromised calving rates. The lower BCS for the youngest cow age group could be explained by continued growth and having not reached their mature weight. The lower BCS for the ≤ 3 year olds and ≥ 11 year old cows could also be due to number and placement of teeth (FSIS).

Conclusion

Mutations associated with the prolactin gene were associated with profitability traits in beef cattle. Two SNP sites located in the enhancer region were related to calf birth weight, calf weaning weight, as well as cow efficiency. This research confirms the effects of cow age on

cattle productivity. These results suggest that polymorphisms associated with the bovine PRL gene may be used as a selection tool for beef cattle sustainability.

Table 1. Mutations associated with the PRL gene of cattle

Polymorphism[a]	Sequence	Reference
C1286T	AGTGAACATGACTGT[C/T]TAGAATTTTGTTTTA	Looper et al., 2010b
A1134T	TCATCTCATTCAGGA[A/T]ATCTCTAAAAGGCAA	
G8398A	CCTAGTCACCGAGGT[G/A]CGGGGTATGAAAGGA	Brym et al., 2005

[a]Single nucleotide polymorphism occurred at the number indicated. First letter indicates the primary allele and the letter following the digits is the minor allele

Table 2. Distribution of SNP in the bovine PRL gene

Polymorphism[a]	Genotype distribution[b]			MAF[c]
	Homo	hetero	homo	
A1134T	129	41	0	12.1
C1286T	59	86	25	40.0
G8398A	84	68	18	30.6

[a]Single nucleotide polymorphism occurred at the number indicated. First letter indicates the primary allele and the letter following the digits is the minor allele

[b]Number of cows that were homozygous for the primary allele (Homo), heterozygous (hetero), and homozygous for the minor allele (homo)

[c]Minor allele frequency expressed as percent

Table 3. Overall calving rate for three consecutive years (2012 - 2014)

Polymorphism[1]	Genotype	*n*[2]	% calved	*P*-value
C1286T	CC	157	94[a]	0.038
	CT	217	88[b]	
	TT	62	95[a]	
A1134T	AA	331	92	0.071
	AT	105	87	
G8398A	GG	229	93	0.148
	GA	163	88	
	AA	44	91	

[1]Single nucleotide polymorphism occurred at the number indicated. First letter indicates the primary allele and the letter following the digits is the minor allele

[2]*n* = total number of animals that calved over 3 consecutive years (2012, 2013, 2014)

[a-b]Means without common superscript differ ($P < 0.05$)

Table 4. Main effects of Prolactin SNP on bovine productivity traits

	C1286T[a]					A1134T				G8398A				
Productivity Traits[b]	CC	CT	TT	SEM	*P*-value	AA	AT	SEM	*P*-value	GG	GA	AA	SEM	*P*-value
No.[c]	148	190	59	-	-	306	91	-	-	214	143	40	-	-
Pre-Breeding														
BCS	5.4	5.4	5.4	0.13	0.96	5.5	5.3	0.13	0.28	5.4	5.5	5.4	0.12	0.64
Weight, kg	516	519	513	9.17	0.89	518	518	8.89	0.96	514	520	496	11.7	0.48
Calving														
Julian	268	270	267	2.72	0.73	269	267	2.60	0.62	267	270	267	2.14	0.50
Birth Weight, kg	35	32	34	0.93	0.12	35[a]	31[b]	0.93	0.007	33	34	34	0.92	0.76
Weaning														
BCS	4.9	5.0	5.0	0.10	0.76	5.0	4.7	0.10	0.11	4.9	5.1	5.1	0.08	0.33
Cow Weight, kg	496	498	486	9.16	0.64	499	489	10.52	0.55	490	501	501	8.66	0.65
Calf Weight, kg	204[yz]	194[z]	208[y]	5.12	0.08	203[y]	190[z]	4.89	0.08	196	200	204	4.29	0.41
Adj. 205 Weight, kg	214[yz]	203[z]	215[y]	4.16	0.07	212[a]	196[b]	4.08	0.01	205	209	214	3.93	0.32
Cow Efficiency	44.4[ab]	41.7[b]	45.5[a]	1.15	0.03	43.7[a]	40.8[b]	0.98	0.04	43.3	42.7	44.4	1.13	0.54

