Genetic Associations of Beef Cattle with Fertility and Rangeland Behavior

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Morgan R. Stegemiller

Major Professor: Brenda Murdoch, Ph.D.

Committee Members: John Hall, Ph.D.; James Sprinkle, Ph.D.

Department Administrator: Robert Collier, Ph.D.

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Authorization to Submit Thesis

This thesis of Morgan R. Stegemiller, submitted for the degree of Master of Science with a Major in Animal Science and titled "Genetic Associations of Beef Cattle with Fertility and Rangeland Behavior," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

Major Professor: _____   Date: _____
                                 Brenda Murdoch, Ph.D.

Committee Members: _____   Date: _____
                                 John Hall, Ph.D.

                                 _____   Date: _____
                                 James Sprinkle, Ph.D.

Department
Administrator: _____   Date: _____
                                 Robert Collier, Ph.D.

Abstract

Selecting for efficient replacement females is a crucial decision for cattle producers. Replacement females need to be reproductively efficient and if the producer pasture grazes their animals, they also need to have efficient rangeland behavior. Genetic associations with important traits can help understand the biology behind the traits and be used to make selection decisions. One study identified genetic associations with cattle fertility traits, antral follicle counts and reproductive tract scores in crossbred heifers. The second study examines cattle rangeland behavior traits, grazing minutes, walking minutes, and maximum slope use while experiencing mild heat stress. In addition, a significant association was identified with the amount of time cows spent on slopes greater than 15 degrees regardless of experiencing heat stress. Identifying the genetic associations can deepen the understanding of the biology behind significant traits as well as enable producers to select for replacement females that will benefit their management strategies.

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Dedication

I would like to dedicate this thesis to my parents and sisters for their love and support no matter the distance. I would also like to thank my friends for always being there for me. Finally, I would like to thank all my new friends here in Idaho for accepting me and taking care of me during this adventure.

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List of Abbreviations

AFC	Antral follicle count
AMH	Anti-Mullerian hormone
CL	Corpus luteum
FSH	Follicle stimulating hormone
GPS	Global positioning system
GWAS	Genome-wide association study
HWE	Hardy-Weinberg equilibrium
LD	Linkage disequilibrium
LH	Luteinizing hormone
MAF	Minor allele frequency
MLMM	Multi-locus mixed model
OR	Ovarian reserve
PCA	Principal component analysis
PCOS	Polycystic ovary syndrome
PVE	Proportion of variance explained
ROS	Reactive oxygen species
RTS	Reproductive tract scores
SLMM	Single-locus mixed model
SNP	Single nucleotide polymorphism
THI	Temperature humidity index

Chapter 1: Literature Review

Introduction

The beef cattle industry is a major agricultural industry in the United States. Beef cattle production alone represents $43 billion (Briske et al., 2021). There are many components to the beef cattle industry. These include cow-calf operations, stocker and feedlots, and beef processing. In the United States the beef cattle industry produces 11.98 million metric tons of beef a year (Briske et al., 2021).

In cow-calf operations, the selection of replacement females is a vital process to ensure the most valued females are selected to become the next generation. Producers need replacement females to have good herd longevity by conceiving at a young age and every subsequent year. For producers that pasture graze, it is also important for them to choose replacements that will be efficient on pasture (Endres & Schwartzkopf-Genswein, 2018). The selection of replacement animals that are efficient both reproductively and on pasture would be beneficial for many producers.

Cows need to have a successful pregnancy every year to prevent the losses producers can accrue by keeping an open female in the herd. Factors that affect cattle reproduction efficiency include age at puberty, conception rates, duration between parturition and conception, and lifetime productivity (Burns et al., 2010). Females that have their first calve at two years of age, on average produce 0.7 more calves throughout their lifetime than females that calve first at three years of age. The economic efficiency between the heifers that calve at two versus three years of age is 6-8% greater (Day & Nogueira, 2013). It is important for producers to have reproductive efficient animals so that their production operations are sustainable.

Raising cattle can also be costly, with one of the largest expenses being feed. In the United States approximately 27% of the land is rangeland that producers can use to raise their cattle. While some producers raise cattle using intensive or semi-intensive systems, others capitalize on more extensive system and graze public and/or private land for their cattle. When raising cattle on pasture it is beneficial for cattle to utilize the whole pasture so that they are the most efficient with the land (Bailey, 2005). For this to occur there needs to be an even grazing distribution so that the land available is used to the greatest advantage.

Reproductive Biology in Female Cattle

Folliculogenesis

Reproduction is essential for the sustainability of the cattle industry and in order for a heifer or a cow to become pregnant she must first be able to ovulate an egg that is competent and able to be fertilized. This begins with a developing oocyte and the follicle that develops around it, a depiction of this process is shown in **Figure 1.1**.
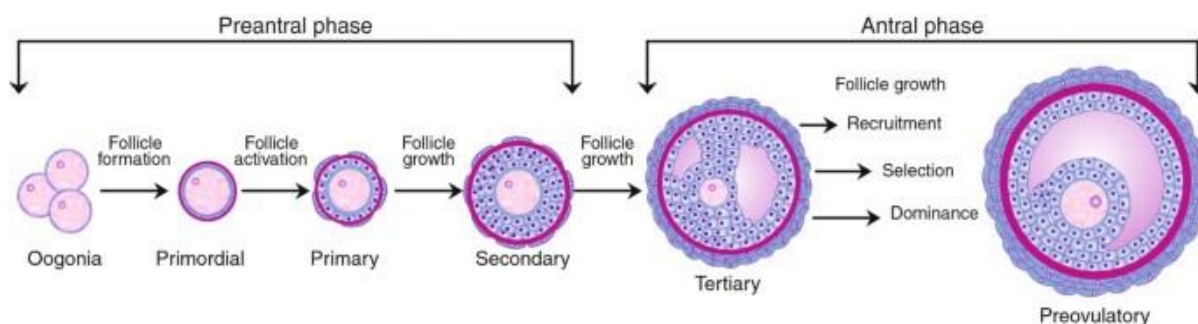
Figure 1.1: Follicular development from oogonia to preovulatory follicle (adapted from Araujo et al., 2014).

The ovarian reserve (OR) is made up of primordial follicles which each contain an oocyte arrested at meiotic prophase I (Aerts & Bols, 2010). The OR in cattle is complete at birth and folliculogenesis can begin before the heifer is born (Aerts & Bols, 2010; Garverick et al., 2010). These primordial cells have a single layer of squamous cells surrounding them

as depicted in **Figure 1.1** (Senger, 2005). Out of the OR a cohort of cells are recruited for development (Gigli et al., 2006). Anti-Müllerian hormone (AMH) functions to inhibit activation of primordial follicles to prevent early follicular growth (Fortune et al., 2013). This is important because without AMH, the OR can be depleted quickly leading to infertility.

After activation, follicles go through gonadotropin independent growth where they do not require gonadotropins to stimulate their growth (Baerwald, 2009). Primordial cells first develop into primary follicles and at this stage the single layer of granulosa cells, become cuboidal in shape (Senger, 2005). Secondary follicles are the next stage and are classified by having two or more layers of cuboidal granulosa cells (Fair, 2003). In addition, the oocyte here has developed a zona pellucida. This helps to enable communication and protect the oocyte.

A secondary follicle develops into an antral follicle which is characterized by the fluid filled cavity called an antrum. At this stage the follicle becomes gonadotropin dependent and requires gonadotropins to continue development into the antral follicle stage (Scaramuzzi et al., 2011). This is shown in the second half of **Figure 1.1** under the antral phase. For the antrum to develop, follicle stimulating hormone (FSH) is needed (Aerts & Bols, 2010). An antral follicle has three layers of cells surrounding them consisting of an external and internal thecal cell layer, and a granulosa cell layer. In this stage, oocytes in antral follicles are meiotically competent (Senger, 2005). Once a follicle reaches the antral stage it can continue developing or at any point can undergo atresia.

Follicular development happens in waves and a cohort of small antral follicles are recruited together to develop. This recruitment wave is proceeded by a rise in FSH (Adams et al., 1992). After follicles are recruited, they can continue growing and be classified as

selected follicles (Baerwald, 2009). Some follicles will progress and eventually develop into dominant follicles. During the recruited and selected stages the follicle requires FSH, however luteinizing hormone (LH) levels increase just before the follicle becomes a dominant follicle and the other selected follicles undergo atresia. The cohort of recruited follicles that did not become a dominant follicle help to make the transition from FSH to LH dependence by secreting inhibin to suppress FSH (Ginther et al., 2001). Dominant follicles produce estradiol and the increase in estradiol concentration provides a negative feedback on FSH and induce granulosa cells to form LH receptors (Beg et al., 2002). The increase of estradiol production during dominant follicle growth continues until it peaks on the day of the LH surge which induces ovulation (Baerwald, 2009). In cattle, there can either be two or three follicular waves in a cycle (Knopf et al., 1989). The waves that include the development of a follicle into dominant follicle are major waves. In cattle all waves are major waves. The first one or two waves per cycle are anovulatory waves because the dominant follicle ends up regressing. The final wave is an ovulatory wave as the dominant follicle will ovulate at the end of the wave (Ginther et al., 1989).

Estrous Cycle

The estrous cycle is the hormonal cycle that pubertal females experience. The estrous cycle is divided into two main phases, the follicular phase and the luteal phase (Figure 1.2). The luteal phase begins with ovulation and the subsequent development of a corpus luteum (CL) and ends with the regression of the CL. The follicular phase begins with the regression of the CL and is characterized by the growing follicles and ends with ovulation.
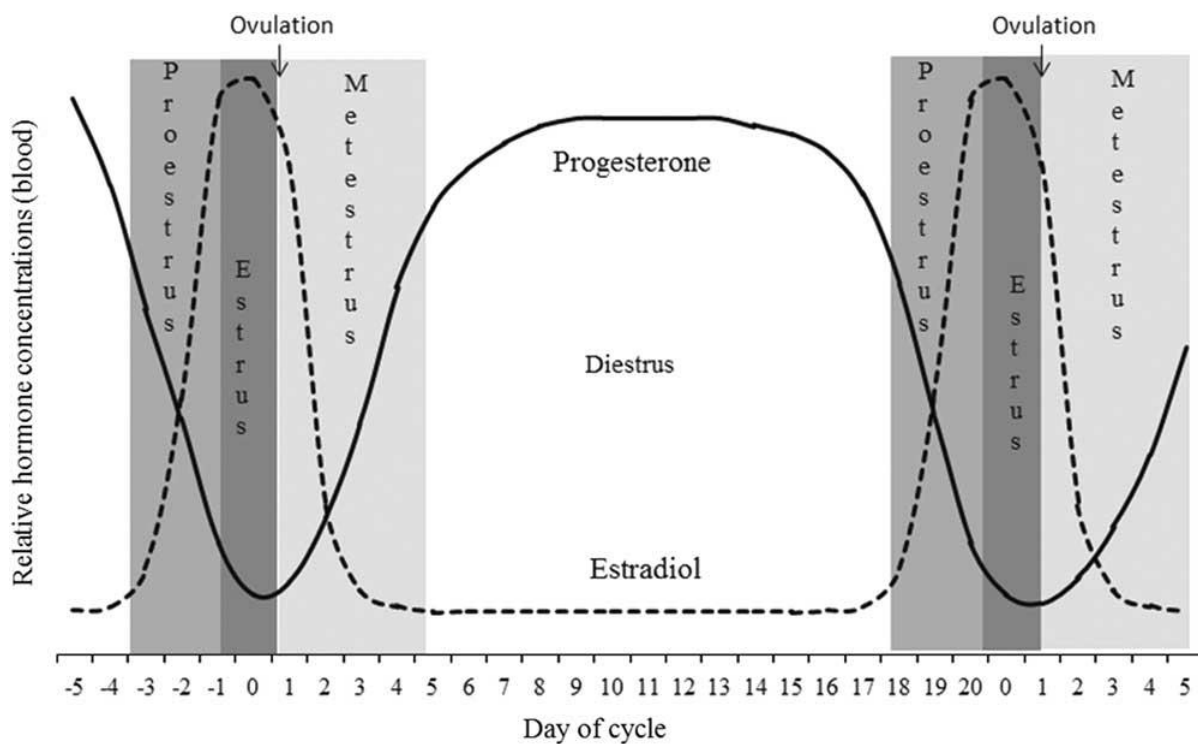
Figure 1.2: Estrous cycle of cattle (adapted from Reith and Hoy, 2017).

      The luteal phase is divided into two stages, metestrus and diestrus. Metestrus is the first stage which follows ovulation and involves the formation of the CL. The CL secretes progesterone so at the beginning of metestrus progesterone levels will be low and then will increase throughout the stage as the CL develops as shown in **Figure 1.2**. Diestrus occurs once the CL is formed and then ends with CL destruction which is called luteolysis. Luteolysis is caused by an increase in prostaglandin $F_{2\alpha}$ which increases towards the end of diestrus (Arosh et al., 2004). As shown in **Figure 1.2** there is a high level of progesterone throughout diestrus but as luteolysis occurs, progesterone levels start to decline at the end of diestrus. During diestrus the two to three follicular waves occur. As these follicular waves require FSH, two FSH waves will occur as well.

      At luteolysis, the follicular phase will begin. The two stages of the follicular phase are proestrus and estrus. During proestrus antral follicles mature and one pre-ovulatory follicle

progresses. Dominant follicles produce estradiol so as progesterone declines there is an increase in estradiol. During estrus there is a peak of estradiol being produced from the follicles. There is also a physical sign of estrus that the female is sexually receptive to the male. This stage ends with ovulation and the luteal phase begins again. The LH surge induces ovulation, so as luteolysis occurs and there is a reduction of progesterone, LH concentration levels decrease and will reach their peak during the estrus phase (Rahe et al., 1980). The whole of the estrous cycle takes between 17 and 24 days in cattle with an average of 21 days. The change in concentration of follicle stimulating hormone during the estrous cycle contributes to the growth and development of antral follicles; however, the number of antral follicles contributes to reproductive success of the female.

