A NUTRIGENOMIC PERSPECTIVE TO SEARCH FOR GENE VARIANTS THAT INFLUENCE CARCASS TRAITS OF FEEDLOT CATTLE

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Saskatoon, SK Canada

Submitted by

Kelsey Evelyn Hamilton

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University of Saskatchewan

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Dean

College of Graduate and Postdoctoral Studies

University of Saskatchwan

116 Thorvaldson Building, 110 Science Place

Saskatoon, Sakatchewan, S7N 5C9, Canada

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ABSTRACT

Vitamin A (VA) has a nutrigenomic effect on intramuscular fat. Discovering variants in genes involved in fat deposition that are also affected by vitamin A could allow feedlots to precision feed to optimize carcass traits. A single nucleotide polymorphism (SNP) in *alcohol dehydrogenase 1C* (*ADH1C*) has previously shown promise for this approach but has yet to be effective at a commercial level; therefore we hypothesized a variant in another gene or its interaction with *ADH1Cc.-64T>C* might be the solution. Genes previously shown to be affected by retinoic acid, a metabolite of vitamin A: *aminopeptidase* (*ANPEP*), *clusterin* (*CLU*), *adipose differentiation-related protein* (*ADFP*), *glutathione peroxidase* (*GPX3*), *secreted protein, acidic, cysteine-rich* (*SPARC*), and *insulin growth factor binding protein 6* (*IGFBP6*) were sequenced and screened for variants. The *ANPEPc.410G>A* SNP was selected for genotyping in a population of mixed breed steers (n=988). This population was fed vitamin A at 100% (100VA) or 50% (50VA the NRC recommended level (2200 IU/kg dry matter). No interaction was found with *ADH1Cc.-64T>C* however, *ANPEPc.410G>A* affected carcass yield (P<0.01; *AA*=2.47±0.03, *GA*=2.36±0.03, *GG*=2.14±0.08), marbling score (P<0.01; *AA*=397.2±2.7, *GA*=388.6±3.3, *GG*=370.4±7.2), and fat (P<0.01; *AA*=8.52±0.17 *GA*=7.58±0.21, *GG*=7.04±0.44; mm). Vitamin A also had an effect on backfat (P<0.05; 100VA= 8.13±0.24, 50VA = 7.35±0.25), and an interaction with *ANPEPc.410c.G>A* affected rib-eye area (P<0.05). The *ANPEP* SNP was genotyped in a second population of mixed breed steers (N=708) fed a standard feedlot ration with the NRC recommended level of vitamin A. There was an association with yield, marbling, fat, and rib-eye area (P<0.01). The AA genotype was more marbled, while GG animals were leaner with higher yields. Interestingly, *ANPEPc.410G>A* is the fourth variant in a haplotype containing twelve SNPs that are in linkage disequilibrium in exon 1 and intron 1. This

was confirmed by sequencing cattle of various breeds from different populations. The three haplotypes could affect gene expression by altering transcription or translation efficiency. Investigation of the functional effects of these variants needs to be completed in order to understand how it alters traits related to feedlot cattle performance.

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LIST OF ABBREVIATIONS

ADFP	Adipose differentiation related protein
ADH1C	Alcohol dehydrogenase 1 C
ANOVA	Analysis of Variance
ANPEP	Aminopeptidase
ATRA	All-trans retinoic acid
BIP	Bovine intramuscular preadipocyte
BSA	Bovine serum albumin
BSU36I	E.coli carried BSU36 gene from Bacillus subtillus 36
C/EBP	CAAT/Enhancer-binding protein
CD	Cluster of differentiation
CLA	Conjugated Linoleic acid
CLU	Clusterin
CRABP2	Cellular retinoic acid binding protein 2
ddH_2O	double distilled water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
E2F2	E2F Transcription factor 2
ECM	Extracellular membrane
ERK	Extracellular regulated kinases
Exo sap	Exonuclease I shrimp alkaline phosphatase
FAT	back fat thickness
GPX3	Glutathione peroxidase 3

H3K27me3	methylation of histone 3 on lysine 27
H3K4/9ac	acetylation of histone 3 lysine 4 or 9
HCW	Hot carcass weight
IARC	International Agency for Research on Cancer
IGF	Insulin growth factor binding protein 6
IGFBP6	Insulin growth factor
IMF	Intramuscular fat
in	inch
IRAP	Insulin-regulated aminopeptidase
IU	International unit
kg	kilogram
LEP	Leptin
LSD	Least significant difference
MAF	Minor allele frequency
MAM	Marker assisted management
MAPK	mitogen-activated protein kinase
microRNA	micro ribonucleic acid
ml	millilitre
μl	microlitre
mRNA	messenger ribonucleic acid
NRC	National research council
PCR	Polymerase chain reaction
PPAR	Peroxisome proliferator-activated receptor

pref-1	Preadipocyte factor - 1
RA	Retinoic acid
RANBP10	Ras-related nuclear protein binding protein 10
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RAS	Renin-angiotensin system
RFLP	Restriction fragment length polymorphism
RXR	Retinoid X receptor
SAGE	Serial analysis of gene expression
SNP	Single nucleotide polymorphism
SOX9	Sex-determining region Y (SRY) -Box 9
SPARC	Secreted protein, acidic, cysteine-rich
sq cm	square centimeter
US	United States
UTR	Untranslated region
VA	Vitamin A
VGMARB	Vision grade marbling
VGYLD	Vision grade yield
Wnt	Wingless (Drosophila) + integration site (mouse)

1.0 GENERAL INTRODUCTION

In the beef cattle industry, from a management standpoint, it is essential for cattle to have reached the desired growth of skeletal muscle and intramuscular fat (IMF) in the most cost-effective way possible. Feed accounts for the major portion of the total cost of cattle production, and, therefore, it is vital to maximize the outcomes from the ration fed to the animals. Although inexpensive in itself, a component of feed that has gained recent attention is vitamin A (VA). The VA metabolites and isomers, such as all-trans retinoic acid (ATRA) have known powerful effects on gene regulation (McGrane, 2007). Nutrigenomics and nutrigenetics can be powerful tools to use to find genes and gene variants that would be profitable in a marker-assisted management (MAM) system. Knowledge of genes that affect traits such as IMF that are also affected by certain nutrients, such as VA, could allow managers to adjust the level of these in the diet to have the most desired outcome. Genes that are affected by VA during the differentiation phase of cells involved in IMF production, such as preadipocytes, could be useful in a search for single nucleotide polymorphisms (SNP)s to use in a MAM system.

The expression levels in six genes: *adipose differentiation-related protein* (*ADFP*), *glutathione peroxidase 3* (*GPX3*), *insulin growth factor binding protein 6* (*IGFBP6*), *secreted protein, acidic, cysteine-rich* (*SPARC*), *clusterin* (*CLU*) and *aminopeptidase* (*ANPEP*), changed greatly in bovine intramuscular preadipocyte cell (BIP) culture after exposure to ATRA during differentiation (Mizoguchi *et al.*, 2014). Gene expression levels were increased after addition of ATRA in five of the six genes, whereas *ANPEP* had decreased expression (Mizoguchi *et al.*, 2014). Additionally, a series of studies on a SNP in *alcohol dehydrogenase 1 C* gene (*ADH1C*; Ward *et al.*, 2012; Krone *et al.*, 2015), interacted with VA in the diet and showed an association with IMF in finished steers when VA was limiting in the diet. Recently, the *ADH1Cc.-64T>C*

genotype was tested in a commercial setting with 50% of the NRC recommended level of VA and included both hormone implanted (Component TE100; 100 mg trenbolone acetate and 10 mg estradiol) and non-implanted steers. They did not observe any significant differences with respect to IMF (Madder *et al.*, 2018) but perhaps this gene interacts with others regulated by VA.

2.0 LITERATURE REVIEW

2.1 Consumer Beef Preferences

Consumer preferences influence their decisions and therefore control what types of products the beef industry must provide to remain competitive. This is important at the storefront but begins at the cow-calf and feedlot levels. Those looking for high-quality beef products will often choose cuts that contain higher marbling content and are willing to pay more for it (Killinger *et al.*, 2004). Marbling, although not the main contributor to beef tenderness is often associated with it and contributes to the eating experience by adding flavor and juiciness (Killinger *et al.*, 2004). Unsaturated fatty acids found in marbling decrease the melting point of fat and increase the palatability (Smith *et al.*, 2016). A study of consumers in the United States showed that locally sourced beef, breed, traceability, and quality of the meat were the most important factors influencing their buying choices (Mennecke *et al.*, 2007). It is reasonable to assume that Canadian consumers would have similar preferences. Since Canada exports approximately 45% of its total beef production, 75% of which goes to the US (Canadian Cattlemen's Association, 2018), it is essential that US consumers are willing to purchase beef produced in Canada. When US consumers were asked to score Canadian AAA and AA along with US Choice and Select steaks for tenderness, taste, flavour, juiciness, and likelihood to buy, there were no significant differences between the steaks of equivalent grades. The higher marbled Canadian AAA and US Choice were equally preferred over the other grades (Tedford *et al.*, 2014). Additionally, the consumers in the Tedford *et al.* (2014) study indicated high perceptions towards the quality and safety of Canadian beef and very few had a concern about country of origin labeling information.

2.1.1 Health Concerns

Although a preference for marbling is based on palatability, consumers are becoming increasingly health conscious and are selecting leaner meat cuts. In studies which investigated disparities of gender when making or selecting food choices reviewed by Mennecke et al. (2007), women are suggested to be more concerned than men about the health effects of meat. Beef is very high in nutrients that are important in a healthy diet such as creatine, conjugated linoleic acid, certain antioxidants, vitamin B12 and zinc among other vitamins and minerals. In particular, iron from red meat is well absorbed and even improves the absorption of iron from vegetable sources when consumed together (Troy *et al*., 2016). There have been claims in the popular media that the fat in beef is a contributor to heart disease. Marbling, however, is different from other fat on the beef carcass; while intermuscular fat contains many saturated fatty acids, intramuscular fat contains many healthy unsaturated fatty acids (Troy *et al*., 2016). Oleic acid can increase beneficial high-density lipoprotein cholesterol but not the harmful low-density lipoprotein and has no risk for increased cardiovascular disease (Smith *et al*., 2016). Beef products are the highest sources of conjugated linoleic acids (CLA) which are known to have powerful anti-carcinogenic effects (Nuernberg *et al*., 2005). Grass-feeding can increase CLA in marbling, but because cattle on grass have lower marbling, the proportion is similar to that in marbling of beef finished on concentrate diets (Nuernberg *et al*., 2005). Despite this information, there is a growing concern that red meats can be carcinogenic to humans. A report on red meats by the International Agency for Research on Cancer (IARC) has classified it as probably carcinogenic to humans based on several epidemiological studies (IARC, 2015). Reviews of this investigation have noted problems with this claim and the way the public has interpreted it. The problems were based on the challenging nature of epidemiological studies to determine cause and

effect (Troy *et al.*, 2016; De Smet & Vossen, 2016). Rather than the meat itself, the preparation and cooking methods that cause charring and the production of heterocyclic aromatic amines is the concern for carcinogenic effects (De Smet & Vossen, 2016). There is much agreement that contributions of the overall diet to either promote or protect against the carcinogenic effects of red meats needs to be investigated but when diets and lifestyles are balanced there is little concern for any adverse health effects (Troy *et al.*, 2016; De Smet & Vossen, 2016).