[a]Single nucleotide polymorphism occurred at the number indicated. First letter indicates the primary allele and the letter following the digits is the minor allele

[b]BCS = Body Condition Score; Cow efficiency calculated by dividing the Adj. 205 Weight by cow weight at weaning

[c]No. = total number of animals over 3 consecutive years (2012, 2013, 2014)

Table 5. Main effects of cow age group and year on bovine productivity traits

	Cow Age Group[a]					Year				
Productivity Traits[b]	≤ 3	4 - 10	≥ 11	SEM	*P*-value	2012	2013	2014	SEM	*P*-value
No.[c]	136	238	26	-	-	115	126	159	-	-
Pre-Breeding										
BCS	5.3[b]	5.7[a]	5.2[b]	0.16	0.01	5.1[b]	5.4[b]	5.7[a]	0.12	0.001
Weight, kg	460[b]	554[a]	534[a]	8.03	0.001	507[b]	494[b]	547[a]	7.91	0.001
Calving										
Julian Date	267	270	266	2.50	0.43	263[b]	268[ab]	273[a]	2.58	0.02
Birth Weight, kg	31[b]	35[a]	36[a]	0.82	0.001	33	34	34	0.78	0.63
Weaning										
BCS	5.0[ab]	5.2 [a]	4.7[b]	0.13	0.01	5.0	4.9	5.0	0.09	0.59
Cow Weight., kg	455[b]	513[a]	513[a]	10.4	0.001	475[b]	502[a]	504[a]	7.67	0.001
Calf Weight, kg	182[b]	219[a]	204[a]	6.67	0.001	205[a]	189[b]	212[a]	4.52	0.001
Adj. 205 Weight, kg	217[a]	206[b]	209[ab]	3.70	0.006	207[b]	199[b]	226[a]	4.84	0.001
Cow Efficiency	49.3[a]	41.1[b]	41.0[b]	1.01	0.001	45.0[b]	40.6[b]	45.9[a]	0.94	0.001

[a]Cow age groups = ≤ 3 years of age, young; 4 – 10 years of age, adult; ≥ 11 years of age, mature adult

[b]BCS = Body Condition Score; Cow efficiency calculated by dividing the Adj. 205 Weight by cow weight at weaning

[c]No. = total number of animals over 3 consecutive years (2012, 2013, 2014)

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Chapter 3

Associations of Single Nucleotide Polymorphisms in the Bovine Melatonin Receptor 1A and Dopamine D_2 Receptor Gene with Hair Coat Scores in Beef Cattle

Introduction

Tall fescue [*Lolium arundinaceum* (Schreb.) S. J. Darbyshire], a cool-season perennial bunch grass, is a prevalent forage grown across the Southeastern United States (Jackson et al., 1984; Roberts et al., 2005). Most tall fescue plants are infected with an endophytic fungus (*Neotyphodium coenophialum*) which enhances the persistence of that plant (Hill et al., 1991); however, ingestion of the ergot alkaloids that are produced by the endophyte are the primary cause of fescue toxicity in grazing animals (Roberts et al., 2005).

Animals grazing pastures with ergot alkaloid contamination demonstrate symptoms such as loss of body condition score, decreased cow productivity (Brown et al., 2000), and rough hair coats during the summer (Aiken et al., 2011; Strickland et al., 1993). On average, cows who shed their winter coats earlier in the summer wean heavier calves (Gray et al., 2011). Decreased serum prolactin is a physiological response to fescue toxicosis (Nihsen et al., 2004). The dopamine D_2 receptor (DRD2) is responsible for the inhibition of prolactin secretion (Civelli et al., 1993). Campbell et al. (2014) examined a polymorphism within the DRD2 gene and found genotype effects on serum prolactin concentrations and hair coat score (HCS) in Angus-based steers grazing endophyte infected tall fescue.