Antral Follicle Count

Antral follicle count (AFC) is one of several ways to measure reproduction in cattle. The benefit of using AFC is that it is highly repeatable within an individual (Jimenez-Krassel et al., 2009). This means that only a single non-invasive ultrasound is required to take the measurement (J. J. Ireland et al., 2007). Within a population however, AFC is highly variable. Additionally, AFC is positively correlated with other indirect measures of fertility. Previous studies provided evidence that AFC is correlated with longer herd productivity, a greater super ovulatory response, and a thicker endometrium (Jimenez-Krassel et al., 2009; Ireland et al., 2007; Walsh et al., 2014; Silva-Santos et al., 2014; Santos et al., 2016; Jimenez-Krassel et al., 2015). Antral follicle count is also correlated with some ovarian anatomical measures such as a greater length, size, and weight of the ovary (Ireland et al., 2008). The OR is important because it consists of all the oocytes that the animal has for their lifetime. A greater number of antral follicles tends to indicate a larger OR (Mossa et al.,

2012). In addition, a smaller OR in cattle has been shown to be correlated with reduced ovarian function (Mossa and Ireland, 2019). One example of this involves studies from superovulation experiments. A higher AFC has been shown to be correlated with better superovulation in *Bos taurus*, *Bos indicus*, and *Bos indicus-taurus* cows (Ireland et al., 2007; Silva-Santos et al., 2014; Santos et al., 2016). In high and low AFC animals, the proportion of healthy to total number of follicles is the same, but there are a larger total number of healthy follicles in high AFC animals (Ireland et al., 2008). Overall, in *Bos taurus* crossbred beef heifers, previous research indicated that animals with high AFC have a higher likelihood of being pregnant by the time breeding season is over.

Polycystic Ovary Syndrome

There is however some contention about the benefits of animals with high AFC. Recent studies in *Bos indicus* animals have seen that conception rates in Nellore cattle were the highest in the low AFC group (Morotti et al., 2017). Similarly, a study in Holstein heifers saw results that high AFC animals had lower pregnancy rates and more days open than in the low and medium AFC group (Jimenez-Krassel et al., 2017). A proposed explanation is that females with abnormally high AFC have polycystic ovary syndrome (PCOS) (Jimenez-Krassel et al., 2017). This syndrome causes decreased fertility because of chronic anovulation in the affected individuals. With this anovulation, more AFC could be present but if they cannot be ovulated there is no chance for the affected animal to conceive (Zawadzki et al., 1992; Wiser et al., 2013). This presents another question of if animals with higher AFC have a longer reproductive lifespan or if instead the increased number indicates that the OR actually is depleting more quickly (Cushman et al., 2018). Understanding the genetic basis of AFC is important to determine genes and variants that affect folliculogenesis.

In this aspect, more knowledge could help to elucidate not only more about folliculogenesis and its requirements but identify more regions to examine for causes of infertility such as with PCOS and reproductive longevity.

Female Cattle Reproductive Tract Anatomy

Having proper structure of the reproductive tract is crucial to conceiving and maintaining a pregnancy. The reproductive tract must be developed and be functioning correctly for a successful pregnancy to occur. To evaluate the development of the reproductive tract it is important to first understand the female reproductive tract anatomy.

The structures to examine on the ovary are the follicles and looking for the presence of a CL (Martin et al., 1992). These structures are located in the ovarian cortex or the inner part of the ovary (Senger, 2005). After the follicle develops, the LH surge causes ovulation and the egg enters the oviduct. The oviduct is segmented into three parts, the infundibulum, the ampulla and the isthmus. The infundibulum captures the oocyte and transports it to the ampulla. The ampulla is where the oocyte is fertilized. As the oviduct transports the oocyte and the sperm it must have a developed muscular layer as well as mucosal layer with cilia to help with transport (Pohler et al., 2020) Then the zygote is transported to the isthmus which is connected to the uterine horns.

Cattle have a bicornate uterus with two moderate uterine horns and a small uterine body. Similar to the oviduct, a cow's uterus has an outer connective tissue layer, the perimetrium, and then a double layer of muscle. Together the outer longitudinal and inner circular muscle layers make up the myometrium. When evaluating the uterus, the resistance of the myometrium contributes to the assessment of the uterine tone (Bonafos et al., 1995). The innermost layer of the uterus is the endometrium made up of a mucosa and submucosa

layer. The mucosal layer surrounds the lumen and secretes materials into the lumen while the submucosa is connective and supporting tissue. The diameter of the uterus has three phases of development by increasing rapidly until 10 weeks of age, again from 12-24 weeks of age and then finally from 32-60 weeks (Honaramooz et al., 2004). Uterine weight also increases rapidly from six to ten months of age (Atkins et al., 2013).

The uterine body opens up into the cervix. Sperm is transported through the lumen which is surrounded by multiple rings that form interlocking projections. The diameter of the cervix increases rapidly from 2-20 weeks of age. This rapid increase also occurs again from 40-58 weeks of age (Honaramooz et al., 2004).

The final parts of the female reproductive tract are the vagina and then the vulva. The vagina is the internal organ which functions as the copulatory organ. The vagina has a muscular layer that surrounds a mucosal epithelial layer. This epithelial layer will thicken during estrus to protect the vaginal and prevent possible microorganism invasion (Wrobel et al., 1986).

Reproductive Tract Scores

To be able to give birth at two years of age a heifer must conceive by 15 months. Heifers that give birth at two years have a longer lifetime reproductive productivity. As previously mentioned, cattle start going through folliculogenesis before they are born, and become physiologically developed during puberty. Puberty is the process of physiologic development that then enables animals to be able to conceive and maintain a pregnancy. Heifers that conceive earlier in the breeding season tend to continue to do so throughout their life (Gutierrez et al., 2014). One way to determine pubertal status which correlates to age at puberty is to use reproductive tract scores (RTS) (Martin et al., 1992). To score a

reproductive tract, the tone and size of the tract is evaluated as well as the ovarian structures. In this way the RTS can categorize the physiological changes that result from puberty. A score of one or two categorizes heifers that are immature and in anestrus. A score of four or five signify heifers that are mature and cycling and a score of three represents the peripubertal heifers. In heifers, it was shown that the ovaries grow three times as fast as the body of the heifer does (Desjardins & Hafs, 1969). The other aspects of the reproductive tract, the cervix, uterus, vagina and luminal epithelium, grow rapidly between about six months and puberty (Desjardins & Hafs, 1969). The gonadostat theory is that the steroids secreted from the ovary inhibit gonadotropin secretion. This occurs through negative feedback to the hypothalamo-pituitary axis. Estradiol will suppress LH secretion but when the sensitivity of this negative feedback diminishes, the gonadotropins will cause an LH surge and follicular maturation occurs. Examining heifers that underwent artificial insemination, those with reproductive tract scores of 4 and 5 had a 17.7% higher pregnancy per insemination (Gutierrez et al., 2014). This trend has been confirmed in a study by Dickinson and colleagues (2019) as they examined RTS with body condition scores (BCS). Heifers with a BCS of six and a RTS of five had an 89% pregnancy rate at the end of breeding season.

Reproductive Genetics

Previous research has revealed genetic variants that affect reproductive phenotypes. Two genes with known genetic variants that affect reproduction are *BMP15* and *GDF9*. In cattle, Tang and colleagues (2013) saw that *GDF9* is very conserved in Chinese Holstein cows but did identify two SNPs associated with superovulation. Another study identified polymorphisms in Maremmana cattle in *GDF9* and *BMP15*. They also identified one of these

SNPs in *GDF9* as being associated with twinning (Marchitelli & Nardone, 2015). In sheep, mutations in *GDF9* and *BMP15* have been identified to affect ovulation rate. For example, homozygous mutations in the *BMP15* gene result in females that do not ovulate; however, heterozygous females have increased ovulation rates (Scaramuzzi et al., 2011). The *GDF9* and *BMP15* genes work together to regulate ovulation and having certain mutations in both genes change ovulation rates (Hanrahan et al., 2004). Although some SNPs have been associated with follicle development and ovulation in cattle, more research can help improve the knowledge of genetic associations with cattle fertility.

It is important to identify these genetic mutations as they can have a drastic effect on the phenotype of the animal. This can determine if animals are kept in the herd or is culled. Another gene that affects fertility is *APC2* which was shown to do this as recently as 2019 (Mohamed et al., 2019). Mice with *APC2* knocked out showed both a reduced rate of ovulation and corpus luteum formation. The identification of the *APC2* gene with ovulation demonstrates that there is more to learn and understand about mammal fertility and the genes and variants that affect it. Identifying genetic variants that affect traits is important not only to identify the variants that cause change, but also to learn more about genes and their functions affecting phenotypes.

Cattle Grazing

In the western United States, there is a variety of rangeland including riparian areas and the surrounding uplands. It is important to have good cattle grazing distribution for multiple reasons. One reason is that overgrazing of the riparian lands can cause damage to the forage and aquatic life that lives there. Another reason is that to fully utilize the rangeland available cattle should eat the forage that grows farther from water. It is important that cattle

are efficient and can utilize the entire range that is being grazed. To be able to increase the rangeland being used it is first fundamental to understand cattle's grazing patterns on the range.

Examining the behavior of cattle on rangeland previously was a hard and labor-intensive task. It required people to traverse the landscape and visually document the location and activity of each individual animal. This documentation would be taken every three hours, but this meant that the activity of the cattle in between these times would not be documented (DelCurto et al., 2005). In addition, some traits are hard to physically observe. With the onset of new technology, evaluating cattle grazing behavior became easier. As GPS and accelerometers became available, phenotypes of cattle activity could be measured more directly and precisely. Halters and ear tags could now be equipped with GPS and accelerometers so that traits such as elevation, distance traveled, grazing time, resting time, bite rate and others could be measured (Bailey et al., 2018). This influx of technology spurred more research into how cattle graze on rangeland.

It has been shown that cattle often tend to congregate closer to water (Bailey et al., 2004). In addition, when the rangeland includes steeper terrain, cattle tend to utilize gentle slopes to avoid climbing steeper slopes (Valentine, 1947). Some strategies implemented to increase use of rangeland farther from natural water sources and on steeper slopes are rotation grazing, providing off-stream water and nutrient supplementation, and selectively breeding for cattle that will use other parts of the pasture.

Some strategies implemented to control cattle grazing are through rotation grazing and herding. Rotation grazing can occur by subdividing a pasture into smaller pastures. During grazing, there is a large number of animals grazing in the same area for a brief

amount of time. This way, areas are grazed more evenly, resulting in better utilization of forage. The other benefit of this type of grazing is the ability to have a higher stocking density because the animals are moved quickly (Manley et al., 1997).

Similar to rotational grazing, herding has many of the same benefits and detriments. As the name implies, this strategy involves herding animals during parts of the day to places with forage growth or supplementation. One of the keys of this strategy is to have an alternate source of water (DelCurto et al., 2005). Cattle will normally graze in the morning and later in the day while preferring to move closer to water in the middle of the day. To be able to coordinate the herding with cattle's natural movement, it is advantageous to have an alternate source of water. Just as with structure of fences and rotating cattle grazing, herding takes increased labor and thus increase of labor costs. Thus, this strategy should only be undertaken when this cost can be made up with either prices of cattle, improved stocking rates, or pasture improvement.

Producers have also looked at supplementation for animals on the range to help mitigate any possible overuse of riparian areas. In addition to this, other factors such as shading also play a role in distribution of grazing cattle. One study provided an animal operated water trough in the pasture to determine its impact on stream water use (Godwin & Miner, 1996). The animals that had access to this alternate source of water visited the stream less often than those that did not. In these pastures where a new water source is developed, it should be placed at least 1 kilometer away from existing water sources (Bailey et al., 2004). This can work to encourage grazing closer to the new water source and provide more even distribution of cattle. Developing a new water source can be costly, so should only be

undertaken if the upfront cost can be covered with increased production that results from improved grazing distribution.

Another supplementation method is to include a low moisture supplement to entice animals to use alternate pasture space. With access to a low-moisture supplement, cattle forage more in the area with the supplement. Cattle also tended to spend more time (30-40%) within 600m of the supplement (Bailey et al., 2001). Strategic low moisture supplement placement increased the uniformity of cattle grazing. These supplements tend to provide supplement for about two weeks and then should be replaced at least 300m from the previous position (Bailey, 2004).

The last strategy that producers have started using to control grazing is to genetically select for individual animals that are better for rangeland grazing. Although many cattle tend to use the lowland riparian areas first, some cattle, called hill climbers, will graze the steeper slopes more than the bottom dwelling cattle will (Bailey, 2004). The goal is to select for these animals and cull the others from the herd to be able to increase the stocking density of the animals using the land. One concern was that when animals that use the riparian rangeland were removed from the herd, the hill climbers would then move down and start using the riparian areas instead. A study separated the hill climbers from the bottom dwelling cows (Bailey et al., 2006). This was done by looking at the top 50% of animals that spent more time on the steeper slopes farther from water and the bottom 50% that used gentle slopes near water. The two groups then grazed separate but similar pastures for three years. Between the two groups, hill climbers traveled farther vertically from water. This study also saw that the first two weeks that cattle were on a pasture were important because as they progressed on a pasture they started using steeper slopes. Hill climbers would use steeper

slopes than bottom dwellers for the first two weeks. Then as the bottom dwellers would deplete the forage on the gentler terrain they would move up into more rugged terrain. This study showed that hill climbing cows graze the terrain more uniformly than cattle that preferred the lowlands, and that behavior will persist even when the cattle are separated. Another aspect of the study was to look at the remaining forage in the lowland area. Between the two groups, lowland stubble height was significantly taller in the pastures used by the hill climbing cattle. This further establishes that lowland cattle may deplete the riparian areas of nutritious forage material and are then forced to then utilize the steeper slopes for grazing. This has economic importance in multiple ways. One is that producers can have more economically efficient animals if they can utilize the whole of the pasture. Also, if stubble heights in sensitive riparian areas fall too low, cattle may have to be moved, which would cause an extra expense to the producers as that pasture is excluded for the duration of the grazing season.

Another aspect that producers should consider is what type of animals are best to have on rangeland. Many different aspects of animals can affect grazing efficiency including breed, age and physiological status of animal. Utilizing the optimal biological type of cow can influence the efficiency of rangeland use. One aspect that affects rangeland use is age of the cattle on the land. Several studies conclude that mature cows will use more rugged and steeper terrain than first time cows (Bryant, 1982). However, a study by Bailey and colleagues saw that three-year-old cows would travel farther from water than older cows (2001). In addition, the study saw a difference that three-year-old cows would climb higher than older animals (Bailey et al., 2001). Conversely the study by Vallentine and colleagues (2000) did not observe a difference in elevation used between ages of cows. A proposed

explanation for this discrepancy is for when older animals are already familiar with the terrain (Vallentine, 2000). Cattle have been shown to have spatial memory and remember locations of water (Bailey et al., 1989). In light of this, older cows graze familiar pastures and use more rugged terrain than the younger animals that have not been on the pasture before.