2.2 Adipogenesis

Adipogenesis is the biological differentiation process of preadipocyte cells to adipocytes (Gregoire *et al.*, 1998). Adipocytes are the specialized cells that are used to store fat throughout the body. Understanding this process in production animals is extremely beneficial as fat quantity and location is an important determinant of carcass quality (Pickworth *et al.*, 2011). Control of adipogenesis is very complex due to the involvement of the coordinated expression of many genes (*pref-1*, *adipogenin*, *leptin*), transcription factors, their genes (C/EBPα, C/EBPβ, PPARs) and other regulatory elements (Figure 2.1). In adult tissues the preadipocyte factor-1 (pref-1) protein allows the preadipocytes to continue to proliferate and increase in number by inhibiting differentiation (Wang *et al.*, 2010). In order for induction of differentiation, growth arrest of the preadipocytes must be signaled by the CCAAT/enhancer binding protein β (C/EBP; Roh *et al.*, 2006; Gregoire *et al.*, 1998). The inhibitory effect pref-1 has on differentiation is due to the upregulation of the ERK/MAPK pathway, which upregulates *SOX9*, that binds to the promotor of *C/EBP* preventing its transcription (Wang *et al.*, 2010).

Recently, a study in mouse 3T3-L1 cell lines found that *pref-1* is regulated by a microRNA (miRNA-143), which is a small non-coding RNA molecule, that binds to a conserved site in the 3'UTR, which causes complete downregulation of *pref-1* and allows expression of *C/EBPβ* to initiate differentiation (Kim *et al.*, 2015). Further regulation by microRNAs, investigated in bovine preadipocytes, revealed that microRNA-378 post-transcriptionally targets two important transcription factors in cell growth, E2F2, and RANBP10, which decreases their expression and promotes differentiation of preadipocytes (Liu *et al.*, 2015). Expression of *PPARγ* follows *C/EBPβ* to bring about growth arrest of the preadipocytes and promotes the maturation stage along with expression of adipogenic genes. As adipocytes begin to mature the *C/EBPα* isomer replaces *C/EBPβ* (Figure 2.2; Roh *et al.*, 2006; Gregoire *et al.*, 1998).

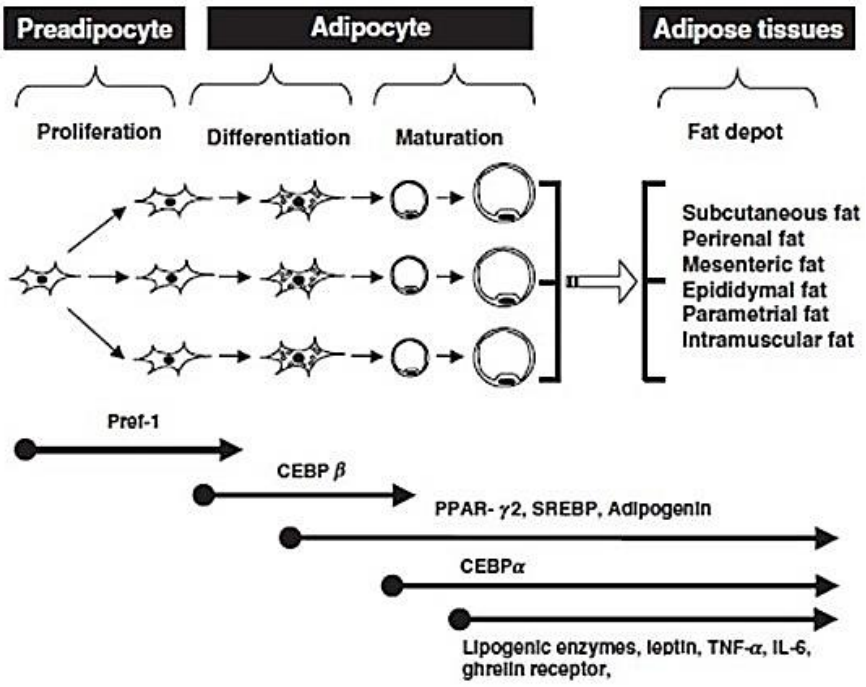

Figure 2.1. Order of the main genes that are expressed throughout the process of adipogenesis in ruminants (Roh *et al.*, 2006 with permission)

Changes to the extracellular matrix (ECM) is also necessary to initiate adipocyte differentiation by allowing room for the adipocytes to change from a fibroblast-like-shape to the rounded shape of mature adipocytes (Nie and Sage, 2009). Prior to adipogenesis, the ECM is rich in type I and III collagen, poly-L-lysine, fibrinogen, and α5 integrin, which have an inhibitory effect on differentiation. The conversion to a basement membrane consisting of type IV collagen, entactin, laminin, and α6 integrin promotes the differentiation of preadipocytes (Nie and Sage, 2009; Lui *et al.*, 2005). Interactions of preadipocytes with the changing ECM components may allow communication with the extracellular environment that are important for signaling the morphological changes during differentiation (Gregoire *et al.*, 1998). In non-ruminants, the absorption of long chain fatty acids and glucose from the diet can have a hormone-like effect that promotes *C/EBP* and *PPAR* transcription and promotes adipogenesis (Azain, 2004). In ruminants, such as cattle, due to bacteria causing biohydrogenation of consumed lipids and fermentation of other nutrients into short chain fatty acids, the nutritional control of adipogenesis may be quite different. For example, acetate, rather than free fatty acids or glucose, is the primary source of lipogenesis in ruminants (Roh *et al.*, 2006; Aso *et al.*, 1995). Another important difference is that lipogenesis in ruminants occurs primarily in the adipose tissue, rather than in the liver (Roh *et al.*, 2006). Although never confirmed in bovine preadipocytes, acetate and propionate have both been shown to positively influence adipogenesis and the expression of *PPAR* through a G-protein coupled receptor in mice 3T3-L1 adipocytes (Hong *et al.*, 2005). There is also evidence that cattle fed a high energy grain diet vs. a forage based diet have higher expression of adipogenic genes and lower expression inhibitory genes (Key *et al.*, 2013).

2.3 Nutrigenomics & Nutrigenetics

Nutrigenomics involves the study of nutrients and how they affect the whole genome. A branch of nutrigenomics is nutrigenetics. Nutrigenetics investigates inherited genetic variation and involves the study of an animal's response to various nutrients, based on genotype (Farhud *et al*., 2010; Fenech *et al*., 2011). Nutrigenomics can act in an epigenetic mannor, with epigenetics being described as heritable changes that involve the regulation of genes more broadly than in the DNA sequence. Specifically factors that determine how and when genes are turned on or off, through DNA methylation and histone modifications (Bar-El Dadon and Reifen, 2017; Fenech *et al*., 2011). Understanding how nutrients act in epigenetic pathways in animals with specific genotypes can be extremely beneficial to understanding health and important traits of livestock in their various environments throughout their lives from on pasture to feedlot management. There are limitations to understanding how nutrigenetics can influence an animal based on varying intake levels across individuals and due to genetic differences in genes outside of the scope of study (Fenech *et al*., 2011).

2.3.1 Vitamin A

Vitamin A is the general term that refers to dietary β-carotene and its metabolite retinoids. Vitamin A is involved in gene regulation due to the ability of retinoids to regulate gene expression. This is an example of nutrigenomics. One of these metabolites, retinoic acid (RA), could potentially regulate expression levels of more than 500 genes (Ambrosio *et al.*, 2011). Isomers of RA such as ATRA and 9-cis-retinoic acid enter into the nucleus via cellular retinoic acid binding protein 2 (CRABP2) and then bind to RA receptors (RAR) and retinoid X receptors (RXR), respectively (Figure 2.2; Bar-El Dadon and Reifen, 2017). These homo- or heterodimers

target response elements, such as the retinoic acid response element (RARE), in the promoter regions of genes, regulating their transcription (Figure 2.2; McGrane, 2007). Regulation by RA in genes that influence growth can have an effect on adipocyte formation by altering differentiation and maturation (Wang *et al.*, 2016). Furthermore, there has been evidence that RA exposure in genes regulated by RAR causes decreased repressive methylation (H3K27me3) and increased activation acetylation (H3K4/9ac) epigenetic marks (Bar-El Dadon and Reifen, 2017). Nutrigenomics effects of VA occur when genetic variation among individuals results in VA affecting these individuals differently. The sequence differences at these critical regulatory regions can alter the binding of RA receptor complexes or a number of other regulatory mechanisms.

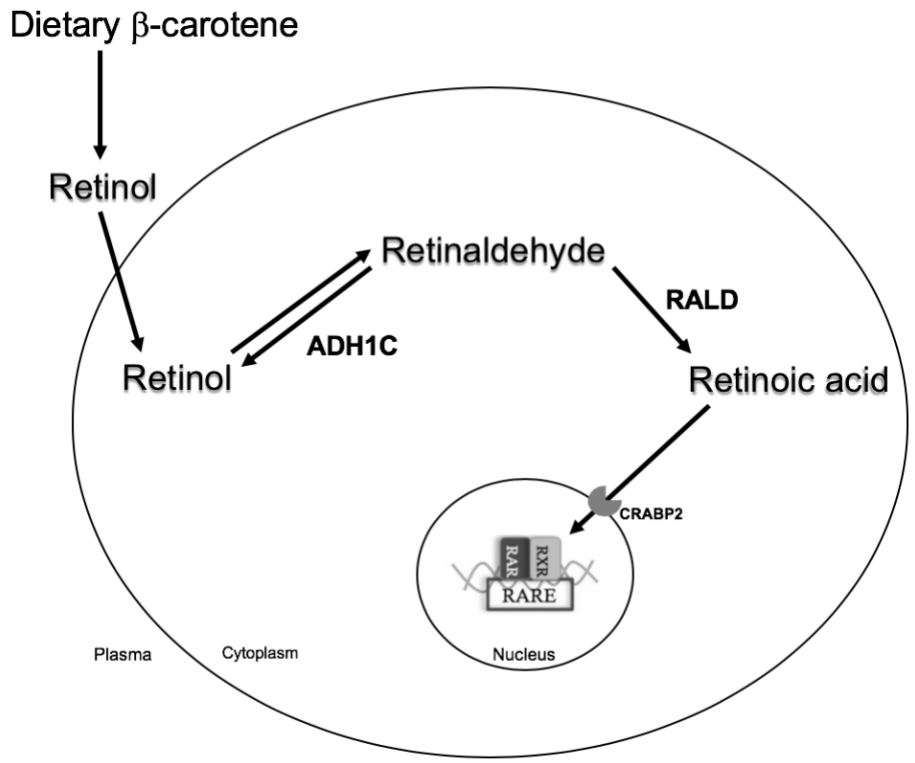

Figure 2.2 Schematic of retinol metabolism in the cell.