Melatonin, the pineal hormone, modulates hair growth in many species (Fischer et al., 2008). Yang et al. (2015) evaluated single nucleotide polymorphisms (SNP) within the melatonin receptor 1a gene (MTNR1A); they determined that MTNR1A could serve as candidate gene for body measurement and meat quality traits in Qinchuan cattle. The linkage between retention

of hair, DRD2 and prolactin concentrations; and MTNR1A and hair growth, suggests these genes may serve as candidates for biomarkers of stress tolerant animals. Associations among MTNR1A polymorphisms and HCS are unknown. Therefore, understanding the phenotype-genotype relationships associated with MTNR1A and DRD2 may lead to profitable selection tools for producers. Objectives of this study were to evaluate mutations within MTNR1A and DRD2 genes, and determine their relationship to hair coat shedding.

Materials and Methods

Animal Management

Crossbred Angus-based cows (n=104), located at the University of Arkansas Cow Calf Unit, were managed on mixed grass pastures of predominantly endophyte-infected tall fescue (E+; *Lolium arundinaceum* (Schreb.) Darbysh and common bermundagrass (*Cynodon dactylon* (L.) Pers.). Animals had a*d libitum* access to water and trace mineral supplement. Cows were managed on a fall-calving schedule.

Data Collection

Hair coat scores (HCS) were collected over three consecutive years, 2012 – 2014. Scores were determined in approximately 30-d intervals by a trained technician. Each cow's coat score was evaluated and scored on a scale from 1 to 5 (Table 1; Turner and Schlegar, 1960; Williams et al., 2006). In 2012, HCS were collected March – November; in 2013, HCS were collected April – December; in 2014, HCS were collected from January – July.

Blood samples were collected from cows by jugular venipuncture in EDTA treated tubes. Samples were centrifuged (2500 *g* for 25 min) and buffy coats harvested and stored at -20°C until DNA extraction.

DNA isolation and sequencing

Buffy coats were used to extract genomic DNA (gDNA) using the DNeasy blood and tissue kit (QIA-GEN, Valencia, CA). Following purification, DNA was quantified using a Qubit® fluorometer (Invitrogen, Carlsbad, California). Specific primers were designed for PCR amplification of an 845-base segment of the bovine MTNR1A gene based on the genome sequence of *Bos Taurus* (GenBank accession number EU716174 region 91 to 1009). Primers [forward (MTNR1A165F; 5'-TAGTTAACGATGGGTGGAGC-3') and reverse (MTNR1A990R; (5'-AAATGAGTAAGGCTTGGAGC-3') (Yang et. al, 2015)] were synthesized and supplied by integrated DNA technologies (IDA; Coralville, IA) and used for DNA amplification via polymerase chain reaction (PCR). A Bio-Rad C1000 Touch™ thermal cycler (Hercules, CA) was used for DNA amplification (Table 2). Amplification (Table 2) of a 793- base segment of the bovine DRD2 gene （GenBank Accession number, NW_003104408.1 regions: 2869399-2870868; Campbell et al., 2014) was performed using the following primers: 5'-TATAGCCCCATTCCTGATTC-3' and 5'-CCCATGCTCTACAACACACG-3' supplied by IDA (Coralville, IA). Included in each PCR was 250 ng of gDNA, 0.20 µM of each primer, and 45 µL of Platinum PCR Supermix (Invitrogen, Carlsbad, CA) for a total volume of 50 µL. All amplification products were verified using 1.2% agarose gels stained with ethidium bromide and purified using the GenScript QuickClean II PCR Extraction kit (Piscataway, NJ). Following purification, PCR products were prepared for forward strand sequencing using Eurofins SimpleSeq (Louisville, KY). Sequences were compared using *MEGA* version 6 (Tamura et al., 2013).