Cattle grazing rangeland pastures are also subject to the climate and changing seasons around them. One aspect of the changing seasons is the maturation of the grasses. As the season goes on, the late forage tends to not be as nutritious as the grasses earlier in the season (DelCurto et al., 2005). Cattle that would distribute evenly across the pasture previously now stay closer to water and the riparian areas to try and prevent the loss of body condition (DelCurto et al., 2005). This could cause increase degradation to this area late in the year. This increases importance to reduce grazing in the lowlands later in the season.

Genetic Associations with Rangeland Behavior

Several studies have examined cattle on rangeland attempt to determine genetic associations with grazing behaviors. The first simply looked at overall terrain use indices between two experiments in lactating and non-lactating cows (Bailey et al., 2015). The first terrain use index is a rough index which combines normalized average of slope and elevation. The second index was the rolling index which uses distance from water in addition to slope and elevation. When combining these experiments, eight SNPs were identified with the rolling index and eight SNPs were identified with the rough index. Bailey and colleagues examined these SNPs and found these SNPs were in relation to genes that contribute to locomotion, motivation, and spatial memory. Other SNPs were located in association with gluconeogenesis, organogenesis and gastrulation. The second study released in 2019 examined a multitude of traits: time feeding, basic activity, high activity, no activity, ear

temperature, welfare index point, welfare index class, milk yield, fat percentage, rumination, and somatic cell score. However, the second study only reported significant genetic associations with rumination, time feeding, and no activity (Yin et al., 2019). The cattle used in this study consisted of 615 animals from five dual purpose breeds in Europe. With the three traits that had significant associations, 22 potential candidate genes were identified. These genes are associated with residual feed intake, the immune system, obesity, transport of nutrients, and energy balance. A more recent study in 2020 used phenotypes of rolling index, rough index, slope, elevation, vertical climb, and distance from water (Pierce et al., 2020). The cattle in this study were beef animals grazed in the western United States. These 330 animals ranged from yearling heifers to mature cows. Pierce and colleagues identified 26 significant SNPs and from these identified eight potential candidate genes that have functions relating to oxygen homeostasis, growth, and feed efficiency (2020). Between these three studies, there were not any overlapping genetic associations. There was some overlap in the studies that identified genes associated with feed efficiency. In addition, there has been associations with pedometer counts and residual feed intake (Connor et al., 2013). This could connect the findings in the study by Bailey and colleagues as they identified a gene associated with locomotion (2015). Throughout these studies, there was less uniformity of the physiological status of the animals in these studies which could confound identification of significant genetic associations.

Mild Heat Load

Cattle on rangeland are exposed to the weather and are at its mercy. In the summer, an increase in temperature can cause heat stress. Stress is a behavioral or physiological response that occurs due to a change in environment (Gwazdauskas et al., 1975). In cattle,

mild heat stress can occur when the temperature humidity index (THI) is greater than 72 (Armstrong, 1994). THI combines both the effects the temperature and humidity in one calculation. This calculation

THI=0.8tdb + RH (tdb – 14.4) + 46.4

uses tdb as the dry temperature and RH is the relative humidity (Thom, 1959). Cattle with heat stress can have reduced reproduction, health, and production levels (Slimen et al., 2016).

Heat stress affects cattle on a cellular level. When experiencing heat stress, cattle will produce excess reactive oxygen species (ROS) (Ganaie et al., 2013). The ROS's can induce oxidative stress and influence cytotoxicity and apoptosis. One way this occurs is through the increase of transition metal ions which will donate electrons to oxygen and form a superoxide anion which is a precursor to most ROSs (Agarwal & Prabakaran, 2005). In addition, heat stress can increase the ratio of NADP+ to NADPH and this change will also generate ROSs (Moon et al., 2010). When the concentration of ROSs changes, the electron transport chain in the mitochondria can be affected and reduce ATP synthesis (Zhao et al., 2006). This heat stress would affect a cow's ability to produce enough energy and they can go into a state of energy depression. The ROSs can also activate the apoptosis pathway in which cytochrome c is released from the mitochondria and causes cell necrosis (Du et al., 2008). Another change that occurs in heat stress is that the adipokine leptin is produced (Morera et al., 2012). This molecule will activate the hypothalamic axis and reduce feed intake (Rabe et al., 2008). These cellular changes during heat stress can cause a change in cattle behavior on rangeland.

Part of the reason that the heat stress will occur is because cattle also have their own heat production. This heat production can be separated into four categories: basal

metabolism, heat of digestion, heat of activity, and production metabolism (Brown-Brandl 2018). Experiencing heat stress can change animal's behavior to try and dissipate some of that heat. As mentioned, one way is that cattle might try to lessen their own heat production by changing their feeding and activity levels. A consequence of this is that some cattle might end up underfeeding (Ratnakaran et al., 2017).

One way for cattle to dispel heat is through latent heat loss. This means that heat will be lost from their body by evaporation either from their skin or from their respiratory tract (Brown-Brandl & Jones, 2016). In severe heat conditions cattle have been seen to increase their water intake by 20-30% (Devendra, 1979). In the case of grazing animals, for this to occur they would need to stay closer to water sources which tend to be in the riparian areas.

Heat loss can also happen through sensible heat loss. This encompasses heat lost through conduction, convection, and radiation. Conduction is heat transferred between solid objects, convection is transfer of heat from a solid object to a fluid, water or air, and radiation is transfer of heat through radiant energy (Brown-Brandl 2018). Sensible heat however can also be accumulated into the body where latent heat can only be lost. The gaining or losing of sensible heat depends on the temperature gradient between the two items. This means that the amount of time lying down can change when the temperature increases. When the ground is warmer than the animal, lying down increases the heat through conduction and radiation into the cow. In this case, standing would help release heat through evaporative heat loss from the cow. However, if cattle stand more than 45% of the day, lameness is more likely to occur (Provolo & Riva, 2009). Lameness can impact the cattle's ability to move and leave them needing to stay closer to water.

Individual cows will handle heat in different ways so the level of stress that they experience will vary. Some factors that cause a change in heat stress even under the same management conditions are coat color, species, sex, health, age, and condition score. One method to judge cattle's susceptibility to heat stress is a model developed by Brown-Brandl and Jones (2011). This model incorporates the environment and the animal's response which in this case is respiration rate. In this way, it is possible to select for cattle that are more tolerant to heat stress.

Genome Wide Association Analysis

One way to elucidate these genetic variants is to begin with genome-wide association studies. Using this method, genetic associations can be identified throughout the genome with a large number of phenotypes. Cattle can be genotyped on 50K genotyping arrays. These arrays analyze about 50,000 single nucleotide polymorphisms (SNPs), or changes in a base pair, throughout the whole genome. However, there are approximately 2.7 billion bases in the cattle genome so 50,000 SNPs encompasses only a portion of them (Rosen et al., 2020). There are some bases that tend to be inherited together and in linkage disequilibrium (LD). One way to measure LD is with squaring Pearson's coefficient of correlation to produce r^2. In cattle, the average r^2 between 100kb and 500kb is reported to be 0.56 (McKay et al., 2007). Due to alleles commonly being inherited together, if some of the alleles are known, others can be inferred. Inferring these alleles is called imputation. In cattle, the LD is relatively high which allows imputation to be done successfully with high accuracy, $r^2>0.99$ (Rowan et al., 2019). Using imputation in studies allows for the genotyping of the animals on a low-density array with a smaller number of SNPs and imputing resulting in a greater number of SNPs to use in the study. **Figure 1.3** shows a sample chromosome that is

genotyped on a low-density array. There is also a reference panel that is genotyped on a higher density array. Using the reference panel chromosomes that are similar to the sample chromosome, more SNPs can be inferred and added to the sample chromosome. With this, genome wide association studies have the ability to examine more of the genome for associations.
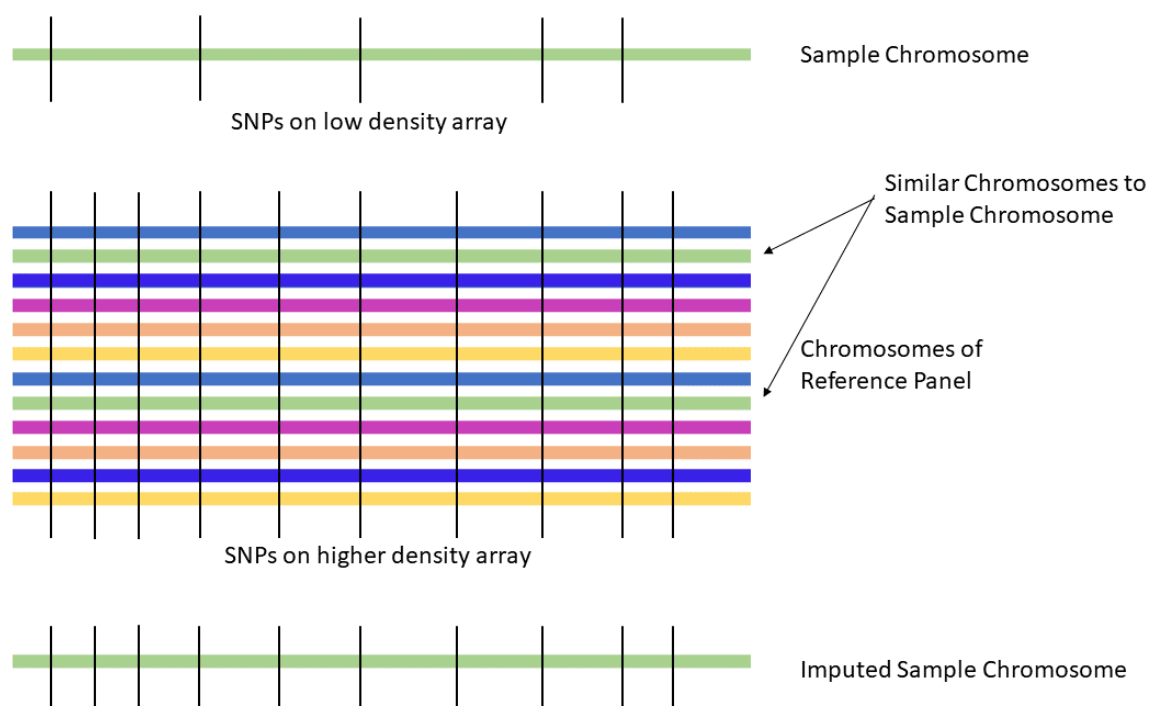
Figure 1.3: Overview of how imputation increases the number of SNPs on the Sample Chromosome.

Summary

Producers require the most efficient animals in the herd to prevent unnecessary losses. In selecting replacement heifers, producers need them to be reproductively efficient. For producers that pasture graze their females, these animals also need to be efficient on the range. Understanding more about female cattle reproduction is important to determine which females should be retained. Similarly, learning more about the behavior of cattle on rangeland and the factors that can change that behavior is crucial to utilize the entirety of the

land available. One way to improve both the reproductive and grazing efficiency of cattle is to select for more efficient replacement animals. Identifying and understanding genetic associations that affect reproductive and grazing efficiency is vital for this change to occur. Increasing the knowledge of these genetic associations can not only assist in selection and culling decisions but also help to understand the biological pathways that influence these traits.

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Chapter 2: Genome-wide Association Analyses of Fertility Traits in Beef Heifers

Abstract

The ability of livestock to reproduce efficiently is critical to the sustainability of animal agriculture. Antral follicle count (AFC) and reproductive tract scores (RTS) can be used to estimate fertility in beef heifers, but the genetic mechanisms influencing variation in these measures are not well understood. Two genome-wide association studies (GWASs) were conducted to identify significant loci associated with these traits. In total 293 crossbred beef heifers were genotyped on the Bovine GGP 50K chip and genotypes were imputed to 836,121 markers. A GWAS was performed with the AFC phenotype for 217 heifers with a multi-locus mixed model conducted with year, age at time of sampling and principal component analysis groupings as covariates. RTS GWAS was performed with 289 heifers using an additive correlation/trend test comparing prepubertal to pubertal heifers. Loci on chromosomes 2, 3 and 23 were significant by AFC GWAS and loci on chromosomes 2, 8, 10 and 11 were significant by RTS GWAS. The significant region on chromosome 2 was similar between both analyses. These regions contained genes associated with cell proliferation, transcription, apoptosis and development. This study proposes candidate genes for beef cattle fertility, although future research is needed to elucidate precise mechanisms.

Introduction

Cattle producers benefit from animals that reproduce reliably and efficiently, making fertility a critical trait in the cattle industry. Improving reproductive efficiency can be accomplished by selecting replacement heifers with higher fertility and a longer reproductive

life span. There are several measures used to estimate fertility, including Anti-Müllerian

hormone concentration, days open and calving performance: this study focuses on antral

follicle count (AFC) and reproductive tract scores (RTS).

Antral follicle count is highly repeatable within individuals although it can vary

within a population [1]. The fertility measurement of AFC has been utilized in cattle and

requires only a single, non-invasive ultrasound examination [2]. The heritability of AFC in

cattle is 0.25 and is positively correlated with other indirect measures of fertility such as

endometrial thickness, super ovulatory response and herd longevity [1-6]. In addition, cattle

with a higher AFC tend to have ovaries of significantly greater length, size and weight [7].

Ovaries with a greater number of antral follicles indicate a larger ovarian reserve (OR) [8]. A

smaller OR has been associated with reduced ovarian function [9]. Although the proportion

of healthy to total number of follicles is the same between animals with either high or low

AFC, the total number of healthy follicles is greater in those with high AFC (>25) [7].

Furthermore, *Bos taurus*, *Bos indicus*, and *Bos indicus-taurus* animals that have higher AFC

also produce more oocytes during superovulation [2,4-5]. Previous studies in *Bos taurus*

crossbred beef heifers and Holstein-Friesian dairy cows have shown that animals with high

AFC are more likely to be pregnant at the end of breeding season [8,10].

In addition to AFC, producers use RTS as a semi-objective measurement of pubertal

status and determine age of puberty which has a heritability of 0.43 [11]. Briefly, scoring is

based on palpation of follicular development, corpus luteum presence, and reproductive tract

tone. A scale from one to five is used, in which a score of one indicates the animal is

immature or in anestrous while five shows the animal is mature and cycling, as described

previously [12]. Unsurprisingly, heifers with a higher RTS are more likely to conceive and to conceive earlier in the breeding season than heifers with a lower RTS [13].