2.4 Gene Expression

Gene expression is the process by which the genetic information results in a gene product, usually a protein. Transcription is required for gene expression by converting the DNA code into RNA. The RNA is then translated into a protein. Interruption at any of these steps will affect gene expression, whether this is a change to the DNA sequence or to the regulatory mechanisms and proteins that control transcription and translation. Simply possessing a gene does not necessarily mean that the gene will be expressed and also does not determine the amount of expression. There are many factors and elements that control and modify the expression of genes. This can be a permanent change such as activation or silencing of a gene, or a minor change within genes can also affect transcription levels or the bioactivity of the resultant protein.

2.4.1 Gene variations

A powerful tool for animal selection is the identification of SNPs in association with production traits. These SNPs can alter the protein in ways that affect production traits. Most often the SNPs that occur in the 5' region of the gene will have the greatest effect on expression, while non-conserved missense mutations within exons have the greatest effect on resultant protein structure and function. Important regulatory elements are found within the 5' region of genes, and therefore any changes to the DNA sequence could change transcription factor binding. An example of a 5' SNP in cattle is *ADH1Cc.-64T>C* studied by Ward et al. (2012). The variant causes the removal of a motif for a binding site for the C/EBPα transcription factor and results in less transcription of *AHD1C* (Ward *et al*., 2012).

Non-conserved missense mutations are a single base change that result in the substitution of an amino acid with another one that belongs to another group. A well known *Bos taurus* non-

conserved SNP is in the *Leptin* gene (*LEPc.73C>T)* resulting in a change of amino acid from arginine (basic) to cysteine (polar and sulfur-containing). Animals with the TT genotype have higher carcass fat, whereas CC animals are leaner (Buchanan *et al.*, 2002). Conserved missense mutations result in a codon corresponding to a different amino acid, but one that remains in the same group according to the side chain. A conserved mutation is found in the appetite regulation gene *Melanocortin-4 receptor* (*MC4Rc.122C>G*) in cattle, where the SNP causes an amino acid change from valine to leucine, both of which are polar (Thue *et al*., 2001). The conserved SNP *MC4Rc.122C>G* tends to affect hot carcass weight (HCW) and an interaction with a SNP in *corticotropin-releasing hormone* (*CRHc.22C>G*) gene results in lower HCW in steers with the CC genotype at both SNPs.

Silent mutations are those that change a nucleotide, often in the third position of a codon, and despite the base change they still code for the same amino acid. It is often thought that since a SNP in the third position of a codon does not result in the change of an amino acid that the SNP has no functional effect on the resultant protein and therefore are relatively ignored. Silent mutations may, however, have a functional role and undergo selection pressures (Chamary *et al.*, 2006; Zhou *et al.*, 2009). In a base change that occurs in *pro-opiomelanocortin* (*POMCc.288C>T*), no amino acid change occurs, and the codon still corresponds to serine (Thue and Buchanan, 2003). An association with *POMCc.288C>T* was found for average daily gain and carcass weight (Buchanan *et al*., 2005). While a silent SNP may not cause an amino acid change that would affect the resulting protein structure, the altered sequence could affect mRNA structure and consequently translation (Shabalina *et al.*, 2013). It is also important to consider that an amino acid has about four codons that correspond to that particular amino acid, and a phenomenon known as codon bias, states that not all codons will be used as often as the others.

(Zhou *et al.*, 2009). A variant in the third position of the codon may result in a less common codon which can interrupt translation (Zhou *et al*., 2009).

2.4.2 Haplotypes

A haplotype is a series of genetic variants that are inherited together from one parent. Commonly, a haplotype refers to variants that span a large section on a chromosome, and are frequently found in inbred or closely related populations. Trait inheritance information can be used to predict performance of an animal in terms of production traits or disease resistance and species evolution (Calafell *et al.*, 2001; Barendse, 2011). Haplotypes can also be isolated to specific genes. Similar to the haplotypes that span a chromosome, those within genes can provide information about gene evolution and inheritance throughout a population, as well as information about specific traits (Ueyama *et al*., 2012). Allele variations within a haplotype can be very useful for identifying frequency of cross-over events and understanding genes at the population levels (Wall and Pritchard, 2003).

2.5 Candidate Genes

2.5.1 Alcohol Dehydrogenase 1 C

Alcohol dehydrogenase 1 C (ADH1C) is the enzyme that oxidizes retinol to retinaldehyde which is later oxidized to retinoic acid. A previous study identified a SNP in the *ADH1C* gene of *Bos taurus* cattle (*ADH1Cc.-64T>C*). This variant eliminated the binding motif for an important transcription enhancer factor (C/EBPα), with the C allele, causing lower levels of transcribed *ADH1C* in animals with one or two copies of this allele (Ward *et al*., 2012). The *ADH1Cc.-64T>C* SNP showed promise in MAM to optimize IMF at low dietary VA levels (Ward *et al*.,

2012; Krone *et al*., 2014). Animals homozygous for the C allele have less ADH1C to oxidize retinol to retinaldehyde and when VA is limiting the next oxidation step of retinaldehyde to retinoic by retinaldehyde dehydrogenase is increased threefold (Ward *et al*., 2012). Consequently, the TT animals had higher IMF (Ward *et al*., 2012; Krone *et al*., 2014). An extensive commercial trial of *ADH1Cc.-64T>C* genotype on 50% of the recommended level of VA did not have an effect on IMF (Madder *et al*., 2018). VA is important for gene regulation by activating RA receptors that combine in either homo or heterodimers and further activate RAREs in the promoter regions of genes involved in adipogenesis. The effect of the *ADH1Cc.-64T>C* SNP on the metabolism of the VA pathway may still effect IMF through an interaction with a gene involved in adipogenesis or required for IMF production.

2.5.2 Adipose differentiation-related protein

In cattle, the *adipose differentiation-related protein* gene is located on chromosome 8 and contains 9 exons. The *ADFP* gene codes for the ADFP protein, which belongs to a lipid droplet-associated protein family. Lipid droplets are the storage form of triglycerides in tissues and are found in association with proteins such as ADFP in skeletal muscle. The more ADFP present, the higher the capacity to store triglycerides (Shaw *et al*., 2009). Despite high expression of *ADFP* mRNA in adipose tissues throughout differentiation, the protein content of ADFP begins to decrease at day three of adipose cell differentiation and is replaced by perilipins, which are predominant in mature adipocytes (Brasaemle *et al.*, 1997).

An *in-vitro* study of BIP cells showed *ADFP* to be upregulated during differentiation (Mizoguchi *et al.*, 2014). Furthermore, this study observed increased expression of *ADFP* when ATRA was present. In a study of Korean cattle, a SNP (*ADFPc.-56-18A>G*) was found to be

associated with marbling (Cheong *et al*., 2009). The same variant may not be present in North American cattle populations, but knowledge of this association with marbling in the Korean cattle make this gene worth investigating.

2.5.3 Glutathione peroxidase 3

The mammalian *glutathione peroxidase* (*GPX*) gene family has four members that contain selenocysteine at the active site (Arthur, 2001). The *GPX3* gene has been sequenced in human, rat, mouse, and cattle (Arthur, 2001). It is found on bovine chromosome 7 and has 5 exons. Glutathione peroxidase serves as an antioxidant protein with one of its main roles to reduce hydrogen peroxide, preventing oxidative damage (Yamasaki *et al*., 2006). During differentiation of BIP *in-vitro, GPX3* was upregulated with a further increase when ATRA was present (Mizoguchi *et al*., 2014). An increased expression of *GPX3* during BIP cell differentiation may be due to GPX acting to reduce oxidative damage during lipid metabolism (Yamaskaki *et al.,* 2006).

2.5.4 Secreted Protein, acidic cysteine-rich

In cattle, the *Secreted Protein, acidic cysteine-rich (SPARC)* gene is found on chromosome 7 and has 10 exons. This highly conserved gene throughout all species has a matricellular protein that regulates cell interactions with the extracellular membrane that can be expressed in many tissues at times of development, cell turnover and tissue repair (Yan and Sage, 1999). The SPARC protein is found to interact with growth factors and is known to have relatively diverse activity (Joseph *et al.,* 2012). In a study comparing *SPARC*-null to wildtype mice, the null mice had a larger number and size of adipocyte cells in their fat pads (Bradshaw *et al*., 2003). This is

consistent with studies that have shown SPARC to activate integrin-linked kinase which led to increased levels of β-catenin (part of the Wnt/β-catenin pathway that determines the tissue fate of mesenchymal stem cells) and inhibits adipogenesis by favoring osteoblastogenesis (Nie and Sage, 2009). Additionally, SPARC enhances preadipocyte growth and proliferation by enhancing fibrinogen in the extracellular matrix and the α5 integrin and prevents expression of α6 integrin and deposition of laminin which is associated with growth arrest (Nie and Sage, 2009). When BIPs undergo differentiation *SPARC* is downregulated, however, with ATRA present during differentiation, SPARC mRNA levels are higher (Mizogouchi *et al.*, 2014).

2.5.5 Insulin growth factor binding protein 6

The bovine *insulin growth factor binding protein 6* (*IGFBP6*) gene, has 4 exons, is located on chromosome 5, and is an important binding protein for insulin growth factors (IGFs). Although IGFBP6 binds both IGF-1 and IGF-2, it is especially important for IGF-2 inhibition, having a 50 fold preferential binding affinity for IGF-2 over IGF-1 (Bach, 2015). Since IGF-2 is still prevalent in adult tissues, while IGF-1 is downregulated after birth (Bach, 2015), the preferential binding of IGFBP6 for IGF-2 may indicate its importance to growth and development.

The regulation of IGFBP6 is cell-specific and is controlled by effectors such as cAMP, IGFs, vitamin D, p53, glucocorticoids and most importantly for our purposes, RA (Bach, 2015). Regulation of *IGFPB6* expression has been shown to be controlled by retinoid X receptor (RXR) and retinoic acid receptor β (RARβ) in the first intron, in which expression is increased by binding of retinoids (Uray *et al.*, 2009). It has also been proposed that the suppression of *IGFBP6* upon adipogenic stimulation may be an indication that this gene controls the proliferation of pre-adipocytes (Mizoguchi *et al.*, 2014).