Statistical analysis

Data were analyzed using mixed model ANOVA (SAS inst. Inc., Cary, NC) with the main effects of year, month, and genotype. Overall means for HCS over 12-months are reported (Figure 1). A repeated measures analysis using the maximum likelihood method was utilized (Jennrich and Schluchter, 1986). The dependent variable was HCS over 4 months (April – July). When *F*-tests for main effects were significant ($P < 0.05$), multiple t-tests and the Tukey's adjustment were performed to separate means

Results and Discussion

Identification of polymorphisms

An 845-base segment of the bovine MTNR1A (Yang et. al, 2015) was amplified and sequenced and five transitions were identified (Table 3). Mutation site identification is based on the base pair number in the NCBI deposited region, the first letter indicates the primary allele and the letter following the digits is the minor allele. The SNP sites A541G, G575A, T679C, and C721T appear to be linked; similarly found by Yang et al. (2015). The SNP sites selected for further analysis were A541G and A583G (adenine transition with guanine). A 793- base segment of the bovine DRD2 gene (Campbell et al., 2014) was amplified and sequenced, and a transition from adenine to guanine was detected at base 534 (A534G) (Table 3).

MTNR1A base position 541

A transition from adenine to guanine was detected at base position 541. Ten animals were either heterozygous or homozygous for the minor allele (Table 4). Neither HCS nor calving rate were affected ($P > 0.12$) by A541G or its interaction with month (Table 5).

MTNR1A base position 583

A transition from adenine to guanine was detected at base position 583. Seventy-two animals were either heterozygous or homozygous for the minor allele (Table 4). Cows with the GG genotype had a greater ($P < 0.05$) HCS in the month of May when compared to cows with the AA genotypes; cows with the AG genotype did not differ from either genotype. However, no other differences between genotypes were detected (Figure 2). Cows with one or two copies of the G allele had a greater ($P = 0.0008$) calving rate when compared to homozygous A cows (Table 5).

DRD2 base position 534

A transition from adenine to guanine was detected at base position 534. Seventy-three animals were either heterozygous or homozygous for the minor allele (Table 4). Hair coat scored tended (P = 0.10) to be affected by month and genotype (Figure 3). Homozygous AA cows had the greatest HCS in the month of June.

Discussion

Melatonin's physiological effects are mediated by specific melatonin receptors, MTNR1A. We observed that cow fecundity was associated with genotype at SNP site A583G within the MTNR1A gene. Those findings are consistent with research in chicken, pig, Sarda goat, and Rasa Aragonesa sheep (Li et al., 2013; Ramírz et al., 2009; Chu et al., 2007; Martínez-Royo et al., 2012). Ewes administered melatonin had decreased atresia rate for large follicles, increased ovulation rate, and increased embryo viability (Bister et al., 1999; Forcada et al., 2006). We did not evaluate circulating melatonin concentrations in this study; however, we did

find SNP effects on calving rates which suggests the reproductive axis may have been altered. Additional studies will be conducted to determine the precise mechanisms linking polymorphisms and calving rates.

Relationships between circulating melatonin concentrations and hair growth is well established (Santiago-Moreno et al., 2004). In this study, we are the first to report an association between MTNR1A genotypes and HCS; however, similar SNP sites have been associated with antler growth in Sika deer (Yang et al., 2014). The precise mechanisms of how melatonin affects HCS were not evaluated, but that will be the subject of future research.

Prolactin is involved in winter hair coat shedding (McClanahan et al., 2008). Circulating prolactin concentrations increase as the day length increases and decreases as the day length becomes shorter (Karg and Schams, 1974). Elevated circulating prolactin concentrations during the summer months leads to winter hair coat shedding (Porter and Thompson, 1992). A low HCS in the summer months allows cattle to better dissipate heat and can reduce the effects of heat stress (Olsen et al., 2003). Gray et al. (2011) demonstrated that cows who shed their winter coats by June 1st weaned heavier calves.

Prolactin is inhibited by dopamine, which indirectly links dopamine to hair growth (Ben-Jonathon and Hnasko, 2001). When cattle were grazing endophyte-infected tall fescue, HCS was associated with genotype at SNP site A534G in the DRD2 gene (Campbell et al., 2014). In our study, we observed that HCS tended to be affected by an interaction between genotype at A534G and month of year. While our results are not in complete agreement with those of Campbell et al. (2014), the two studies suggest that genotype within the DRD2 gene may be

associated with HCS. Many factors affect HCS and cattle productivity such as calving season and forage management (Caldwell et al. 2013; McClanahan et al., 2008). Those factors may have led to the inconsistencies in our work and Campbell et al. (2014). Future research will be designed to account for the potential interactions between genotypes and management techniques.