Understanding the biological mechanisms contributing to increased AFC and earlier reproductive tract development will allow producers to select for more reproductively efficient animals as replacements in their herd. This study used genome-wide association studies (GWASs) to investigate if AFC and RTS exhibit significant genetic associations with genetic variation and has identified potential biological pathways involved. Previous GWASs in cattle such as Neupane et al. and Cole et al. have identified genes associated with fertility and reproductive traits in cattle; however, neither examined AFC or RTS [14,15]. Determining the mechanisms contributing to follicular and reproductive tract development will provide a basis for future fertility studies.

Methods and Materials

Animals and Phenotype Collection of Antral Follicles and Reproductive Tract Scores

This study examined a total of 293crossbred heifers over a two-year period, 139 from year one and 154 from year two. The heifers were sired by Angus, Hereford, Simmental, Simmental-Angus (SimAngus) or Shorthorn bulls. These animals were raised at the University of Idaho Nancy M. Cummings Research, Education and Extension Center in Carmen, ID. The heifers ranged from 10.5-13.5 months of age and had a body condition score between five and seven at the time of evaluating the antral follicles and reproductive tracts. AFC data was collected on heifers (n=220) by performing ovarian ultrasound imaging with an Ibex, EVO portable ultrasound with a 7.5 MHz linear probe [7,10]. The recorded ultrasounds were examined to identify follicles ≥ 3mm, which were counted for total AFC [16]. Reproductive tracts were scored in heifers (n=293) using palpation and confirmed with

ultrasound [12,16]. Data from the first year of heifers has been previously published by Reynolds and colleagues in 2018 [16].

Genotyping

Blood was collected from 293 heifers at NMCREEC and shipped to the University of Idaho where DNA was isolated using the phenol chloroform method as previously described [17]. DNA for each animal was genotyped with the Bovine GGP 50K chip that consisted of 47,843 Single Nucleotide Polymorphism (SNP) markers (Neogen, Lincoln, NE). In total 293 samples were genotyped, however four samples were removed as they had a call rate <0.9, therefore 289 samples were analyzed, however only a subset of these samples (n=217) had AFC data. Further, non-autosomal markers and those with a call rate <0.9 were removed. The remaining 45,436 variants were phased using Eagle (v2.4.1) [18], and then imputed up to 836,121 SNP markers with Minimac3 [19] using the methods and reference panels described in Rowan et al. [20]. Briefly, the imputation reference panel contained 9,629 animals genotyped on the Illumina HD array (777K SNPs), 28,183 animals genotyped on the GGP-F250 array (~227K SNPs), and 2,718 animals genotyped on both high-density assays. The multi-breed imputation reference contained between 354 (Shorthorn) and 16,703 (Angus) high-density individuals from the component breeds represented in the genotyped dataset. Rowan et al. (2019) observed high individual imputation accuracies ($r^2 > 0.99$) for crossbred animals using this same multi-breed reference panel [20]. After the imputation SNPs with minor allele frequency (MAF) <0.01 were discarded and the remaining 712,666 SNPs were used in the subsequent analyses.

Genotypic Analyses

A principal component analysis (PCA) was performed to examine the genetic relatedness of heifers. A Fisher's Exact Test was run in R version 3.6.2 and used to determine if the PCA groups had a difference in proportion of heifers classified in the high, medium and low AFC groups [21]. An association analysis was performed for 217 heifers with AFC data. A kinship matrix was first created to correct for any population structure that may exist in sample set. Year of collection, age at time of AFC (to the nearest half month) and PCA groupings was used as covariates. A multi locus mixed model additive association test was performed [22]. A separate association test with 289 heifers and RTS phenotype was also performed. For this GWAS, RTS of 1 and 2 (prepubertal heifers) were classified as cases, and the RTS of 3-5 were classified as controls. Subsequent to a genetic relationship matrix correction, an additive correlation/trend association test was performed. The SNP & Variation SuiteTM version 8.7.2 software was used for the PCA and GWASs analyses (Golden Helix, Inc., www.goldenhelix.com).Genome wide significance threshold of p<1.00E-05 was set based on previous species specific research [23-25].

Results

Principal Component Analysis

In order to examine the genetic relationship of the heifers used in the AFC analysis, we performed a principal component analysis (PCA) and plotted the first two eigenvalues. The PCA plot depicts that the heifers separate into four distinct groups (**Figure 2.1**). Group one consists of heifers that were sired by a single SimAngus sire and group two are heifers sired by six Hereford bulls. Group three consists of heifers sired by a single Angus bull and group four, the largest group, consists of heifers out of 18 Angus, SimAngus, Simmental and

Shorthorn sires. Groups one and three were comprised of heifers that belong in the year two cohort, and their sires were not used previously for year 1. Groups two and four consisted of heifers sired by animals that were used for both years. The individual heifers in this plot are colored based on AFC categories of low (≤15), medium (16-24) and high (≥25) [16]. Interestingly, heifers in the high AFC category are not evenly distributed between PCA groups. Over half (55%) of all high AFC heifers are grouped in PCA group two, and although PCA group four contains 50% of the population, only 22% of group four heifers had a high AFC. Using a Fisher's Exact Test, there was a significant (p=0.004) difference in proportion of heifers classified as high AFC vs those classified as medium and low between the PCA groups.
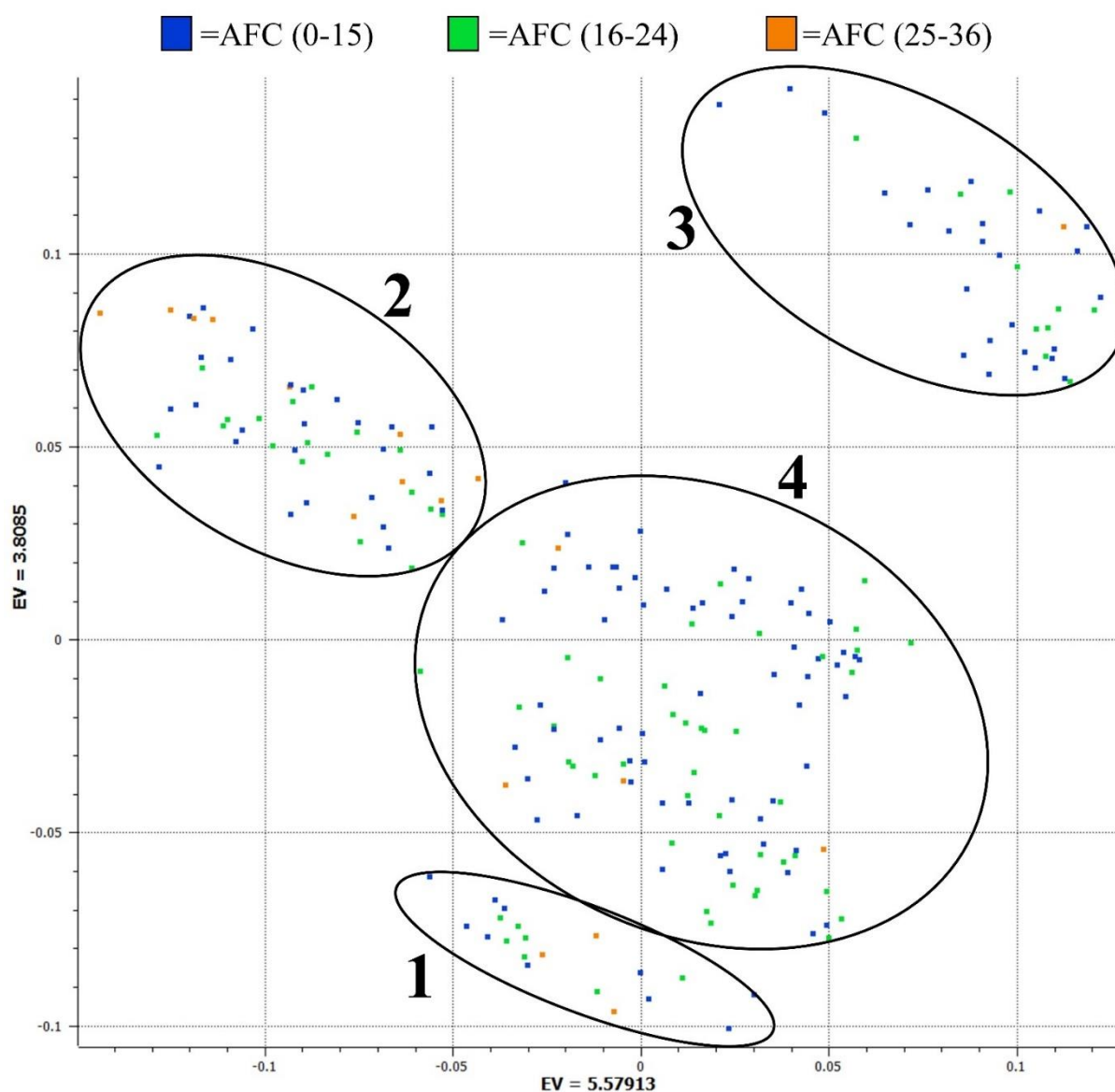
Figure 2.1: Principal Component Analysis plot to group animals by genotype. Group 1 are heifers of a single SimAngus sire, group 2 are all Hereford heifers, group 3 is heifers of a single Angus sire and group 4 are the heifers of the remining Angus, Simmental, SimAngus, , and Shorthorn sires. The individual heifers are color coded based on the number of AFC observed, with blue exhibiting lower (0-15) , green medium (16-24), and orange the highest number of (25-36) AFC observed.

GWAS with antral follicle count

A GWAS was performed to test for genetic associations in 217 heifers with different

AFC. Chromosomes 2, 3 and 23 exhibited loci that are significantly associated with

differences in AFC (**Table 2.1**). In total there were 14 significant SNPs with seven on

chromosome 2, five on chromosome 3 and two SNPs on chromosomes 23. The results from

the GWAS are displayed in a Manhattan plot in **Figure 2.2**.

Table 2.1: Chromosome, rs number, Position, P-Value, -log(P-Value), Percentage of Variance Explained, and the Candidate Genes for significant SNPs associated with AFC.

Chromosome	rs number	Position (bp)	P-Value	-log(P-Value)	Percentage of Variance Explained (%)	Candidate Genes
23	42333762	48495249	7.20E-08	7.14	12.9	*BMP6, RREB1*
	42333752	48497351	8.03E-08	7.10	12.8	
2	110145174	96774781	2.53E-07	6.60	11.9	*CREB1, FZD5, PLEKHM3, IDH1, PIKFYVE, MAP2*
	109967601	96714406	3.67E-06	5.43	9.7	
	109066756	96804827	3.68E-06	5.43	9.7	
	109574474	96807982	3.68E-06	5.43	9.7	
	110145277	96801078	4.16E-06	5.38	9.6	
	109609461	96801273	4.16E-06	5.38	9.6	
	135894326	96802055	4.16E-06	5.38	9.6	
3	133573457	104450767	7.58E-06	5.12	9.1	*FOXO6*
	43366810	104451813	7.58E-06	5.12	9.1	
	43367756	104420695	9.79E-06	5.01	8.9	
	110027403	104422767	9.79E-06	5.01	8.9	
	43367746	104423898	9.79E-06	5.01	8.9	
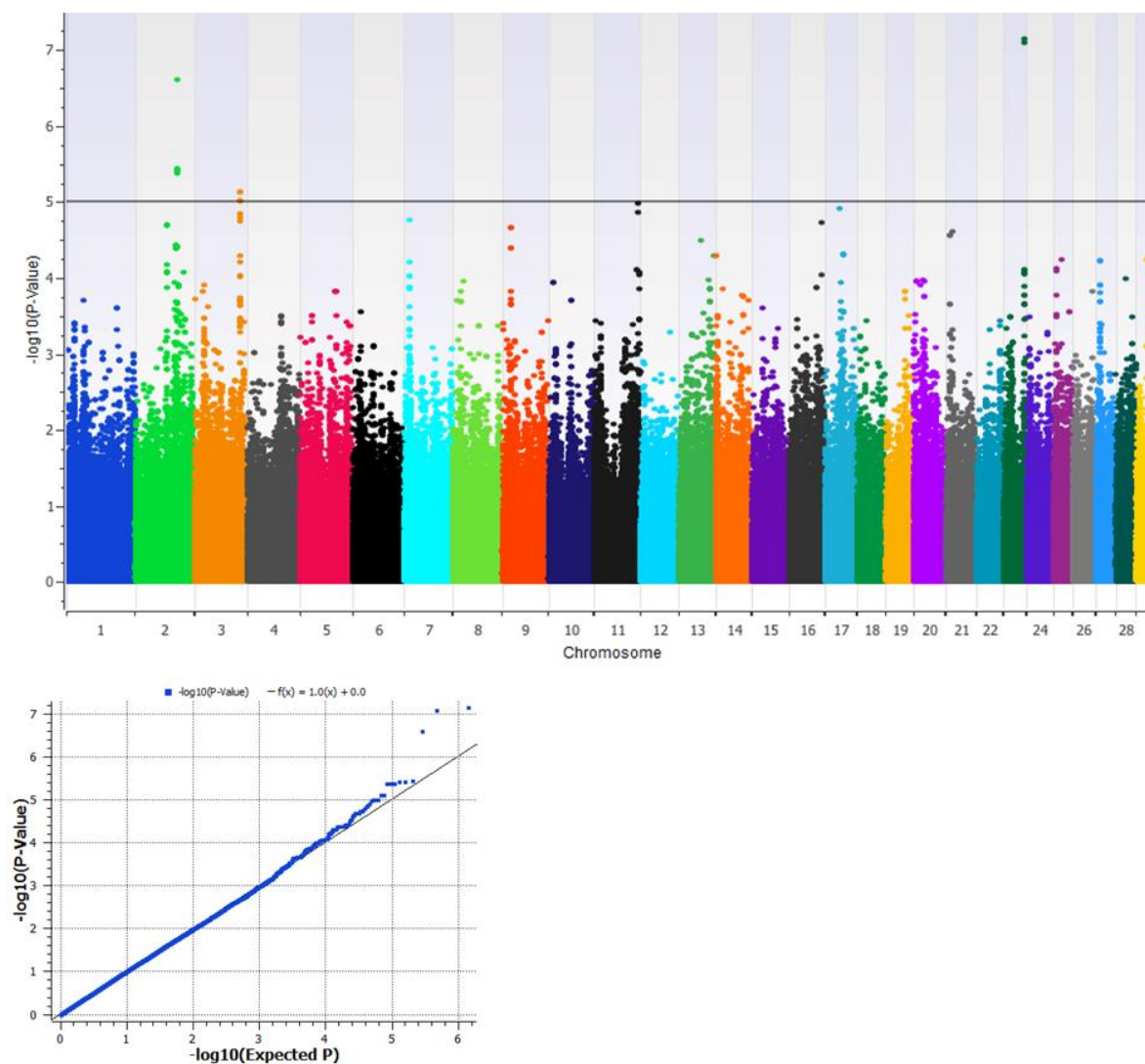
Figure 2.2: Manhattan plot of GWAS for variation of AFC in heifers in the left panel and the corresponding qq plot in the right panel. The solid line denotes genome wide significance at -log(p-value)=5.