2.5.6 Clusterin

The *Clusterin* (*CLU*) gene, is located on chromosome 8 and contains 11 exons in *Bos taurus*. The gene is highly conserved, and expression is found in many mammalian tissues; in humans, two distinct mRNA transcripts have been found, due to regulation by two different promoters (Rizzi *et al.*, 2009). The CLU protein, also known as apolipoprotein J, is most commonly found in the cell cytosol under stress conditions (Poon *et al*., 2002). Determining the exact physiological role of CLU has been problematic due to the fact that the protein appears to be involved in many different processes, interacting with many molecules, including itself, often acting as a chaperone protein (Jones and Jomary, 2002). Most of the research on CLU has been in humans on cancer, Alzheimer's and male fertility, although some research has investigated CLU in the area of obesity (Oberbach *et al.*, 2011). An important role of CLU may be in its association with high-density lipoproteins which reduces the level of low-density lipoproteins and therefore helps to reduce inflammation and oxidation (Park *et al.*, 2017; Brites *et al.*, 2017). *In-vitro* study of BIP cells showed increased expression of *CLU* during differentiation, and even more expression occurred when ATRA was present when the BIP cells were maturing (Mizoguchi *et al*., 2014).

2.5.7 Aminopeptidase

Aminopeptidase (*ANPEP*), also known as *CD13*, *APN*, and *P150*, has 20 exons and is found on chromosome 21 in *Bos taurus*. The *ANPEP* gene is conserved throughout many species. Exon one is highly conserved as it contains the vital cytoplasmic domain as well as the transmembrane anchoring region (Luan and Xu, 2007). Expression of ANPEP is found in many different tissues throughout the body but shows the highest expression in the brush border membranes of the

kidney, mucosa of the small intestine and in the liver (Chen *et al.*, 2012). The ANPEP protein is known for having many different biological roles, however, its extent of importance and involvement is not well known. It is a membrane-bound member of the M1 zinc aminopeptidases, cleaving amino acids from the N-terminal ends of proteins, especially favouring alanine (Sato, 2003). A function of ANPEP may be influencing angiogenesis (Rangel *et al*., 2006; Sato 2003).

Aminopeptidase may influence the amount of adipose tissue present throughout the body due to its role in the Renin-Angiotensin System (RAS). The most well-known role of RAS is in the maintenance of blood pressure and electrolyte balance in the body through controlling water and solute filtration of the kidneys. Recent research, however, has focused on how the role of RAS in other tissues may affect feed intake and adipose tissue growth and metabolism (Sunagawa *et al.*, 2001). There are several aminopeptidases involved in the RAS pathway that result from different genes. Aminopeptidase N has been identified as the enzyme that converts angiotensin III to angiotensin IV. Angiotensin IV is one of the final products of the RAS pathway and activates insulin-regulated aminopeptidase (IRAP), which downstream regulates glucose uptake. The main hormone of the RAS pathway, angiotensin II has been shown to inhibit adipogenesis (Slamkova *et al*., 2016). There is evidence that angiotensin III function very similarly to angiotensin II and in some cases may be the preferred ligand (Yugandhar and Clark, 2013).

2.6 Hypothesis

I hypothesized that based on function, one or more of the six candidate genes (*ANPEP, CLU, ADFP, IGFBP6, GPX3,* and *SPARC*) will contain a variant that will affect marbling content and overall carcass quality of steers. The candidate gene may also have an interaction effect with *ADH1C* when vitamin A is limited.

3.0 *ANPEP* VARIANTS AFFECT CARCASS TRAITS OF BEEF CATTLE

3.1 Introduction

Identification of gene variants that modify the performance of beef cattle at any stage of production is a valuable tool that producers can use to maximize the outputs of their management practices. Knowledge of the effects of gene variants can be of particular importance at the feedlot as this is the point in production where management practices make a large impact on the well-being and growth of the animals. Since management at the feedlot level is a highly controlled and expensive process, it can be advantageous if genotype based management improves the efficiency of current practices in North American feedlots; this is termed marker-assisted management (MAM). At the feedlot, diets are tightly regulated and formulated specifically to the growth stage of the animal. North American practices of raising beef with hormone implants to increase lean meat yield can negatively affect the intramuscular fat (IMF) or marbling of these animals (Wang *et al.*, 2016). Therefore, it is important to find other ways to generate marbled carcasses for consumer demands. Marbling is important because it adds value to meat cuts by contributing to tenderness, flavor, and juiciness. Consumers in North America find higher marbled beef more preferable and are willing to pay more for it (Killinger *et al.*, 2004).

Vitamin A is an important component of a mammalian diet. It is necessary for many processes within the body, including vision, immune function, reproduction, adipogenesis and growth (Li and Tso, 2003). According to NRC beef (1996), the recommended level of VA in the diet of feedlot cattle is 2200 IU/kg dry matter. Vitamin A is consumed in the pro-retinoid carotenoid form, known as β-carotene, which is later converted to retinol in the intestine (D'Ambrosio *et al*., 2011). The bioactive metabolites of VA are retinol and retinoic acid (RA). The isomers of RA, all-trans-retinoic acid (ATRA) and 9-cis-retinoic acid, bind to retinoic acid

receptors (RAR) and retinoid X receptors (RXR), respectively. In either homo- or heterodimers these then bind to RA response elements (RARE) in the promoter regions of genes regulating their transcription and affecting adipogenesis (McGrane, 2007). The ability of retinoids to regulate gene expression is an example of nutrigenomics.

Nutrigenomics has been described as the study of the interaction of nutrients and the whole genome. Nutrigenetics considers genetic variation and involves the study of an animal's response to various nutrients, based on genotype (Farhud *et al.*, 2010). It is thought that RA could regulate expression levels of greater than 500 genes (D'Ambrosio *et al.*, 2011). Nutrigenomic effects of RA regulate gene expression affecting adipocyte formation, and differentiation or maturation (Wang *et al.*, 2016).

Many research projects have been conducted to identify genes involved in IMF or associated with the amount of IMF (Ward *et al*., 2012; Buchanan *et al*., 2005; Buchanan *et al*., 2002). One example is a gene variant in *leptin* (*LEP*) that has been implemented into feedlots (Buchanan *et al*., 2002; Kononoff *et al.*, 2005) in North America. The genotypes from *LEPc.73C>T* are sorted by genotype in North American feedlots to optimize IMF utilizing MAM. Another promising gene variant was in *alcohol dehydrogenase 1C* (*ADH1C*; Ward *et al*., 2012; Krone *et al*., 2015), which interacts with VA in the diet. The *ADH1Cc.-64T>C* SNP had an association with IMF in finished steers when VA was limiting in the diet. Recently, the *ADH1Cc.-64T>C* genotype was tested in a commercial setting with 50% of the NRC recommended level of VA and included both hormone implanted and non-implanted steers. They did not observe any differences with respect to IMF (Madder *et al*., 2018). We are now considering other genes affected by RA that might have a greater effect on IMF, perhaps also having an interaction effect with *ADH1C*.

Identification of genes that affect traits such as IMF that are also affected by certain nutrients, such as VA, could allow managers to adjust the level of these in the diet to have the most desired outcome. A preliminary study evaluated differentially expressed genes in response to ATRA in bovine intramuscular preadipocyte (BIP) cells from Japanese Black cattle (Mizoguchi *et al*., 2010). In this study, serial analysis of gene expression (SAGE) was performed that identified 878 genes that were differentially expressed during intramuscular adipocyte differentiation (Mizoguchi *et al*., 2010). Sixteen genes from this study were selected for further investigation due to the magnitude of change observed in expression levels, in the presence of ATRA (Mizoguchi *et al*., 2014). Gene expression levels for each of the genes was compared with and without exposure to ATRA (Mizoguchi *et al*., 2014).

Six of these genes were selected: *adipose differentiation-related protein* (*ADFP*), *glutathione peroxidase 3* (*GPX3*), *insulin growth factor binding protein 6* (*IGFBP6*), *secreted protein, acidic, cysteine-rich* (*SPARC*), *clusterin* (*CLU*) and *aminopeptidase* (*ANPEP*) whose expression levels changed the most in BIP cell culture after exposure to ATRA (Mizoguchi *et al*., 2014). These genes were screened for variants in Canadian beef cattle, and association studies with carcass traits were assessed. The nutrigenetic and nutrigenomic effect of VA with the gene variant was evaluated.

3.2 Materials and Methods:

3.2.1 Animals

3.2.1.1 Discovery population

Sixteen cattle were used for gene sequencing to search for SNPs. These samples consisted of five purebreds (Limousin, Hereford, and three Simmental) and seven crossbred animals from the Canadian Beef Reference Herd (Schmutz *et al.*, 2001) and an additional four crossbred steers (Pugh *et al.*, 2011).

3.2.1.2 Population 1

This population of 1000 steers was finished at Cattleland Feedyards Ltd (Strathmore, AB) and was used to determine the effect of SNPs and their interaction with VA and/or *ADH1Cc.-64T>C*. This population was previously described by Madder *et al*. (2018). Briefly, 1000 TT steers and 1000 CT steers at *ADH1Cc.-64T>C* were each further divided into 500 hormone implanted (Component TE100; 100mg trenbolone acetate and 10mg estradiol, at sorting and after 70 days on feed) and 500 not implanted. Within each group of 500, 250 were fed 50% and 250 were fed 100% of the NRC recommended level of VA during finishing (Figure 3.1). Animals were slaughtered at an average pen weight of 612 kg. For the purpose of this study only the implanted animals of both TT and CT genotypes were investigated (n=1000).

3.2.1.3 Population 2

This population of 700 steers was representative of feedlot cattle in Canada. Steers were finished at Cattleland Feedyards Ltd. (Strathmore, AB) and were fed a standard feedlot diet with 100% NRC recommended level of vitamin A. The steers were grouped in pens according to standard feedlot procedures.