Conclusion

Our results demonstrated that A583G genotypes in the bovine MTNR1A gene were associated with HCS and calving rate in Angus-based crossbred cows. This suggests that mutations in MTNR1A could be used as molecular markers for selection of HCS and productivity traits in beef cattle.

Table 1. Description of hair coat scores (HCS)

HCS	Description
5	Full winter coat, 0% shed
4	Initial shedding, 25% shed
3	Half way shed, 50% shed
2	Almost shed, 75% shed
1	Slick summer coat, 100% shed

(Gray et al., 2011; Turner and Schlegar, 1960; Williams et al., 2006)

Table 2. Summary of PCR conditions for the bovine *MTNR1A* and *DRD2* genes

Gene	Accession number[a]	Primer sequence[b]	Cycle	Reference
MTNR1A	EU716174 region: 91 - 1009 Length: 845bp	F 165[c]: 5'-TAGTTAACGATGGGTGGAGC-3' R 990: 5'-AAATGAGTAAGGCTTGGAGC-3'	94°C for 5min (94°C for 1 min 62° for 1 min 72°C for 1 min) x35 72°C for 10 min 8°C infinite hold	Yang et al., 2015
DRD2	NW_003104408.1 region: 2869399 - 2870868 Length: 793bp	F 1[d]: 5' TATAGCCCCATTCCTGCTTC – 3' R 793: 5' – GCCCATGCTCTACAACACACG – 3'	94°C for 2 min (94° for 30 sec 58°C for 30 sec 68°C for 30 sec) x35 68°C for 10 min 4°C infinite hold	Campbell et al., 2014

[a]Based on the GenBank accession number (NCBI)
[b]Primers provided by Integrated DNA technologies (Coralville, IA)
[c]Based on the sequence region
[d]Based on the first base of the forward primer sequence

Table 3. Mutation sites within MTNR1A and DRD2 genes

[a]Polymorphism	Sequence
[b]MTNR1A	
A541G	GAGAATCTGGGCCCT(A/G) GTTCTTCAGGTCAGA
G575A, A583G	AGGGTGAAACCTGAC(G/A)ACAAACC(A/G)AAACTGAAGCCCCAG
T679C	GAACTTCATTGGTCT(T/C)GTTGTGGCCTCGGAC
C721T	GGCACCCAGGATCCC(C/T)GAGTGGCTGTTTGTG
[c]DRD2	
A534G	ATCCAGGAGACCGGA(A/G)TCACCCTGACCCAGG

[a]Single nucleotide polymorphism occurred at the number indicated. First letter indicates the primary allele and the letter following the digits is the minor allele.

[b]SNPs are shown relative to the GenBank accession number: EU716174 REGION: 91 – 1009. Numbering is based on the sequence region. (Yang et al., 2015).

[c]SNPs are shown relative to the GenBank Accession number, NW_003104408.1 regions: 2869399-2870868. Numbering based on the first base of the primer sequence. (Campbell et al., 2014).

Table 4. Distribution of SNPs in the bovine *MTNR1A* and *DRD2* genes

	Genotype distribution[b]			
Polymorphism[a]	Homo	hetero	homo	MAF[c]
MTNR1A				
A541G	94	10	0	4.8
A583G	32	48	24	46.2
DRD2				
A534G	29	51	22	46.6

[a] Single nucleotide polymorphism occurred at the number indicated. First letter indicates the primary allele and the letter following the digits is the minor allele.

[b] Number of cows that were homozygous for the primary allele (Homo), heterozygous (hetero), and homozygous for the minor allele (homo).

[c] Minor allele frequency expressed as percent.