The proportion of variance explained (PVE) identified in **table 2.1** was calculated in SVS to show how much variation can be explained by the effects of the marker [22]. The two significant SNPs on chromosome 23 explained most of the variation at 12.9% with the seven SNPs on chromosome 2 explaining 11.9% of the variation. Further, the five SNPs on chromosomes 3 explained 9.1%. In relation to the reference genome ARS-UCD 1.2, the homozygous reference markers on chromosomes 2 and 23 are associated with increased AFC while the reference markers on chromosome 3 are associated with decreased AFC [26].

Subsequently, annotated genes within 1 Mb upstream and downstream of significant SNPs were identified. An enlarged view of the genomic regions containing significant SNPs as well as all markers within 1 Mb upstream and downstream are illustrated in **Figure 2.3**. Within this region, there are a total of 12 genes located on chromosomes 2, 22 genes on chromosome 3 and 13 genes on chromosome 23 (**Appendix A Table 1**).
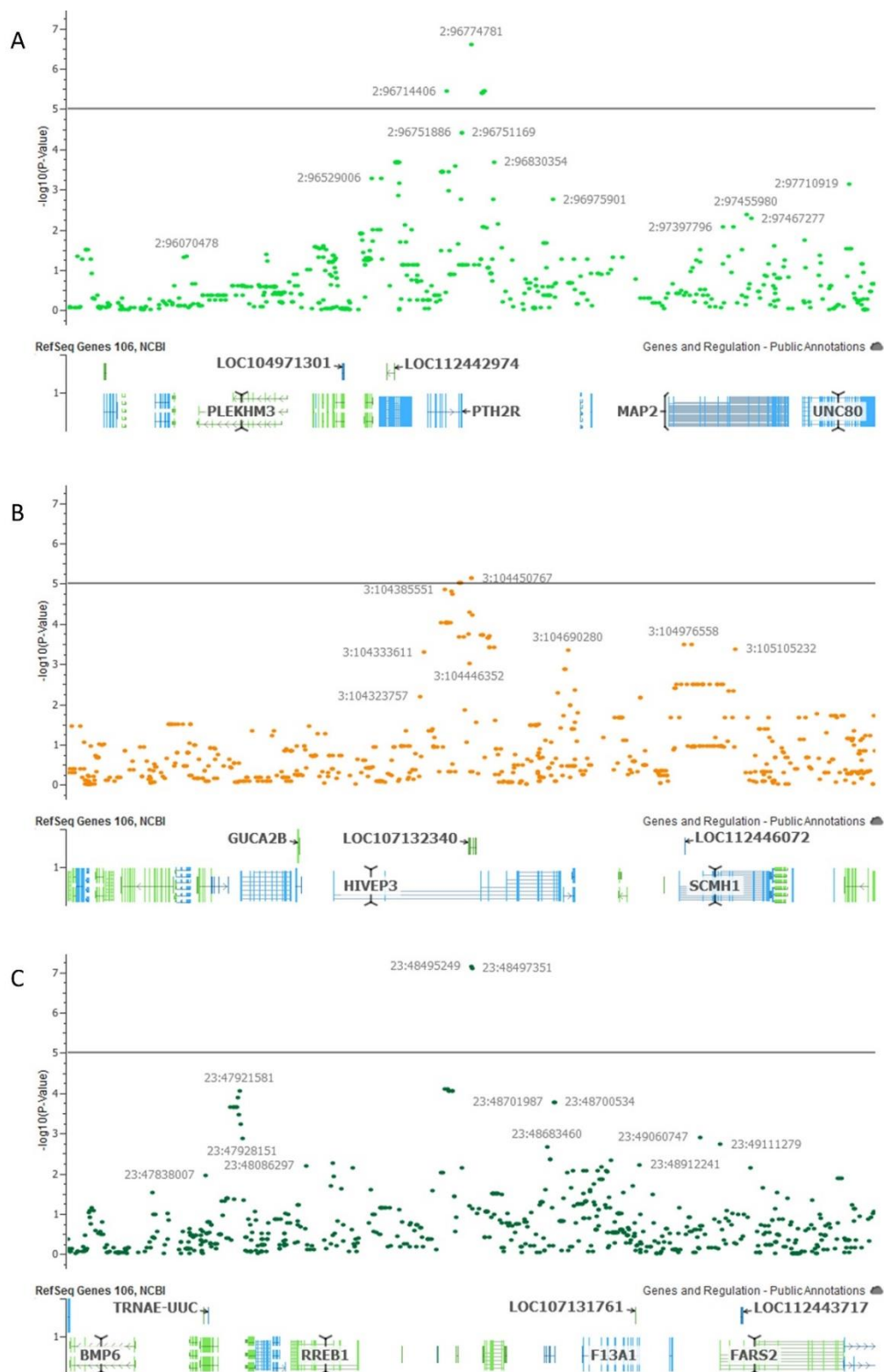
Figure 2.3: Regions within 1 Mbp of the most significant SNP for the antral follicle count GWAS results for A) chromosome 2, B) chromosome 3, C) chromosome 23

The genes in the regions of interest identified as candidate genes are noted in **Table 2.1**. The genes were identified as candidate genes because they have been identified in pathways that have known effects or the potential to affect follicle development. The most significant region on BTA 23 had two candidate genes identified. On BTA 2 a total of six candidate genes were identified while on BTA 3 one gene was identified. Of these nine identified genes, six are currently known to contribute to or affect the process of three well-known biological pathways linked to reproduction. These pathways are the PI3K/AKT, WNT signaling and MAP Kinase (MAPK) pathways, all of which have been identified as affecting folliculogenesis and demonstrates prior knowledge of candidate gene pathways.

GWAS with reproductive tract scores

A separate GWAS was performed using 289 heifers to investigate genetic associations with the RTS phenotypes (**Figure 2.4**). The focus of this analysis was to examine the pre-pubertal animals in comparison to the pubertal heifers. There are four significant loci above the genome-wide significance threshold of $p < 1.00E-05$. Chromosomes 2 and 8 had two significant SNPs each and chromosomes 10 and 11 had one significant SNP each (**Table 2.2**). There was the same area of significance between the two fertility traits on BTA 2 at 96.8 Mb.
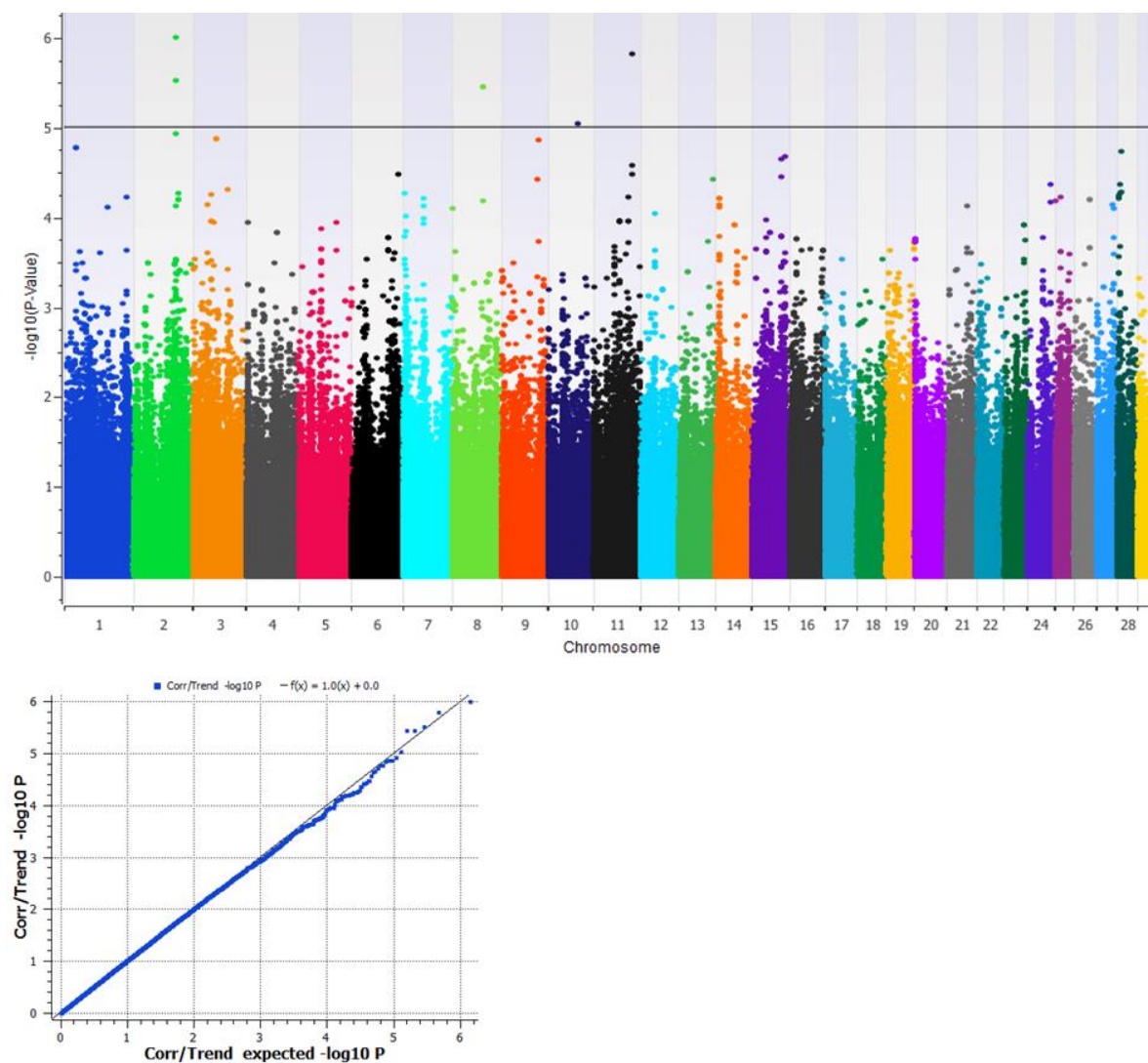
Figure 2.3: Manhattan plot of GWAS for the variation in RTS in heifers in the left panel and the corresponding qq plots in the panel on the right. The solid line is at significance level –log(p-value)=5.

Table 2.2: Chromosome, rs number, Position, P-Value,-log(P-Value) and Candidate Genes for significant SNPs associated with RTS.

Chromosome	rs number	Position (bp)	P-Value	-log(P-Value)	Candidate Genes
2	110658876	96879125	9.99E-07	6.00	*CREB1, FZD5, PLEKHM3, IDH1, PIKFYVE, MAP2*
	136255286	96870517	3.05E-06	5.52	
8	110172413	71343554	3.56E-06	5.45	*LOXL2, STC1*
	137380598	71359794	3.56E-06	5.45	
10	134739799	68971489	9.11E-06	5.04	*PELI2, TMEM260, OTX2*
11	111004666	90023995	1.55E-06	5.81	*RNF144A, CMPK2, SOX11*

From these four locations, regions of significance were defined as 1 Mb upstream and downstream of each significant SNP. These regions were investigated using ARS-UCD 1.2 reference genome (**Figure 2.5**) [26]. The significant SNPs identified were in gene rich regions. In the significant regions there are a total of 14 genes on chromosome 2, 11 genes on chromosome 8, nine genes on chromosome 10 and four genes on chromosome 11 (**Appendix A Table 2**).
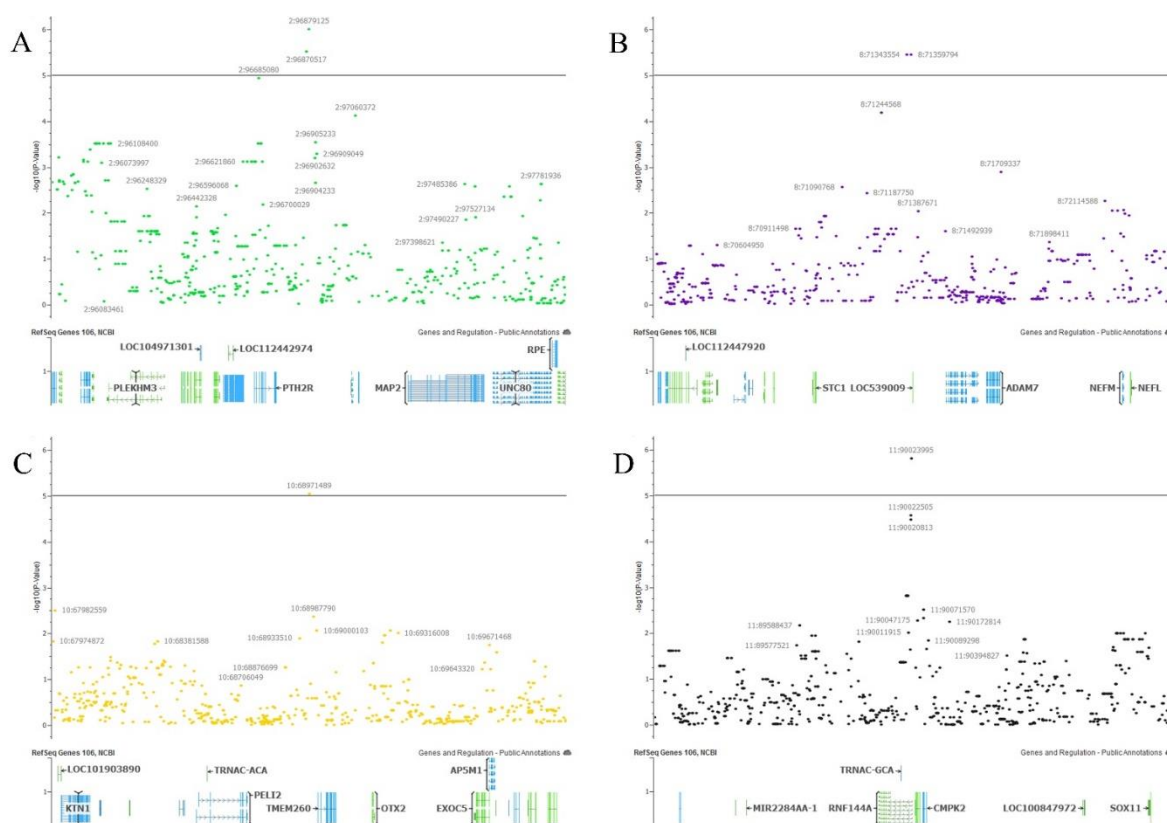
Figure 2.5: Regions within 1 Mbp of the most significant SNP for the RTS GWAS results for A) chromsome 2, B)chromosome 8, C) chromosome 10, D) chromosome 11

Of the genes listed in the regions of interest, 15 genes were identified as candidate genes (**Table 2.2**). The genes were identified as candidate genes because they have been identified in pathways that have known effects or the potential to affect the development of the reproductive tract. From the 15 candidate genes, six are located in proximity with the

most significant loci on BTA 2. These six genes on BTA2 are located in the proximity of significant SNPs identified in both the AFC and RTS GWASs. The other significant regions on BTA 8, 10 and 11 have three candidate genes each for a total of nine candidate genes. Two of these candidate genes are involved with pathways previously mentioned, the WNT signaling and the MAPK pathway.