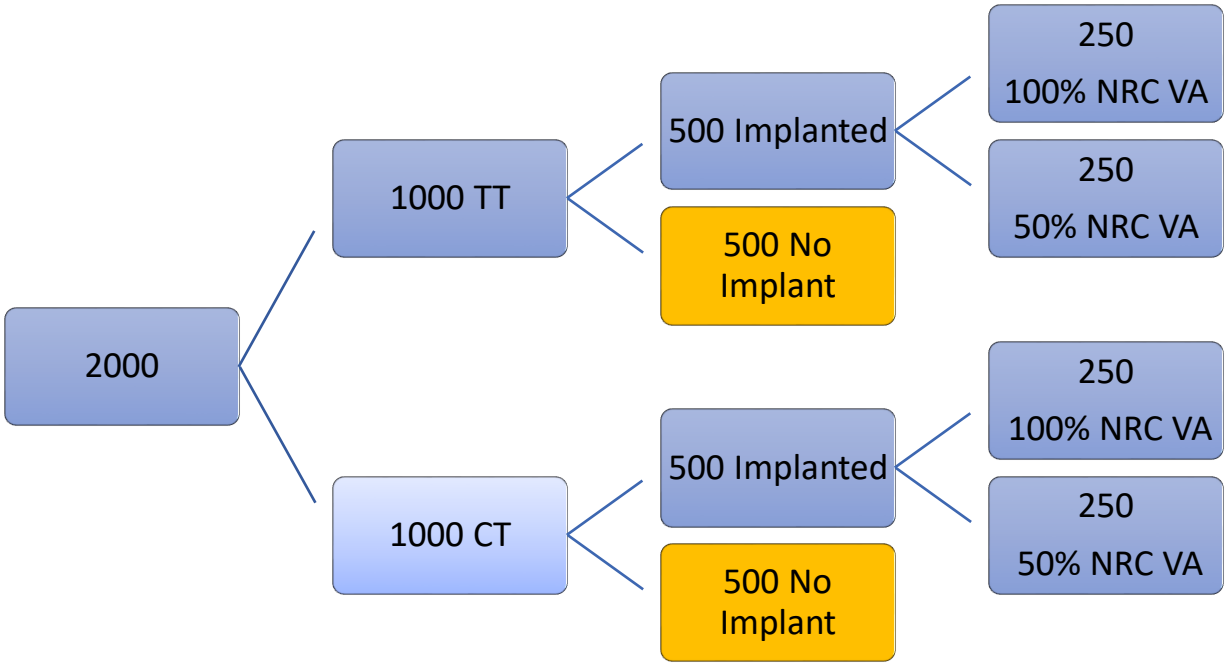

Figure 3.1 Demonstration of how steers were separated into treatment pens based on *ADH1C.-64T>C* genotype, implants and vitamin A treatments (percentage of NRC 1996 recommendation). The orange cell depicts the group of animals that would follow the same pattern as shown for the implanted animals but were excluded from this study.

3.2.2 Carcass Evaluation

Both populations were slaughtered at JBS Food Canada (Brooks, AB) where carcass data was collected. This included hot carcass weight (HCW) and further carcass evaluation was performed via a VBG 2000 (e+v Technology GmbH & Co. K.G, Oranienburg, Germany) vision camera grading system for vision grade yield (YLD; 1.0-5.9), vision grade marbling (MARB; 00-900), ribeye area (REA) and fat thickness.

3.2.3 DNA Extraction

Skin tissue samples were collected from all animals and the DNA extraction was performed at Quantum Genetix (Saskatoon, Sk). The tissue samples were placed into 96 well plates and 0.2 M

NaOH was added followed by an incubation period of 15 mins at 58-62°C. Following incubation, 1.6% concentration HCl + 0.1M Tris was added to each well. Plates were then centrifuged to ensure sufficient mixing of the solutions.

3.2.4 SNP Discovery & Selection

The selected candidate genes, *ADFP*, *GPX3*, *CLU*, *SPARC*, *IGFBP6*, and *ANPEP* were screened for SNPs. The *Bos taurus* reference sequence was obtained for each gene (GenBank NC_007306, AC_000165, NC_007305, NC_007305, AC_000178, AC_000162 respectively). Primers were designed using Primer 3 software (ELIXER) and purchased from IDT (Coralville, Iowa). The primers were designed so that sequence of the coding regions, as well as the 5' UTR and the 3' UTR of each gene, could be searched for variants (Appendix A).

Polymerase chain reaction (PCR) was performed to amplify genomic DNA in 16 animals from the discovery population. The 25 μl PCR cocktail was made with 0.2 pmol forward and reverse primers (Integrated DNA Technologies, Coralville, IA) 2mM $MgCl_2$, 0.2mM dNTP (Burlington, ON), 10X taq buffer with $(NH_4)_2SO_4MgCl_2$), 2 μl Taq polymerase (Fermentas, Burlington, ON) and 0.5 mg/ml BSA and ddH_2O. The PCR was performed on a T100 Thermocycler (Biorad, Mississauga, ON) at 52-62°C for 30 cycles. The program began with initial denaturation at 95°C for 2 minutes followed by 29 cycles of 30 seconds at 95°C, 30 seconds at the annealing temperature (52-62°C) and 45 seconds at 72°C. Finally, an extension period completed the program at 72°C for 10 minutes.

Post-PCR samples were cleaned using an Exonuclease I, FastAp protocol (Thermo Fisher, Waltham, MA) before being sequenced by Sanger sequencing at the National Research Council of Canada (Saskatoon, SK). Sequences were aligned to the *Bos taurus* reference

sequence and analyzed for SNPs with Sequencher 4.9 software (Gene codes corporation, Ann Arbor, MI).

3.2.5 Genotyping

In order to genotype, a polymerase chain reaction-restriction fragment polymorphism (PCR-RFLP) was designed for the *ANPEPc.410G>A* SNP which is part of an 12-SNP haplotype in exon 1 and intron 1 of *ANPEP*. These SNPs were in linkage disequilibrium so this SNP effectively genotyped 12 SNPs at once. Due to lower quality DNA samples extracted from ear tissue samples in population one and two, as opposed to DNA extracted from blood in the discovery population, a different forward and reverse primer set (forward 5'- ACC TTG GAC CAG AG CAA GC-3'; reverse 5'-CTG CTT CCA GGG AGC TC TT-3') had to be used to obtain a product. Amplification by PCR was performed as described above, with an annealing temperature of 61°C and BSA as the additive. The PCR product was then digested with the restriction endonuclease *BSU36I* (New England Biolabs, Ispwich, MA) overnight at 37°C, and visualized via gel electrophoresis on an ethidium bromide stained 3% agarose gel.

3.2.6 Haplotype confirmation

In order to determine if the *ANPEP* haplotypes identified in the initial SNP discovery population still held in the experimental population, 14 randomly selected animals from population one were selected for sequencing. The primer set (forward 5'- ACC TTG GAC CAG AG CAA GC-3'; reverse 5'-CTG CTT CCA GGG AGC TC TT-3') for a shorter amplicon had to be used to allow for amplification in the experimental population. Sequencing was performed as described above and analyzed using Sequencher 4.9 software (Gene codes corporation, Ann Arbor, MI).

3.2.7 Amino Acid Alignment

The haplotype region of *ANPEP* exon 1 in the population one animals was aligned to nucleotide sequence for *Bos taurus* (AC_000162), *Bos indicus* (NC_032670.1) and nine other species: *Bison bison* (NW_011494727), *Ovis aries* (NC_019475), *Capra hircus* (NC_030828), *Mus musculus* (NC_000073), *Odocoileus virginianus* (NW_018336177), *Oryctolagus cuniculus* (NW_003159364), *Canis lupus familiaris* (NC_006585), *Sus scrofa* (NC_010449), and *Homo sapiens* (NC_000015) using Sequencher 4.9 software. The amino acid sequence was then obtained for each species and aligned in MS Excel.

3.2.8 Statistical Analysis

In Population one, an initial search for an interaction between *ANPEP* (*AA, GA, GG*) and *ADH1C* (*TT, CT*; Madder *et al.*, 2018) used a 3x2 factorial design. Additionally, to uncover any nutrigenomic influence of VA, data were analyzed as a 3x2 factorial design for the three genotypes of *ANPEP* (*AA, GA, GG*), by two VA treatments (H, L). The proc mixed procedure of SAS (SAS version 9.4; SAS Institute, Inc, Cary, NC, USA) was used to complete an analysis of variance (ANOVA).

In Population two the three genotypes of *ANPEP* (*AA, GA, GG*) were analyzed relative to traits. The experimental unit was the individual. This was also performed using the proc mixed procedure of SAS (SAS version 9.4; SAS Institute, Inc, Cary, NC, USA) to complete an analysis of variance (ANOVA). Standard errors were adjusted using a Kenward-Roger adjustment and means were separated using Tukey's LSD. Significance was set at $P \leq 0.05$ and trends at $0.05 > P \leq 0.01$.

3.3 Results and Discussion

3.3.1 SNP Discovery and Selection

Seventy-six SNPs were found throughout the six candidate genes (Table 3.1) sequenced in the discovery population. Many of these SNPs were in non-coding areas such as introns (n=50), 3' (n=3) and 5' (n=1). Of the SNPs identified in the exons fourteen were silent (did not change the amino acid), five were conserved missense mutations (the amino acid substituted was from the same grouping according to the side chain), and two were non-conserved missense mutations (amino acid substituted was from a different group according to side chain properties). Allele frequencies were calculated (Table 3.1) and taken into consideration when selecting a gene SNP for genotyping. To consider a marker for genotyping the minor allele frequency (MAF) had to be greater than or equal to 30%.

Table 3.1. *Single nucleotide polymorphisms identified in the six candidate genes in the discovery population.*

Gene	Location	Annotation	Amino acid change	Allele Frequencies
ADFP	Exon 1	*ADFPc.17C>T*	Ala>Val[1]	C=0.17 T=0.83
	Intron 1	*ADFPc.30+21G>A*	-	
	Intron 1	*ADFPc.30+27T>C*	-	
	Exon 2	*ADFPc.53A>G*	Gln>Gln	A=0.76 G=0.24
	Intron 2	*ADFPc.226+54G>T*	-	
	Intron 3	*ADFPc.309+758A>G*	-	
	Exon 4	*ADFPc.462C>T*	Ser>Ser	C=0.92 T=0.08
	Intron 4	*ADFPc.595+23A>G*	-	
	Intron 4	*ADFPc.595+865A>G*	-	
	Intron 4	*ADFPc.595+931T>C*	-	
	Intron 4	*ADFPc.595+944A>T*	-	
	Intron 5	*ADFPc.777+64T>C*	-	
	Intron 5	*ADFPc.777+107A>G*	-	
	Intron 6	*ADFPc.912+33G>C*	-	
	Intron 6	*ADFPc.912+1180T>C*	-	
CLU	5'	*CLUc.-53C>G*	-	C=0.70 G=0.30
	Intron 1	*CLUc.79+1060C>G*	-	