Table 5. Overall calving rate for three consecutive years (2012-2014)

Polymorphism[1]	Genotype	[2]*n*	% calved	*P*-value
[3]MTNR1A				
A541G	AA	282	93	0.12
	AG	30	100	
A583G	AA	72	97[a]	0.0008
	AG	144	88[b]	
	GG	96	99[a]	
DRD2				
A534G	AA	87	93	0.76
	AG	153	93	
	GG	66	95	

[1]Single nucleotide polymorphism occurred at the number indicated. First letter indicates the primary allele and the letter following the digits is the minor allele

[2]*n* = number of animals that calved over 3 consecutive years (2012, 2013, 2014)

[3]Melatonin Receptor 1A; Dopamine Receptor D2.

[a-c]Means without common superscript differ ($P < 0.05$).

Table 6. Hair coat score[a] minimum and maximum over three years (2012 – 2014).

Month	Minimum	Maximum	SE
January	5	5	0
February	5	5	0
March	4	5	.12
April	2	5	.44
May	1	5	.86
June	1	5	1.1
July	1	5	.56
August	1	4	.47
September	1	3	.33
October	1	4	.67
November	1	5	1.29
December	3	5	.49

[a]Based on a scale of 1-5; 5- 0% shed, 1-100% shed. (Turner and Schlegar, 1960; Williams et al., 2006).

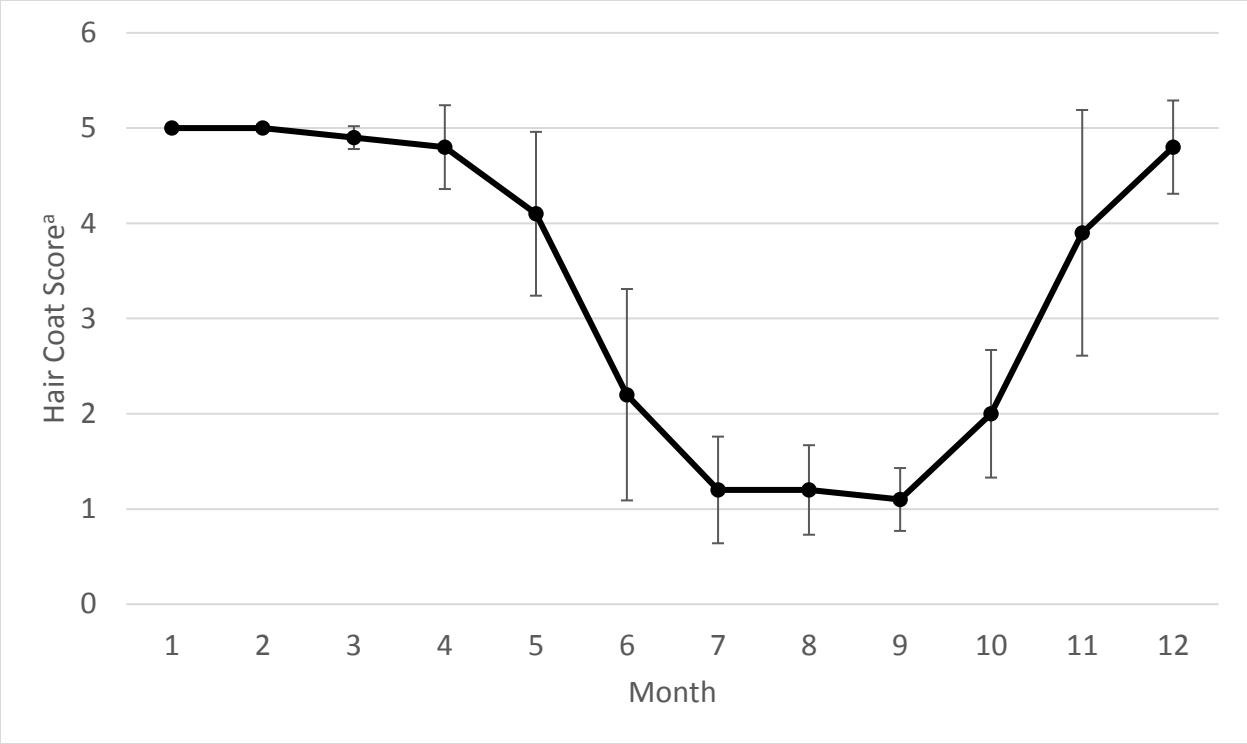

Figure 1. Average hair coat score over three consecutive years (2012 – 2014).
[a]Based on a scale of 1-5; 5- 0% shed, 1- 100% shed.