Discussion

Fertility is a highly variable trait but is of major importance in the cattle industry. Cattle fertility can be measured by AFC and RTS, although little is understood of the genetic variation associated with these measurements. In order to examine the genetic associations with these traits we conducted two GWASs using crossbred heifers.

GWAS related to antral follicle count

Genes located within the significant regions of the AFC GWAS that have previously been described in important biological pathways are reported in **Figure 2.6**. One notable pathway is the PI3K/AKT pathway, as it is known to be involved in the activation of AKT in granulosa cells in rats and we can predict a synonymous role in the granulosa cells of cattle [27]. Two candidate genes identified in this study, pleckstrin homology domain containing M3 (*PLEKHM3*) and forkhead box O6 (*FOX06*) are closely related with the PI3K/AKT pathway. *PLEKHM3* codes for a scaffold protein for AKT that localizes AKT to the plasma membrane, an essential step in AKT activation, whereas FOXO6 is a transcription factor that is regulated by AKT [28,29]. Little is known about FOXO6 in tissues other than the brain; however, other genes in this family are involved in cell metabolism and death of oocytes [30,31].
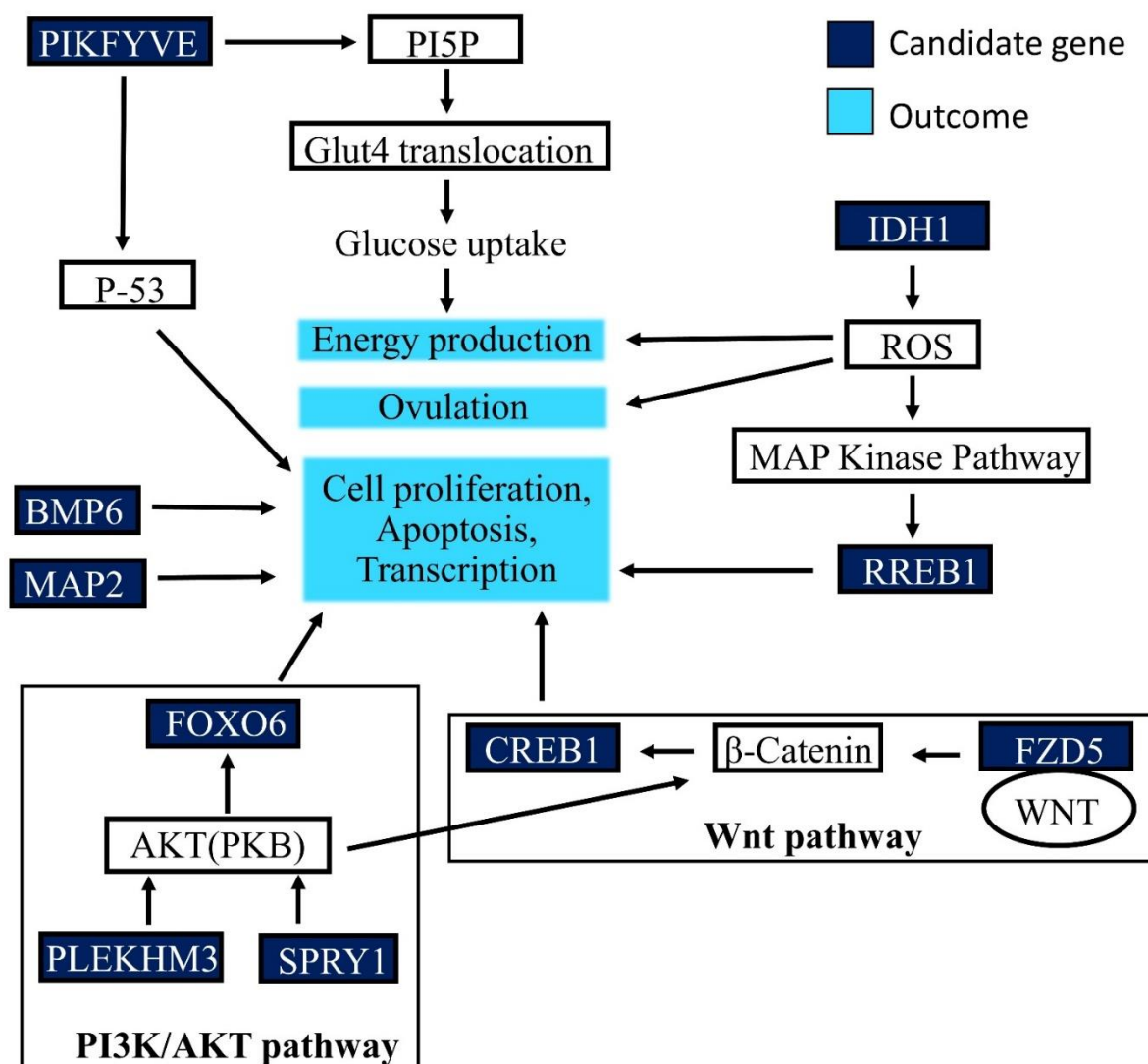
Figure 2.6: Proposed pathway(s) for candidate genes in regions of significant SNPs associated with AFC. Arrows indicate effect not increase or decrease.

The candidate gene phosphoinositide kinase, FYVE-type zinc finger containing

protein (*PIKFYVE*) directly affects the level of the cellular phosphoinositides; PI(3,5)P2 and

PI5P [32]. When PI5P levels increase this triggers the translocation of glucose transporters to

the cell membrane which leads to an increase in intracellular glucose (energy) levels [33].

PI5P further regulates the p-53 dependent apoptotic pathway, and it is plausible that

preventing apoptosis supports folliculogenesis and maturation [33]. *PIKFYVE* has been

identified as a putative target of microRNA that is differentially expressed in dominant theca

cells [34]. It is conceivable that the association of this pathway with reproductive traits relates to the influence on cellular energy levels as well as regulation of apoptosis.

The WNT signaling pathway contributes to oocyte development [36]. The canonical WNT pathway uses β-catenin to affect gene transcription [30]. Previous studies show that WNT proteins are required for ovarian follicle development via regulating FSH and LH signaling [35]. WNT2 induces an accumulation of β-catenin which through the canonical pathway causes an increase in estradiol production [36]. Alternatively, for normal female fertility WNT5a suppresses the canonical pathway [35]. The critical role of Wnt5a in folliculogenesis is supported by the report that Wnt5a-null mice experience an increased rate of follicular atresia [35]. Two candidate genes are involved in this pathway, frizzled receptor 5 (*FZD5*) and cAMP responsive element binding protein (*CREB1*). FZD5 is a receptor for several WNT ligands including WNT5a and WNT2. β-catenin is also affected by the PI3K/AKT pathway mentioned previously. β-catenin can be phosphorylated by AKT which increases its transcriptional activity [37]. CREB1 is associated with the canonical pathway and is shown to have decreased levels in this process when the canonical pathway is suppressed [35]. CREB1 is also a putative target of microRNA that is differentially expressed in dominant granulosa cells [34].

The candidate gene isocitrate dehydrogenase 1 (*IDH1*) codes for a protein involved in the TCA cycle and regulates oxidoreductase activity as well as metabolism. A study in rats engineered to mimic PCOS saw a reduction in *IDH1* levels, indicating that *IDH1* is involved in follicular growth and ovulation [38]. Due to its involvement in the TCA cycle, *IDH1* may affect energy and reactive oxygen species (ROS) levels [39]. ROS are necessary to induce ovulation [39]. In addition, ROS are mediators of the MAPK pathway which has been shown

to affect follicle maturation [39,40]. Another candidate gene ras responsive element binding protein (*RREB1*) is a downstream effector of the MAPK [41]. Overall, change in ROS levels can affect the outcomes of the MAPK pathway, thereby influencing cell proliferation and the likelihood of ovulation.

The last two candidate genes are bone morphogenic protein-6 (*BMP6*) and microtubule-associated protein 2 (*MAP2*). Several bone morphological proteins and their receptors are involved of cell proliferation, apoptosis, and cell differentiation [42]. In the ovary, *BMP6* works to regulate FSH through decreasing cAMP levels [43]. The last gene, MAP2 codes for a protein that helps to stabilize microtubules during growth, a step which is important for proper separation during mitosis [44]. This protein is also shown to be upregulated as follicles mature normally from primordial to secondary follicles, in mice [45].

A study by Fortes and colleagues examined genes associated with puberty in tropical breeds of cattle [46]. The authors of this study identified two genes, HIVEP zinc finger 3 (*HIVEP3*) and *RREB1* which are also found in our regions of significance for our AFC GWAS on chromosome 3 and chromosome 23 respectively. The previous association of these regions with onset of puberty supports our findings with fertility traits in beef cattle.

Identifying the underlying genetic variation that contributes to the biological mechanisms of AFC can help improve knowledge of the aspects of germ cell development that are currently not understood. Previous studies have enumerated the benefits of animals that present a high AFC. However, more recent studies in various dairy and *Bos indicus* animals have shown contrasting results. In a study with Holsteins, heifers in the high AFC group had more days open and less percent pregnant at the end of lactation than either the low or medium groups [47]. Similarly, a study in Nelore cattle reports the highest conception

rates in animals with the lowest AFC, and the lowest conception rates in animals with the highest AFC [48].

One possible explanation is that some animals with high AFC have polycystic ovary syndrome (PCOS) which negatively impacts fertility and skews the relationship between follicle number and fertility [47]. Females with PCOS may have a higher AFC, but chronic anovulation results in subfertility [49,50]. In addition, the question remains as to whether increased AFC indicates a longer reproductive lifespan or alternatively that the OR is depleted more quickly [51]. This study has identified nine candidate genes that are associated with differences in AFC. These genes warrant further investigation with respect to their influence and associations with PCOS, as well.

GWAS related to reproductive tract scores

This study identifies regions on four chromosomes that are significantly associated with RTS. The region on chromosome 2 is the same region also associated with AFC. A component of RTS determination is ovarian structure and either the presence or absence of ovulating follicles. Thus, it is reasonable to have the same region be significant as both GWASs include follicle development. Two candidate genes identified in RTS GWAS influence pathways previously discussed the context of AFC. These include Pellino2 (*PELI2*), which is associated with the MAPK pathway and Sex determining region Y-box transcription factor 11 (*SOX11*), which is associated with the WNT signaling pathway [52]. PELI2 can activate MAPK pathway which as previously discussed can affect the maturation of ovarian follicles [40,53]. *SOX11* is expressed in ovarian cells and can dampen the WNT signaling pathway [52,54]. As mentioned previously, WNT proteins are crucial to regulate FSH and LH signaling for follicle development [35].

Multiple candidate genes also affect follicle maturation in pathways not previously discussed. Although first identified in maintenance of mineral levels, Stanniocalcin (*STC1*) has been shown to be highly detectable in the ovaries of mature mice [55]. It has been reported that overexpression of *STC1* in mice is significantly associated with a reduction in litter size [56]. Although this mechanism remains unclear, it is evident that *STC1* affects reproduction in mammals. RNF144A is a transmembrane ligase that interacts with epidermal growth factor receptor (EGFR) and thereby affects EGFR stimulated cell proliferation [57]. Previous studies in mice demonstrate that EGFR is required for cumulous-oocyte complex maturation [58]. Through this interaction, change in RNF144A can affect the maturation and development of follicles.

Several of these candidate genes affect mammalian reproductive ability. Lysyl oxidase-like protein-2 (*LOXL2*) functions in extracellular matrix production by collagen IV assembly [59]. *LOXL2* has been identified in human female reproductive tracts and in cells with advanced aging so its presence in mammalian reproductive tracts is important in our study when comparing heifers that mature less quickly than their cohorts [60,61]. Orthodenticle homeobox 2 (*OTX2*) affects GnRH, LHB and FSHB levels in pituitary tissue of mice. In addition, mice without Otx2 had delayed vaginal opening and fewer litters than mice with a functioning Otx2 [62]. It is known that mice without Otx2 have smaller litters, abnormal estrous cycles and lack of corpus lutea [62]. Any change in OTX2 could affect not only follicle development and ovulation but also reproductive physiology.

Two final genes of note in our significant region: Transmembrane protein 260 (*TMEM260*), and cytodine/uridine monophosphate kinase 2 (*CMPK2*). TMEM260 is a putative transmembrane protein with an unknown function. According to NCBI the

expression of *TMEM260* is high in ovary implying a function in mammal reproduction [63]. CMPK2 is a mitochondrial protein that phosphorylates dCMP and dUMP during mtDNA synthesis, however it has not been observed in all tissues [64,65]. *CMPK2* as it functions in the mitochondria, may be involved in the regulation of essential nucleotide during mitochondrial biogenesis and influence energy production and growth [64].