	Intron 1	*CLUc.79+1144A>G*	-	
	Intron 1	*CLUc.79+1260C>T*	-	
	Exon 4	*CLUc.448C>T*	Asn>Asn	C=0.80 T=0.20
	Exon 4	*CLUc.634C>G*	Leu>Leu	C=0.95 G=0.05
	Intron 5	*CLUc.910+3318G>A*	-	
	Intron 5	*CLUc.910+3380G>A*	-	
	Intron 5	*CLUc.910+3384G>A*	-	
	Exon 6	*CLUc.930G>A*	Pro>Pro	G=0.75 A=0.25
	Intron 6	*CLUc.1140+9C>T*	-	
	Intron 6	*CLUc.1140+666A>G*	-	
	Intron 7	*CLUc.1310+7T>G*	-	
	Intron 7	*CLUc.1310+10T>C*	-	
	3'	*CLUc.*1320+28Gindel*	-	
SPARC	Intron 1	*SPARCc.57+716T>C*	-	
	Intron 2	*SPARCc.120+40T>C*	-	
	Intron 8	*SPARCc.886+355G>C*	-	
GPX3	Exon 1	*GPX3c.33C>T*	Leu>Leu	
	Intron 1	*GPX3c.89+110G>A*	-	
	Intron 3	*GPX3c.361+112T>C*	-	
	Intron 4	*GPX3c.465+94G>A*	-	
	Intron 4	*GPX3c.465+106A>C>T*	-	
	Intron 4	*GPX3c.465+251T>A*	-	
	Intron 4	*GPX3c.465+360A>G*	-	
	Exon 5	*GPX3c.573C>T*	Asp>Asp	
	3'	*GPX3c.*681+85G>A*	-	
	3'	*GPX3c.*681+149T>C*	-	
IGFBP6	Exon 1	*IGFBP6c.87C>T*	Gly>Gly	C=0.97 T=0.03
	Intron 3	*IGFBP6c.663+615A>G*	-	
	Intron 3	*IGFBP6c.663+722T>C*	-	
ANPEP	Exon 1	***ANPEPc.57T>C***	Gly>Gly	T=0.50 C=0.50
	Exon 1	***ANPEPc.201A>G***	Pro>Pro	A=0.50 G=0.50
	Exon 1	***ANPEPc.404T>C***	Val>Ala[1]	T=0.50 C=0.50
	Exon 1	***ANPEPc.410G>A***	Arg>Lys[1]	G=0.50 A=0.50
	Exon 1	***ANPEPc.483C>G***	Val>Val	C=0.50 G=0.50
	Exon 1	***ANPEPc.516G>A***	Thr>Thr	G=0.50 A=0.50
	Exon 1	***ANPEPc.519T>C***	Tyr>Tyr	T=0.50 C=0.50
	Exon 1	***ANPEPc.555C>T***	Asp>Asp	C=0.50 T=0.50
	Intron 1	***ANPEPc.606+18A>C***	-	
	Intron 1	***ANPEPc.606+53T>C***	-	
	Intron 1	***ANPEPc.606+123G>A***	-	
	Intron 1	***ANPEPc.606+478G>A***	-	
	Intron 6	*ANPEPc.1284+48T>C*	-	
	Intron 6	*ANPEPc.1284+51G>A*	-	

Intron 7	*ANPEPc.1428+13T>C*	-	
Intron 7	*ANPEPc.1428+125G>C*	-	
Intron 8	*ANPEPc.1494+79G>A*	-	
Exon 12	*ANPEPc.1946G>C*	Arg>Pro[2]	G=0.81 C=0.19
Exon 14	*ANPEPc.2065A>C*	Met>Leu[1]	A=0.88 C=0.12
Intron 14	*ANPEPc.2154G>A*	-	
Intron 14	*ANPEPc.2154+1533C>G*	-	
Intron 15	*ANPEPc.2246+39T>G*	-	
Intron 17	*ANPEPc.2525+105G>A*	-	
Intron 17	*ANPEPc.2525+1026T>G*	-	
Intron 17	*ANPEPc.2525+1042G>A*	-	
Exon 18	*ANPEPc.2610C>T*	Ile>Ile	C=0.86 T=0.14
Intron 18	*ANPEPc.2666+103A>G*	-	
Intron 18	*ANPEPc.2748+82G>A*	-	
Exon 20	*ANPEPc.2875A>C*	Asn>His[2]	A=0.96 C=0.04
Exon 20	*ANPEPc.2889C>G*	Asp>Glu[1]	C=0.94 G=0.06

[1] conserved missense mutation [2] non-conserved missense mutation. Bold indicates the SNPs involved in the ANPEP haplotype.

The SNPs were selected based on MAF and the likelihood to cause an effect in the resulting protein. Out of all of the SNPs found in the six sequenced candidate genes, a grouping of twelve SNPs spanning exon one and intron 1 of *ANPEP* had the highest MAF at 0.50 (Table 3.1). Initial sequence analysis of *ANPEP* in the discovery population (n=16) revealed that this is a 12-SNP haplotype. This haplotype was in linkage disequilibrium with three distinct genotypes, (one homozygote was the same as the reference sequence, an alternate homozygote, and heterozygous genotype). Eight of the SNPs are in exon one while four are in intron one. Two of the SNPs within the exon are conserved missense mutations (*ANPEPc.404T>C* (Val>Ala) and *ANPEPc.410G>A* (Arg>Lys)) while the other six are silent mutations (Table 3.1). The same MAF of 0.50 (Table 3.2) at all twelve variants combined with the presence of only three haplotypes confirmed linkage disequilibrium. The haplotype was also of interest due to its span across exon one, the largest of *ANPEP*, and into intron one (Figure 3.2). Although only two missense mutations were present (V135A; R137K), they were found within the beginning of the

polypeptide chain of the catayltic domain and could affect structure of the protein (Figure 3.2). The eight exonic SNPs occur in the membrane-spanning (1), supporting stalk (1), and polypeptide chain of the catalytic domain regions of the protein (6, Figure 3.2). The two missense mutations are in the polypeptide chain of the catalytic domain and could affect the protein structure. The SNPs found in *ADFP*, *CLU*, *SPARC*, *GPX3*, and *IGFBP6* either did not have a great enough minor allele frequency or a variant that was likely to have an effect on the resultant protein structure, and therefore none were selected for further analysis.

We genotyped the two populations of steers using a PCR-RFLP test for *ANPEPc.410G>A* SNP, using it as a tag for the haplotype. This SNP was chosen as it was one of the conserved missense mutations within the haplotype and a restriction endonuclease was identified that could distinguish the alleles. Due to the shorter fragment amplified in population one and two, the twelfth SNP of the haplotype (*ANPEPc.606+478G>A*) was not included in the amplicon. Genotyping of *ANPEPc.410G>A* in both populations one and two resulted in lower MAFs of 0.27 and 0.28 respectively as comparared to the discovery population (Table 3.2).

Table 3.2 *Allele frequencies and number of animals genotyped at ANPEPc.410G>A for each population*

	Discovery	Population 1	Population 2
A allele	0.50	0.73	0.72
G allele	0.50	0.27	0.28
Animals (n)	16	968	674

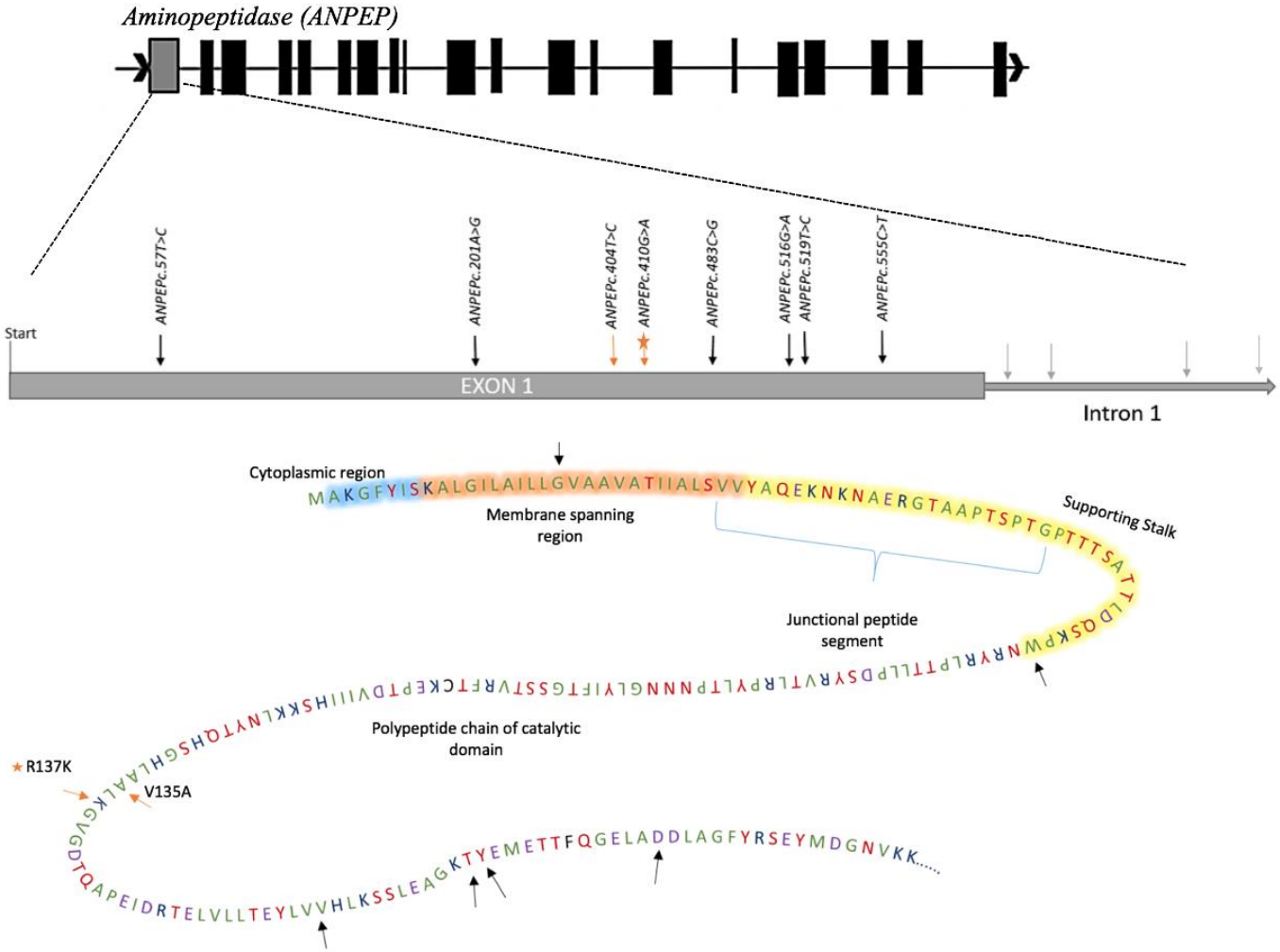

Figure 3.2 The twenty exons of *ANPEP* with a close-up view of exon 1 to show the positions of SNPs within the haplotype and the ANPEP peptide pertaining to exon one. The intronic SNPs are identified by the grey arrows. Arrows point to the SNP positions in the amino acid sequence. Orange arrows indicate the positions at which the SNP causes an amino acid change. The orange star indicates the SNP that was used for genotyping by PCR-RFLP.