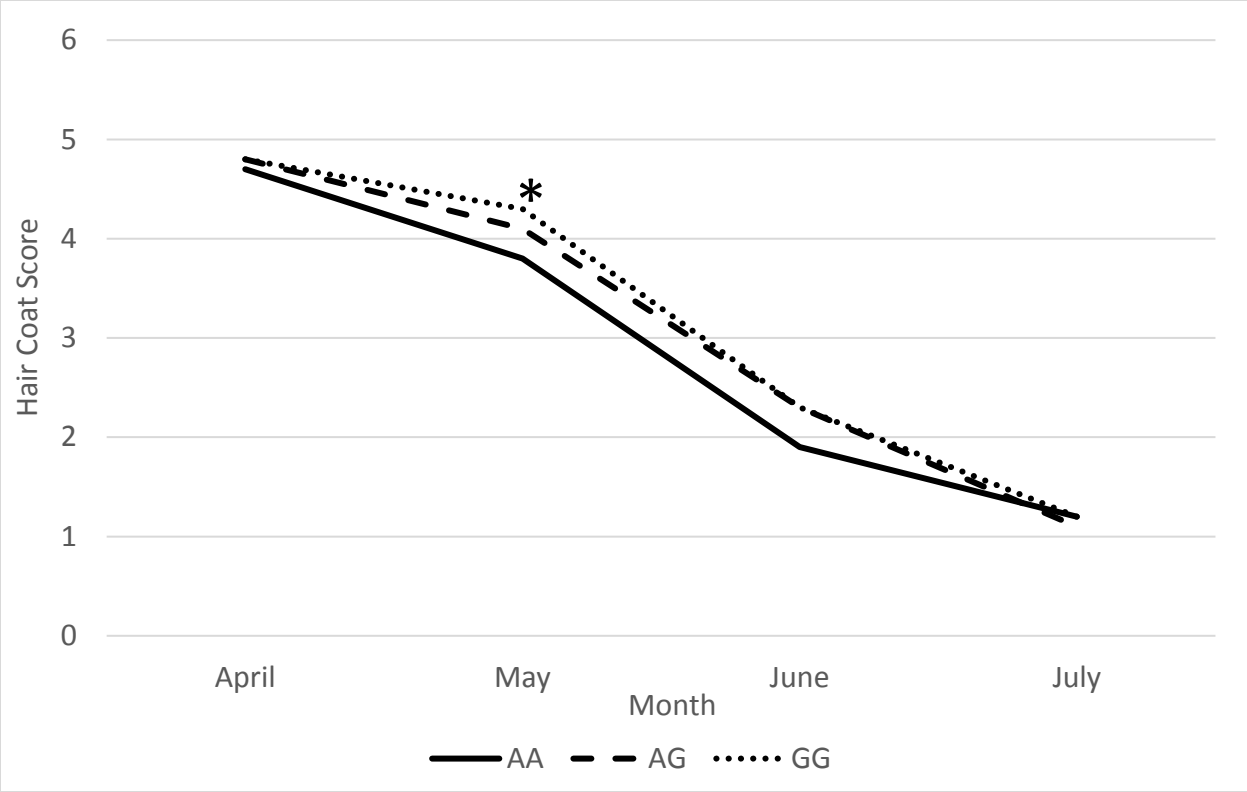

Figure 2. Association of MTNR1A SNP A583G with hair coat score over a 4-month period (April – July) averaged over three consecutive years (2012, 2013, and 2014). *Denotes a difference between genotype within month, *P* – value = 0.0024 SEM = 0.09.