Conclusions

The GWASs identify three regions significantly associated with AFC and four associated with RTS in crossbred heifers. One region on BTA 2 is significantly associated with both fertility phenotypes and contains six candidate genes. In addition to these six genes, three candidate genes relating to AFC are associated with pathways that contribute to follicle growth through energy production, cell proliferation, transcription or apoptosis. In addition to the six candidate genes on BTA 2, nine more candidate genes are in proximity to the regions associated with RTS and could affect the reproductive development of heifers. This study reports an association between these regions and AFC or RTS; however, additional work with larger sample sizes is needed to establish causal variants and the mechanisms of the effects they have on follicle development and fertility in cattle.

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Chapter 3: Identifying Genetic Variants Affecting Cattle Grazing Behavior Experiencing Mild Heat Load

Abstract

In the Pacific Northwest United States, the terrain can make it difficult for grazing cattle to effectively make use of all available rangeland. Differing grazing behavior and terrain utilization, with different forage availability, can result in a dramatic unpredictability in how well cattle thrive. The climate can also change cattle grazing behavior and effect the forage they use. It is important to identify and understand which cattle can efficiently utilize the rangeland. Determining genetic associations with grazing behavior can help identify cows that will effectively use rangeland pastures. This study used genome-wide associations to identify single nucleotide polymorphisms (SNPs) associated with grazing time, walking time, and maximum slope use while experiencing mild heat load. It also identified genetic association with time spent at slopes greater than 15 degrees independent of experiencing heat stress. Genetic, grazing, walking and slope data were collected from Angus X Hereford crossbred two-year-old beef cows over two consecutive years. Genotypes were obtained using a Bovine GGP 50K SNP marker array. Two SNPs on chromosome 11 reached significance ($P=5.01e-7$, $P=6.46e-7$) for grazing minutes and a proportion of variance explained (PVE) of 0.52. Additionally, one SNP on chromosome 3 is significant for walking minutes with a p-value of $1.91e-6$ and PVE of 0.48. With slope, there is a SNP on chromosome 14 that reached significance for maximum slope use ($P=8.50e-6$) and had a PVE of 0.51. Also, regardless of experiencing heat stress, there is a SNP on chromosome 5 associated with time spent on slopes greater than 15 degrees. Some cattle exhibit different grazing behavior which enables them to use certain terrain more effectively. Identifying

genetic variants associated with grazing behavior while under heat stress can enable producers to select for cattle that can best fit the rangeland available to them.

Introduction

In the Pacific Northwest United States, there is varying availability and quality of forage even within the same pasture. Many cows tend to graze on the riparian areas and not utilize the forage available on steeper slopes or farther from water (Valentine, 1947). Studies have identified that some cows in Idaho mountain pastures differentially used the upland and riparian areas (Howery et al., 1996). Producers can then select for cattle that will effectively utilize the upland areas of the pasture.

In addition to natural differences in cattle grazing, the environment also effects how they access rangeland (Wyfels et al., 2018). In the summer, cattle on the range can experience warmer temperatures which affects their behavior. When the temperature humidity index (THI) is greater than 72 and less than 79, *Bos taurus* cattle experience mild heat load (Armstrong 1994). This stress can change an animal's behavioral response. Metabolism can change and affect an animal's feeding behavior as many cattle tend to eat less during heat stress (Ratnakaran et al., 2017). Mild heat load can also affect cattle's behavior by changing how much time is spent standing and lying down (Ratnakaran et al., 2017). One study showed that some cattle had differing grazing patterns when the THI is greater than 72 (Sprinkle et al., 2021a).

Previous studies have identified genetic associations with different grazing behaviors. Associations have been identified with ruminating, feeding, non-activity, and terrain use indices (Bailey et al., 2015). The objective of this study was to determine if there are any genetic markers associated with grazing behavior in the spring and summer months.

Materials and Methods

Phenotypic Data Collection

Animal grazing and walking data collection and analysis has been described in Sprinkle et al. (2021a). Briefly, data collection took place over 2 years using 48 two-year old cows, 12 inefficient and 12 efficient cows from each year. These cows were classified as efficient or inefficient based on residual feed intake (Hall et al,. 2015). The cows were equipped with grazing halters containing both 3-axis accelerometer (USB Logger Model XB, Gulf Coast Data Concepts, LLC, Waveland, MS) and global positioning system (**GPS**) logger (iGotU GT-120, Mobile Action Technology, New Taipei City, Taiwan). Both the accelerometer and the GPS logger had a rechargeable Li-ion 3.7 V, 5200mAh battery (Tenergy Li-ion 18650, Freemont, CA) soldered to the equipment to extend data logging to 30 d (Sprinkle et al., 2021b). Daily grazing and walking time were estimated every 5 s using the 3-axis accelerometer (Sprinkle et al., 2021b). Using these data, the maximum slope individual cattle used and the amount of time cattle spent on slopes greater than 15 degrees was calculated. The accelerometer monitored head movement for 25 data points every s (25 hz) and the observations were averaged to every 5 s. Data were compiled using Python coding (https://www.python.org/). The methodology for processing GPS data is well established (Bailey et al., 2018). Some animals had days removed from the study when they escaped out of their pasture.

In order to compare similar environmental data, grazing and walking minutes and maximum slope use data on days with a THI > 72 were averaged and used for the association analyses. In addition to examining behavior in heat stress, the amount of time cattle spent on slopes greater than 15 degrees was also examined for genetic associations. The data from

three different time segments; in all spring and summer days, days in August, and days with a THI > 72 were individually averaged and examined.

Genotyping Data

Blood was collected and DNA isolated for 43 cows using a phenol chloroform method (Sambrook 1989). DNA was genotyped using the Bovine GGP 50K chip array (Neogen, Lincoln, NE). Genotypic data were examined and one sample with a call rate <0.85 was removed. Markers that were duplicates, had no position or had a call rate < 0.9, Minor Allele Frequency <0.01 or Hardy-Weinberg Equilibrium < 1e-6 were removed. In total 41,686 SNPs were used in the association analyses and the sample size for each study is listed in **Table 3.1**.

Table 3.1: Trait, Sample Size, Chromosome, Position, rs number, p-Value, -log(p-Value), and Proportion of Variance Explained for the significant SNPs associated with each trait.

Trait	Sample Size	Chromosome	Position (bp)	rs ID	p-Value	-log(P-Value)	Proportion of Variance Explained
grazing minutes	37	11	29207614	rs109119871	5.01187E-07	6.30	0.52
		11	29228506	rs43048540	6.45654E-07	6.19	0.51
walking minutes	37	3	70547786	rs43312675	1.90546E-06	5.72	0.48
max slope	38	14	39608062	rs134260807	8.50E-06	5.07	0.51
time spent on slope > 15 degrees, all spring and summer	38	5	6652812	rs109611881	4.05E-07	6.39	0.51
time spent on slope > 15 degrees, August	37	5	6652812	rs109611881	2.03E-07	6.69	0.44
time spent on slope > 15 degrees, THI > 72	38	5	6652812	rs109611881	4.88E-07	6.31	0.43

Genotypic Analysis

The SNP & Variation SuiteTM version 8.9.0 software was used to perform the GWAS analyses (Golden Helix, Inc., www.goldenhelix.com). A kinship matrix was used to correct for any potential relationship structure in the data. A single locus mixed model (SLMM) was used for grazing minutes, walking minutes, and max slope. A SLMM was also

used for all three-time segments for minutes spent at slope greater than 15 degrees. After multiple significant markers using a single locus module were associated with walking minutes, a multi locus mixed model (MLMM) was used for this analysis. Genome wide significance of p<1.00E-05 was set based on previous species-specific research (Seabury et al., 2017). From these SNPs a significant region defined as 1 Mbp upstream and downstream of the significant SNP was investigated for potential influential genes using reference UMD3.1.

Results and Discussion

This study identified loci associated with grazing minutes, walking minutes, and maximum slope use of terrain in cattle. There are also loci identified associated with time spent at slopes greater than 15 degrees. The traits, and significant markers identified are listed in **Table 3.1**. In examining the significant regions, genes of interest were identified; however, all of the genes in the significant regions are located in **Table 3.2**. **Figure 3.1** demonstrates the phenotypic distribution of each genotype for each trait.

Table 3.2: Significant region and Genes for each Trait.

| Trait	Significant Region	Genes
grazing minutes	11 (27.9-30.1Mb)	PRKCE, EPAS1, TMEM247, ATPGV1E2, RHOQ, CRIPT, PIGF, SOCS5, MCFD2, TTC7A, C11H2orf61, CALM2, EPCAM, MSH2, KCNK12, MSH6, FBXO11
walking minutes	3 (69.6-71.0Mb)	SLC44A5, LHX8, CRYZ, TYW3, ERICH3, TNNI3K, FPGT, LRRIQ3
max slope	14 (38.7-40.5Mb)	C14H8orf89, RPL7, RDH10, MIR2284L, STAU2, UBE2W, TCEB1, TMEM70, LY96, JPH1, GDAP1, PI15, CRISPLD1
time spent on slopes > 15	5 (5.8-6.8Mb)	BBS10, OSBPL8, ZDHHC17, CSPR2, E2F7, NAV3
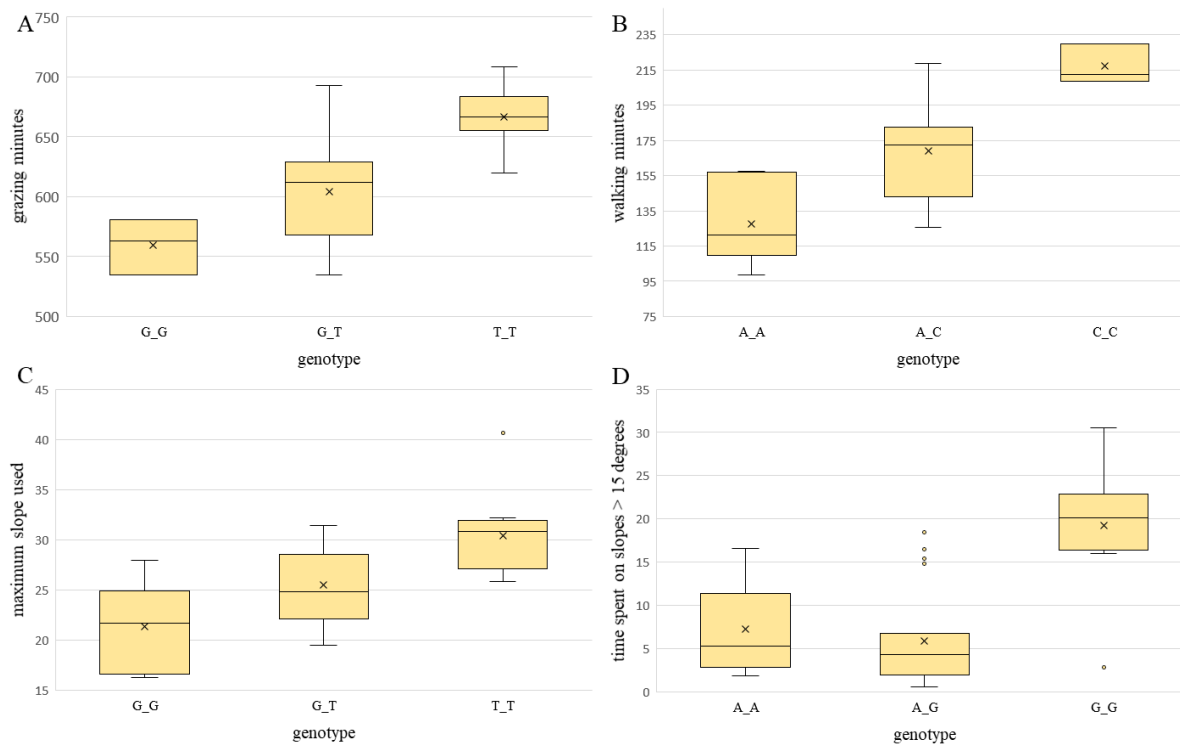
Figure 3.1: Boxplot distribution by genotype for the significant SNPs of each trait. A. Grazing minute B. Walking minutes C. Maximum slope use D. time spent on slopes > 15 degrees.

In association with grazing minutes, two SNPs at one locus on chromosome 11 were identified as significant (**Figure 3.2**). With the most significant SNP, the alternate allele is associated with increased grazing during days with a mild heat load. Within the significant region three genes are associated with intestinal health; tetratricopeptide repeat domain 7A (*TTC7A*), epithelial cell adhesion molecule (*EPCAM*), and mutS homolog 2 (*MSH2*). *TTC7A* was identified to have a significant role in the intestines when mutations were identified to affect multiple cell atresia (Bigorgne et al., 2014). With this the role of *TTC7A* regulating epithelial cell polarity, growth, and differentiation was discovered (Bigorgne et al., 2014). *EPCAM* encodes for a protein that is involved in cellular communication, tight junctions, and is highly expressed in the intestines in mice and humans (Kozan et al., 2015). Without *EPCAM*, proper barrier maintenance and ion transport in the intestines is not possible (Kozan

et al., 2015). MSH2 is an important protein that works to help repair DNA damage caused by oxidative stress (Piao et al., 2014). In mice under oxidative stress without MSH2, there was a significant increase in the number of intestinal tumors present (Piao et al., 2014). These three genes effect intestinal health and then could have the potential to influence how much time a cow would need to spend grazing.
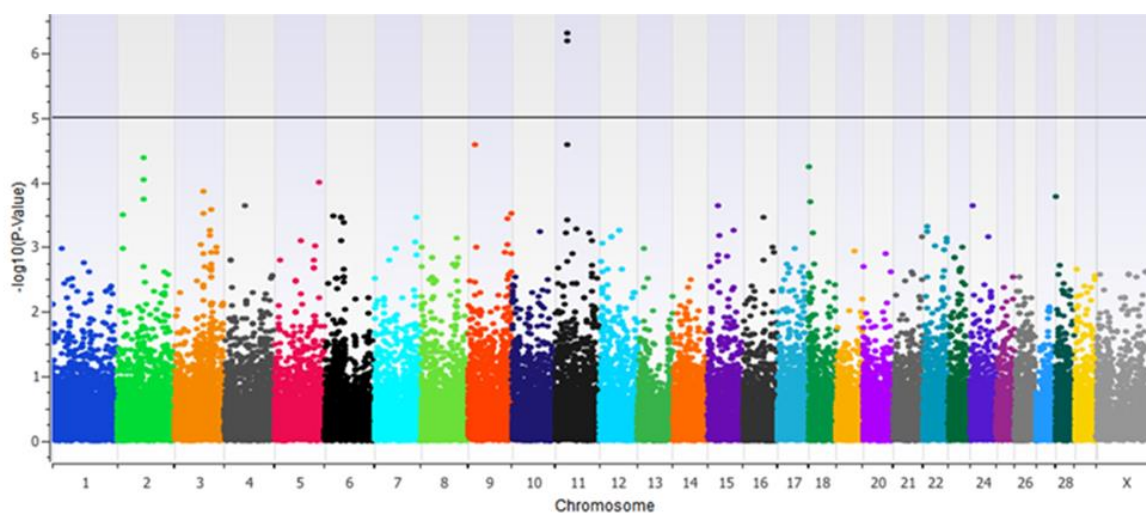

Figure 3.2: Representative Manhattan plot of the GWAS. This plot is for grazing minutes and the solid line denotes the genome-wide significance at -log(p-value)=5.