3.3.2 Association studies with production and carcass traits

3.3.2.1 Population 1 (ANPEP, ADH1C, and vitamin A)

The *ANPEP* haplotype was first genotyped in Population one (n=1000). There was no effect of *ADH1Cc.-64T>C* nor was there an interaction between *ANPEPc.410G>A* and *ADH1Cc.-64T>C* with carcass traits (data not shown). Madder *et al.* (2018) did not observe an effect of *ADH1Cc.-64T>C* or an interaction between *ADH1C* genotypes and VA with carcass traits. Subsequently, *ADH1C* was left out of any further analysis using this population. This population was also used to determine the nutrigenomic relationship between *ANPEP* genotypes and vitamin A level in the diet for an effect on carcass traits of the feedlot steers. Vitamin A had an effect on backfat thickness (FAT) and an interaction of *ANPEP* and VA was found for ribeye area (REA; Table 3.3). A significant effect of *ANPEP* was found for all carcass traits except hot carcass weight (HCW; Table 3.3).

Table 3.3 *Statistical results of ANPEPc.410G>A genotype, VA and the interaction on camera graded carcass traits in Population 1.*

Variable	HCW	YLD	MARB	REA (sq inch)	FAT (inch)
ANPEP	0.253	**<0.001**	**0.001**	**0.027**	**<0.001**
VA	0.618	0.627	0.203	**0.008**	**0.023**
*ANPEP**VA	0.063	0.388	0.743	**0.016**	0.247

Bold values indicate significance (P<0.05). HCW = hot carcass weight, YLD = vision grade yield grade, MARB = vision grade marbling score, REA= ribeye area (inch^2), FAT= back fat thickness (inch), VA= vitamin A treatment, *ANPEP= ANPEPc.410G>A* genotype

Hot carcass weight (HCW) is important to feedlot producers since most abattoirs pay producers based on this value. No association for HCW with ANPEP was found (P = 0.253) however, feedlot producers do have the opportunity to receive premiums for carcass quality. The vision grade yield (YLD) is a scale from 1.0 to 5.9, with a lower score being more desirable. This

calculation considers back fat, internal fat (kidney, pelvic and heart fat), REA, and HCW. The steers with the *GG* genotype had the lowest YLD (2.15 ± 0.075), the *GA* were intermediate (2.36 ± 0.034) and *AA* had the highest (2.48 ± 0.029; Figure 3.3A). Since all of the genotypes are significantly different from each other this would suggest an additive mode of action. A low YLD would make *GG* animals the preferred genotype when it comes to YLD.

In Canada, beef that grades 'AAA' and "prime" are the most desirable to consumers and therefore cost the most at the grocery store (Killinger *et al*., 2004). Canadian "prime" beef is described as having slightly abundant marbling (MARB score between 700-799 by camera grading systems at the processing plant). Canadian 'AAA' is the next best grade of beef and is described as having small marbling and scores between 400-699. The two lower grades are Canadian 'A' and Canadian 'AA' score 200-299 and 300-399, respectively (Canadian beef grading agency). The effect of *ANPEP* on MARB revealed the *AA* genotype had higher (397.60 ± 2.68) MARB than the *GG* genotype (370.76 ± 7.02), while *GA* animals were intermediate (388.40 ± 3.22; Figure 3.3). Although *ANPEPc.410A>G* SNP statistically influences MARB these results may not be of economic importance. The MARB score only changes the quality grade (Canada A to "prime") every100 points with the exception being within "AAA" where there is a 300-point difference. On average there would be no change in quality grade between *ANPEPc.410A>G* genotypes except for potentially some animals with a *AA* genotype that might grade Canadian 'AAA' as opposed to Canadian 'AA'.

Since animals develop back fat before intramuscular fat (Roh *et al*., 2006), a certain amount is necessary, but it is only optimal within a range of 0.20-0.50 in (Aalhus *et al*., 2014). The FAT in population one was within this range for all genotypes of *ANPEP*, but the *AA* genotype was higher (8.52 ± 0.17 in) than *GG* genotype (7.12 ± 0.44 in) and the heterozygous

animals (7.58 ± 0.20 in; Figure 3.3C). This further supports the *A* allele is associated with higher fat production.

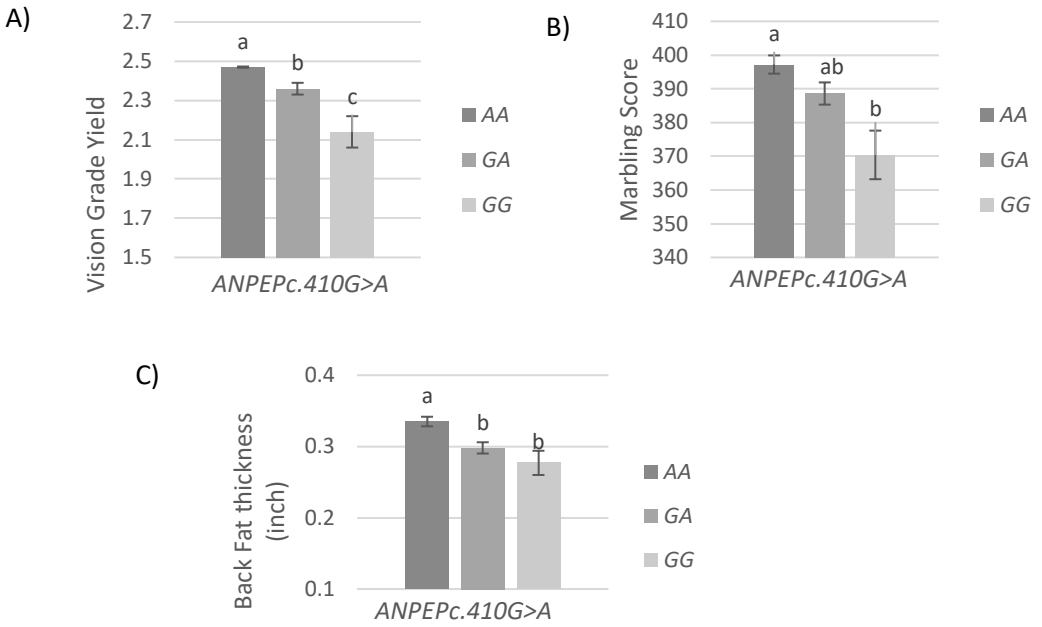

Figure 3.3 The *ANPEPc.410G>A* genotype effect on carcass traits in population one steers (n=1000), A) Vision grade yield (YLD), B) Marbling score (MARB), C) Backfat thickness (FAT; inch). Bars with differing superscripts are significantly different (P<0.05). Error bars indicate the SEM.

The interaction of *ANPEP* and VA for REA (Table 3.3; Figure 3.4) is likely a type I error (false positive) as only the *GG* animals on the high vitamin A diet (n=39 out of 1000) showed a statistical difference. The main effect of VA on FAT resulted in higher FAT in the animals on the high VA diet (0.32 ± 0.0095 inches) as compared to animals on the low VA diet (0.29 ± 0.0097 inches). Since limited VA is typically associated with higher fat content (Ward *et al.*, 2012; Krone *et al.*, 2015), not high VA, this result was unexpected.

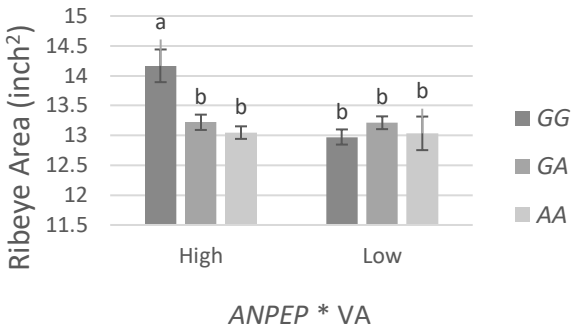

Figure 3.4 Interaction of *ANPEPc.410G>A* genotypes (*AA, GA, GG*) and VA (High = 100% NRC recommended VA and Low = 50% NRC recommended VA). Bars with differing superscripts are significantly different (P<0.05). Error bars indicate the SEM.

3.3.2.2 Population 2

The effect of *ANPEPc.410G>A* genotype on carcass traits was validated in population two. Again there was a genotype effect (P<0.01) for all of the investigated carcass traits, YLD, MARB, REA, FAT, with the exception of HCW. The analysis of these traits in population two were similar to that observed in population one. In regards to MARB, results were similar to those found in population one. The AA animals (448.72 ± 4.1) and heterozygote (435.83 ± 4.66) animals had higher marbling than GG animals (409.41 ± 10.1). Although statistical significance was found there may not be any change to the quality grade among genotypes. In population one, the steers were all "AA" while in population two they are all "AAA" carcasses. Consequently, a few AA in population one may have graded AAA while in population two some of the *GG* animals may have graded 'AA' (Figure 3.5B). In terms of YLD, the *AA* genotype again were the poorer animals, having the higher YLD grade (2.98 ± 0.045) than both GA (2.822 ± 0.051) and GG genotype animals (2.62 ± 0.111; Figure 3.5A). For the traits of REA and FAT, the *GG* genotype is more desirable. The *GG* genotype has the highest ribeye area (14.90 ± 0.24 in^2) and

the lowest amount of back fat (0.39 ± 0.02 in) compared to AA genotype animals (14.22 ± 0.91 in^2 and 0.46 ± 0.01 in, respectively) while *GA* genotype is the intermediate for these two carcass traits (14.55 ± 0.11 in^2 and 0.43 ± 0.01 in, respecitively; Figure 3.5C; D).