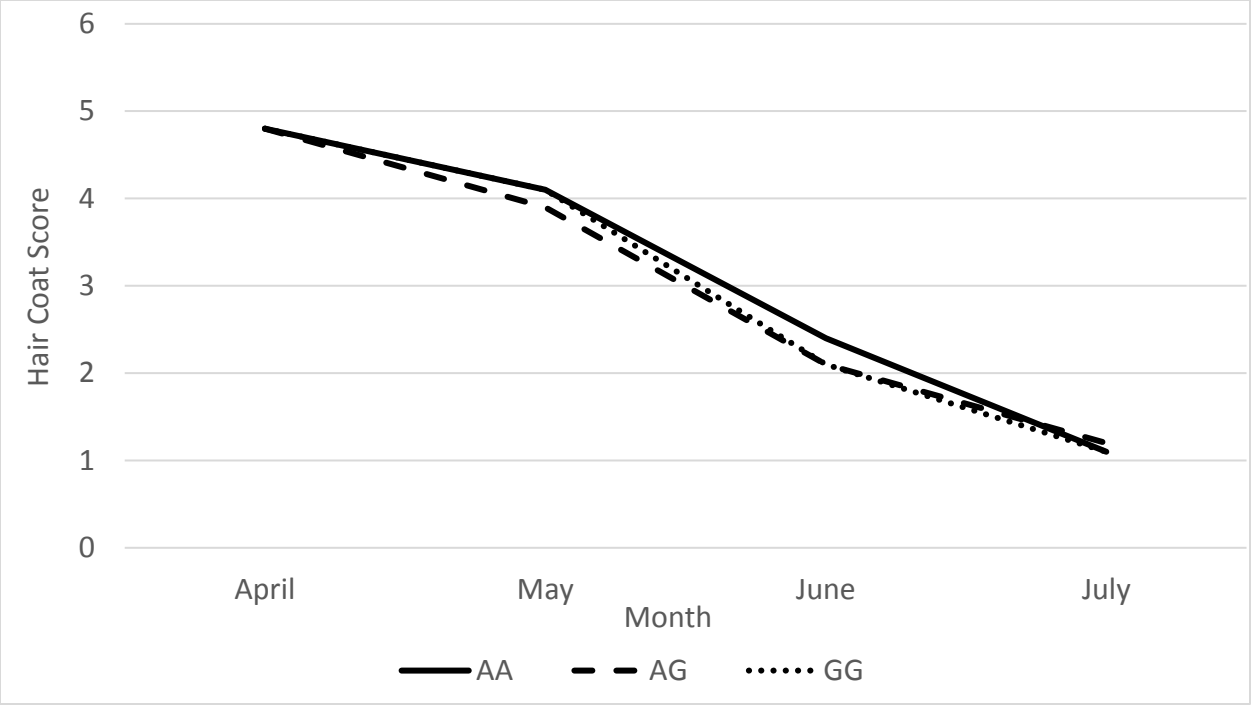

Figure 3. Association of DRD2 SNP A534G with hair coat score over a 4-month period averaged over three consecutive years (2012, 2013, and 2014), *P* – value 0.10 SEM = 0.09.

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Chapter 4

Conclusion

Three SNP sites (C1286T, A1128T, and G8398A) associated with the prolactin gene, two SNP sites (A541G and A583G) within the melatonin receptor 1a gene, and one SNP site (A534G) within the dopamine receptor D_2 gene were evaluated in this study. Two SNP sites located in the enhancer region of the prolactin gene were related to calf birth weight, calf weaning weight, as well as cow efficiency. The A583G SNP site in the bovine MTNR1A gene was associated with hair coat score and calving rate in Angus-based crossbred cows. These results suggest that polymorphisms associated with these genes can be used as molecular biomarkers for selection of productivity traits and hair coat score in beef cattle.

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: A. H. Brown

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: July 3, 2013

SUBJECT: IACUC Protocol **APPROVAL**
Expiration date : **July 2, 2016**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#13062 - "Genetic Considerations for Beef Cattle Production in Challenging Environments"**. You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **07-02-2016** you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

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