The walking minutes association identified one SNP on chromosome 3 that is significant (**Appendix B Figure 1**). In examining this SNP, the alternate allele is associated with an increased amount of walking minutes. In the significant region, there are eight genes. One gene in this region, glutamate rich 3 (*ERICH3*) encodes a protein that has been identified in regulating serotonin metabolic pathway (Gupta et al., 2016). Another gene, LIM homeobox 8 (*LHX8*) is in the same family as *LHX7* which effects differentiation of forebrain neurons (Fragkouli et al., 2005). Mice with mutations in Lhx7 showed impaired spatial learning (Fragkouli et al., 2005). Previous research in cattle examining genetic relationships with terrain use indices also identified a region in which a gene was associated with spatial memory and motivation for narcotics which then influence serotonin levels (Bailey et al.,

2015). With this analysis, we identified biologically relevant genes in our significant region that have a similar function to other genes that have been identified in affecting grazing behavior.

With maximum slope used a SNP on chromosome 14 was identified (**Appendix B Figure 2**). This loci's reference allele was associated with steeper slope use. Two genes in the significant region are associated with heart disease, transmembrane protein 70 (*TMEM70*) and lymphocyte antigen 96 (*LY96*). *LY96* has been associated with increased mortality risk of dilated cardiomyopathy. The other gene, *TMEM70*, has been associated with mitochondrial encephalocardiomyopathy (Cizkova et al. 2008). The third gene of interest in the significant region is staufen double-stranded RNA binding protein 2 (*STAU2*). This gene is associated with spatial learning and explorative activity in mice (Popper 2018). All three of these genes could affect the maximum slope of terrain that is used by cattle on rangeland.

Finally, the amount of time cattle spent on slope greater than 15 degrees had one SNP significant across all three time points (**Appendix B Figure 3**). This SNP was on chromosome 5 and had a dominant affect where the reference allele was associated with the least amount of time spent on steeper slopes. Located in the significant region is the gene zinc-finger DHHC-type palmitoyltranferase 17 (*ZDHHC17*) which has been associated with spatial memory in mice (Milnerwood et al. 2013). Affecting spatial memory could influence cattle's willingness to spend time on slopes that are greater than 15 degrees.

Implications

Terrain use in the western United States is important for efficient use of rangeland. Some cattle differentially use rangeland and it is important for producers to be able to utilize these animals so that the land can be used to its fullest potential. Studies have shown that

there are genetic associations with cattle grazing behavior. In this study, loci were identified

with grazing time, walking time, maximum slope use, and minutes of use at slope greater

than 15 degrees. In the significant regions are several genes that could influence these traits.

While this study did identify genetic variants associated with these traits, it is important to

acknowledge that the sample size in this study is limited and it would be beneficial to include

a larger number of cows over multiple years to increase the power of this study.

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Appendix A - Genes in Significant Region for Fertility Traits

Appendix A Table 1: Location of significant SNPs and the genes located within 1 Mbp of the most significant SNP in the chromosomal region for AFC.

Chromosome	Significant SNP Locations	Gene	Gene Location
2	96774781 96714406[a] 96804827 96807982 96801078 96801273 96801273	CREB1	95864818-95897170
		METTL21A	95908907-95918927
		CCNYL1	95992629-96029603
		FZD5	96034762-96042102
		PLEKHM3	96095228-96318519
		CRYGA-D	96395739-96435050
		C2H2oef80	96437056-96460429
		IDH1	96510381-96531400
		PIKFYVE	96546574-96628760
		PTH2R	96667750-96751783
		MAP2	97263427-97562142
		UNC80	97594858-97825043
3[b]	104450767 104451813 104420695 104422767 104423898	YBX1	103520065-103539625
		PPIH	103540363-103565504
		LOC 112445912	103568648-103580856
		CCDC30	103584645-103713873
		ZMYND12	103720824-103755638
		PPCS	103716209-103720494
		RIMKLA	103772438-103807695
		LOC101907688	103807843-103851717
		F0XJ3	103880144-104005470
		GUCA2A	104015493-104031213
		GUCA2B	104021061-104024316
		HIVEP3	104109087-104674365
		LOC107132340	104442728-104464286
		EDN2	104679329-104706954
		FOXO6	104815304-104837023
		SCMH1	104964994-105189644
		SLFNL1	105189750-105198784
		CTPS1	105201090-105232559
		CITED4	105348797-105350138
		KCNQ4	105374630-105433410
		MIR30C,E	105457746-105460971
		NFYC	105445059-105510431
23	48495249 48497351	BMP6	47502057-47662629
		SNRNP48	47797829-47816534
		DSP	47824109-47868043
		CAGE1	47960663-47997767
		RIOK1	47936864-47960629
		SSR1	48004708-48034860
		RREB1	48048382-48215277
		LY86	48528635-48576190
		LOC112443817	48676267-48702082
		F13A1	48770276-48913854
		NRN1	48986030-48994877
		FARS2	49111514-49417402
		LYRM4	49417555-49509666

a. SNP on chromosome 2 at location 96714406 is located within PTH2R gene

b. All five SNPS on Chromosome 3 located within HIVEP3 gene

Appendix A Table 2: Location of significant SNPs and the genes located within 1 Mbp of the most significant SNP in the chromosomal region for RTS.

Chromosome	Significant SNP Locations	Gene	Gene Location
2	96870517 96879125	CREB1	95864818-95897170
		METTL21A	95908907-95918927
		CCNYL1	95992629-96029603
		FZD5	96034762-96042102
		PLEKHM3	96095228-96318519
		CRYGA-D	96395739-96435050
		C2H2oef80	96437056-96460429
		IDH1	96510381-96531400
		PIKFYVE	96546574-96628760
		PTH2R	96667750-96751783
		MAP2	97263427-97562142
		UNC80	97594858-97825043
		RPE	97826468-97847708
		KANSL1L	97846946-97972643
8	71343554 71359794	CHMP7	70374034-70387496
		R3HCC1	70404873-70413064
		LOXL2	70414275-70527420
		ENTPD4	70548411-70586238
		SLC25A37	70670484-70711535
		NKX2.6	70823594-70828041
		NKX3.1	70788982-70791605
		STC1	70977493-70990673
		ADAMDEC1	71496993-71622274
		ADAM7	71653464-71706037
		NEFM	72181208-72186703
		NEFL	72213269-72217537
10	68971489	KTN1	68006271-68119073
		PELI2	68531911-68731213
		TMEM260	69005149-69075631
		OTX2	69215035-69224752
		EXOC5	69613361-69671092
		AP5M1	69671083-69696378
		NAA30	69768396-69789540
		CCDC198	69823216-69853335
		SLC35F4	69899835-69931916
11	90023995[a]	RNF144A	89897594-90026495
		RSAD2	90038987-90056291
		CMPK2	90068004-90085403
		LOC100847972	90692236-90701372
		SOX11	90946223-90955282

a. The SNP marker on chromosome 11 at 90023995 is located within the RNF144A gene.

Appendix B: Manhattan Plots for Grazing Behavior Traits
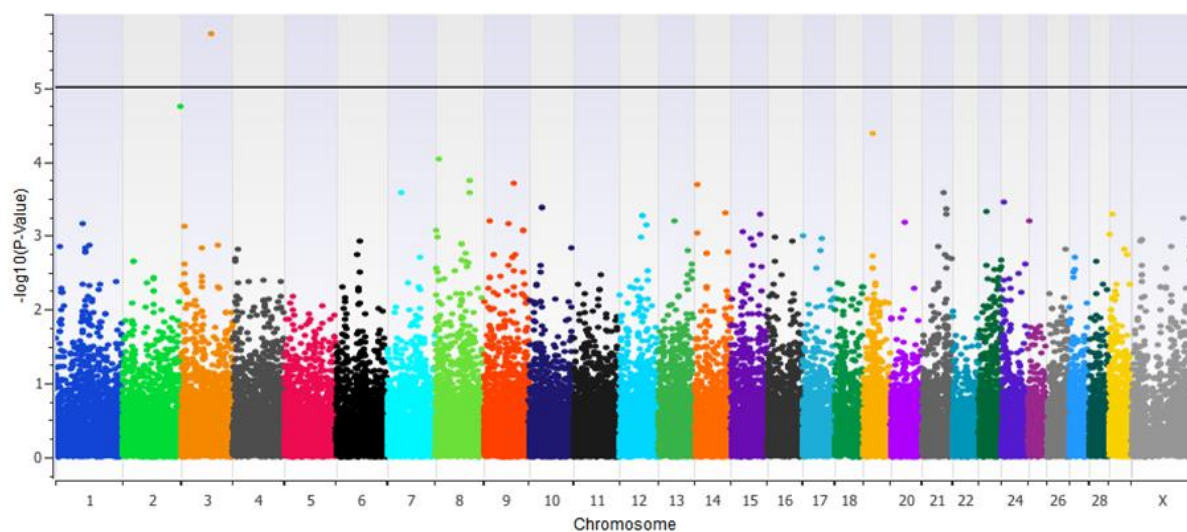
Appendix B Figure 1: Manhattan plot of the GWAS for grazing minutes The solid line denotes the genome-wide significance at -log(p-value)=5.
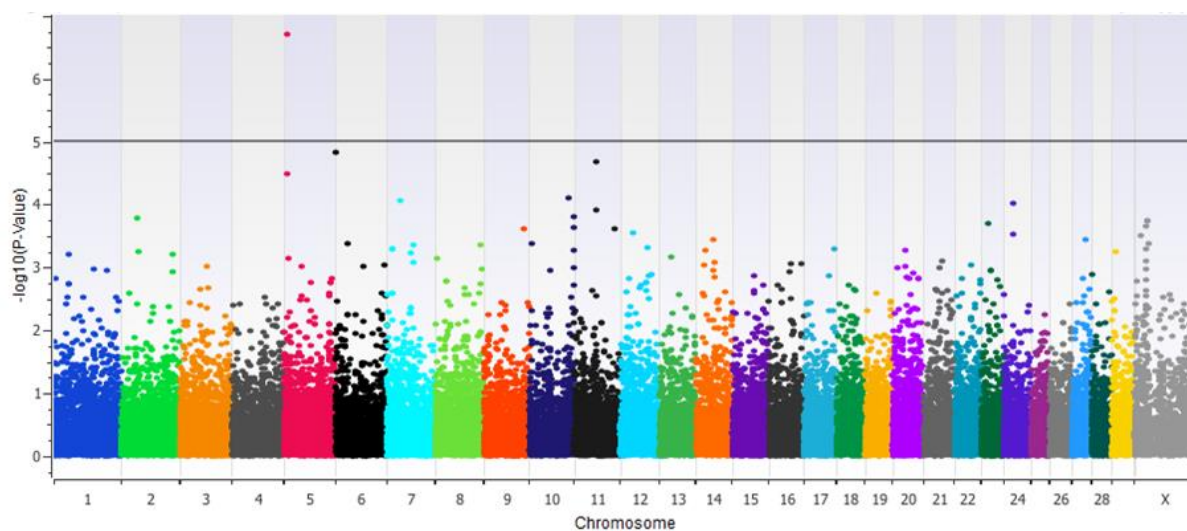
Appendix B Figure 2: Manhattan plot of the GWAS for maximum slope use. The solid line denotes the genome-wide significance at -log(p-value)=5.
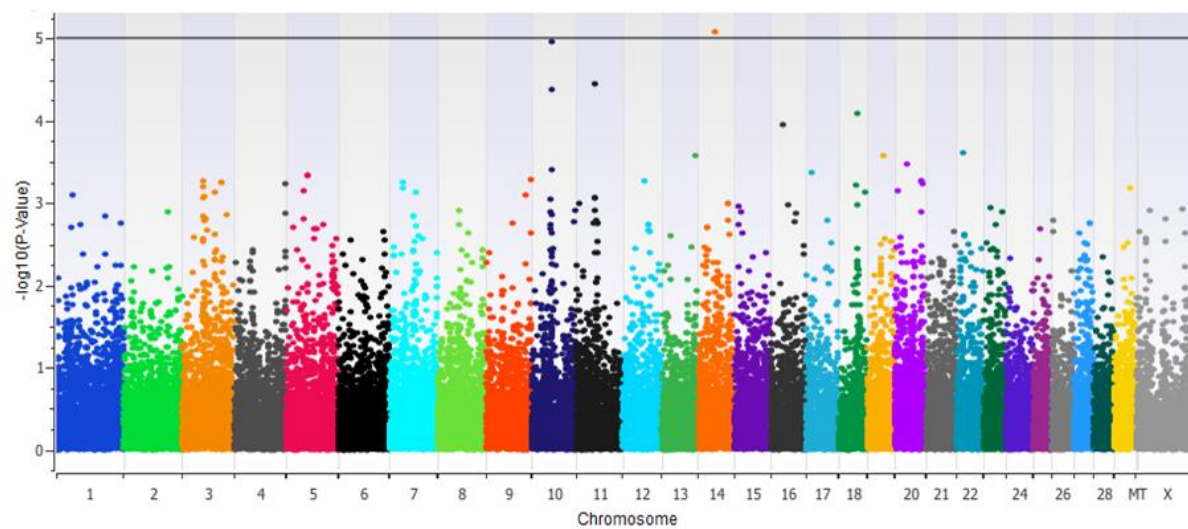
Appendix B Figure 3: : Manhattan plot of the GWAS for time spent at slope greater than 15 degrees. The solid line denotes the genome-wide significance at -log(p-value)=5.