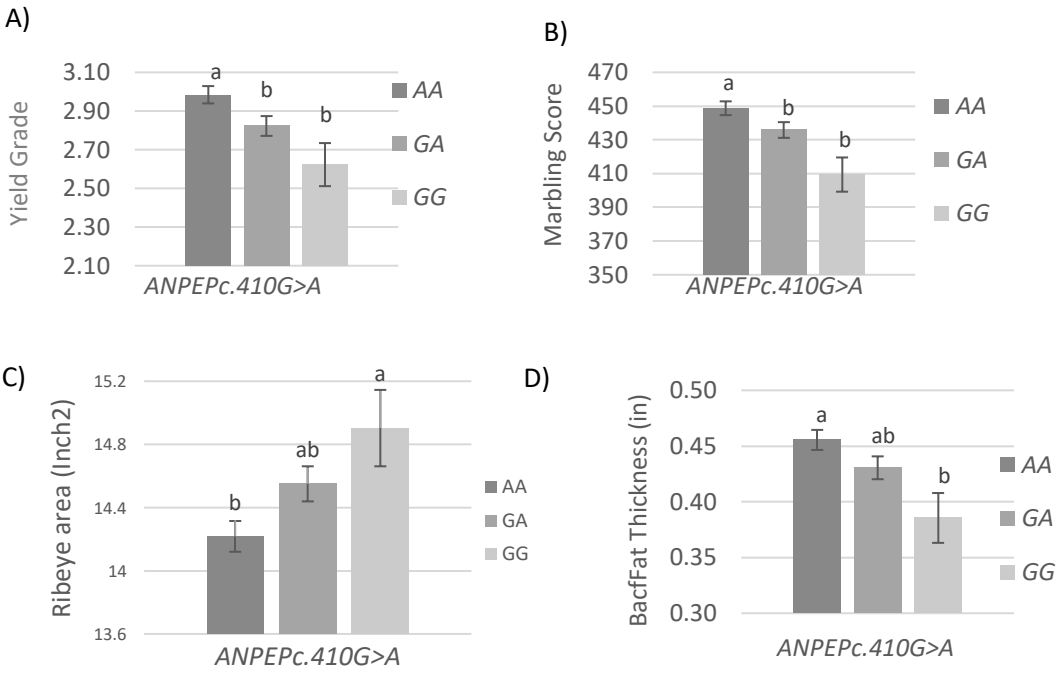

Figure 3.5 The *ANPEPc.410G>A* genotype effect on carcass traits in population two steers (n=700) for the A) yield, B) marbling score, C) ribeye area, D) backfat. Bars with differing superscripts are significantly different (P<0.05). Error bars indicate the SEM.

3.3.3 Haplotype sequencing confirmation

In order to confirm that the haplotypes found in the discovery population held the same linkage disequilibrium, fourteen animals from population one were sequenced. Due to a lower quality source DNA, primers were redesigned to amplify a smaller fragment. The shorter fragment only contained eleven of the twelve SNPs in the haplotype, with *ANPEPc.606+478G>A* being outside of the amplicon. There was still strong evidence that the haplotypes held. As shown in

Table 3.4 animals were either homozygous for the reference alleles, homozygous for the alternate alleles, or heterozygous, giving only three options for the haplotype genotype. This confirmed that by selecting one tag SNP for genotyping, in this case, *ANPEPc.410G>A*, we could then accurately predict the genotype at the other positions of the haplotype.

Animal	*ANPEPc.57T>C*	*ANPEPc.201A>G*	*ANPEPc.404T>C*	*ANPEPc.410G>A**	*ANPEPc.483C>G*	*ANPEPc.516G>A*	*ANPEPc.519T>C*	*ANPEPc.555C>T*	*ANPEPc.606+18A>C*	*ANPEPc.606+53T>C*	*ANPEPc.606+123G>A*
1	CC	GG	CC	AA	GG	AA	CC	TT	CC	CC	AA
2	CC	GG	CC	AA	GG	AA	CC	TT	CC	CC	AA
3	CC	GG	CC	AA	GG	AA	CC	TT	CC	CC	AA
4	CC	GG	CC	AA	GG	AA	CC	TT	CC	CC	AA
5	CC	GG	CC	AA	GG	AA	CC	TT	CC	CC	AA
6	CC	GG	CC	AA	GG	AA	CC	TT	CC	CC	AA
7	TC	AG	TC	GA	CG	GA	TC	CT	AC	TC	GA
8	TC	AG	TC	GA	CG	GA	TC	CT	AC	TC	GA
9	TC	AG	TC	GA	CG	GA	TC	CT	AC	TC	GA
10	TC	AG	TC	GA	CG	GA	TC	CT	AC	TC	GA
11	TT	AA	TT	GG	CC	GG	TT	CC	AA	TT	GG
12	TT	AA	TT	GG	CC	GG	TT	CC	AA	TT	GG
13	TT	AA	TT	GG	CC	GG	TT	CC	AA	TT	GG
14	TT	AA	TT	GG	CC	GG	TT	CC	AA	TT	GG

Table 3.4 Confirmation of *ANPEP* haplotype. The haplotype was confirmed by sequencing 14 randomly selected animals in population 1. Pink = homozygous (reference alleles), Green = heterozygous, Orange = homozygous (alternate alleles)

3.3.4 Amino acid alignment

The *ANPEP* gene is highly conserved across species (Olsen *et al.*, 1988; Jongeneel *et al.*, 1989; Luan and Xu, 2007). When comparing sequence of *ANPEP* it is clear that exon one is the largest throughout all of the species and alignment is very similar. The positions of exons start to differ further downstream in the gene due to variation in the length of introns between species. In order to further analyze the haplotype found within exon one of *ANPEP* in *Bos taurus* cattle, the amino acid sequence of exon one was aligned to other closely related or common used species (Appendix B2). The species include: *Bos indicus* (zebu cattle), *Bison bison* (American bison), *Odocoileus virginianus* (white-tailed deer), *Ovis aries* (sheep), *Capra hircus* (goat), *Sus scrofa* (pig), *Homo sapiens* (human), *Mus musculus* (house mouse), *Oryctolagus cuniculus* (European rabbit), and *Canis lupus familiaris* (dog). The amino acid alignment shows the similarity between all of the species but mostly with those that are more closely related. There is a possibility that the haplotype found in exon one of *Bos taurus* will be present in the other species as well, specifically more closely related the ruminant species. In fact, the reference sequence for *Bos indicus* currently indicates the presence of the same SNP at *ANPEPc.410G>A*. Perhaps the other SNPs of the haplotype are also present in Bos indicus. Further investigation to identify the haplotype in Bos indicus is required.

4.0 GENERAL DISCUSSION

At this time it cannot be determined for certain how the *ANPEP* haplotype could be affecting carcass traits. Since any true nutrigenetic interaction with VA and the haplotype genotypes is unlikely, there must be a difference in the structural function of the ANPEP protein or the mRNA transcript that would allow higher fat content in animals with the *AA* genotype at the SNP (*ANPEPc.410G>A*), and therefore the alternate alleles at the other positions of the haplotype. There are many phenomena that occur at the transcription and translation levels that can affect expression. Polymorphisms of the haplotype could cause exon skipping during splicing of RNA transcription splicing out the exon, such as in the *cone opsin* gene of humans (Ueyama *et al*., 2012). Altered mRNA structure and codon bias could also affect the translation of *ANPEP* (Shabalina *et al*., 2013; Zhou *et al*., 2009).

As described in the literature, ANPEP plays an important role as a membrane protein in the intestinal wall (Maroux *et al*., 1973). Altered nutrient absorption in the gut could potentially influence the carcass traits investigated. A role in nutrient absorption could also explain the differences seen in the amino acid sequence of monogastric versus ruminant species (Appendix B2). The activity of ANPEP as an enzyme cleaving amino acids (Chen *et al*., 2012) could also potentially differentially affect metabolism. Additionally, ANPEP has been shown to have associations with angiogenesis which is vital for growth as it brings blood flow to areas of growth (Fukasawa *et al.*, 2006). Since feedlot steers are youthful animals and undergo a large amount of growth at the feedlot, an altered ANPEP and its role in angiogenesis affecting adipogenesis (Sato, 2003; Cao, 2007; Guanghong *et al.*, 2015) could contribute to differences seen among genotypes for carcass traits.

5.0 CONCLUSION

The purpose of this study was to find a gene variant that could potentially be used in a marker-assisted management program in a commercial feedlot. Specifically, gene variants that have a nutrigenomic influence on carcass traits based on vitamin A inclusion level or are associated with traits. Sequencing all of the candidate genes yielded *ANPEP* as the gene with the most promising variant, or in this case, twelve variants found within a haplotype, that would most likely have an effect on carcass traits. There were no interactions with *ADH1C* (previously shown promise in experimental nutrigenomic based precision feeding trials) or more importantly with the level of VA inclusion useful for a management program. The *ANPEP* genotypes did effect carcass traits with the exception of hot carcass weight, and may have the potential to impact the the marketing of cattle based on lean yield for the heterozygote animals. An adjustment to the grid marketing system using an animal that can have high yields as well as good marbling triats may benefit feedlot producers. The discovery of the haplotype in strong linkage disequilibrium in exon one of *ANPEP* is extremely interesting and may be important from an evolutionary perspective. Further investigation into the functional role of this region of the ANPEP protein could provide insights into why it is inherited in this manner, as well as studying how the haplotype may affect expression by altered transcription and translation.

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APPENDICIES

APPENDIX A

Table A: Primers used for amplification of genes sequenced in the discovery population

Target	Forward Primer	Reverse Primer	Annealing (◦C)	Size (bp)
ANPEP				
5' UTR & Exon 1	GAGCTCCCTCCCCATTCTC	CCAAGGTGGCTTTCATTGTT	60	846
Exon 2 & 3	GTAAGGGGACAGGGATGAGG	GACTCAGAGCTGGGGTTCAC	60	666
Exon 4 & 5	CCCCTCCCTATGACTCCAAG	ATCCTGACTCCTCCCCAAAC	60	675
Exon 6 & 7	GGGGAGGAGTCAGGATGAAG	CAGGAAATTCGAGAGCATCC	60	698
Exon 8 & 9	TCCCATGCTGGTCCTTAGAC	GAGGAGCAGCCAGTAAGCAG	60	568
Exon 10	CCAGTGCTCCTCCACTACCT	TGAGATGGGAACAATCCACA	59	590
Exon 11	AGAGTGGTGGGTGTCAGTCC	TCAATGGGGGTAGATGTTGTC	60	498
Exon 12 & 13	GGCCACTGAGGTTCACAGAT	TGGAGTTCCAGAGTTGGACA	60	845
Exon 14	TGAGCAAGGGAATGTGACTG	CCCAACTTGCTCACCTTCTC	59	775
Exon 15	AGCACACGGGATCTTCGTT	GTTCACAGGCCACATGGAG	60	715
Exon 16 & 17	AAAGCACCCAAAGGGTAGGT	TCTGAGCCTCTGTGTCCTCA	59	809
Exon 18	CAGGAAAGGACGAAGGATGA	GAGTCTGAACCCTGGACCAC	60	497
Exon 19	CTGCTCATGGCTCCTCTCTC	TGGATACCCAGCTTCCTCTG	60	277
Exon 20 & 3'UTR	ACACATAGGAGCCTGGTTGG	AGTGAACATGGGCCAGAGAC	60	473
CLU				
5' UTR & Exon 1	TCTTTTTAAAGTGCGCATTGG	GGAACAGCTGAAGGAGATGC	60	500
Exon 2	GCTCCAGTGTTTGCTTTTCTG	GAAATCTCACCCCTTGAAGC	59	600
Exon 3	GCAGGATGAAGATGGGAGGA	CCTGAACTTGTGGGGTCTGA	59	794

Exon 4	CCTGACGGGTCTTGTGACTT	TAAGGCTCCCTGCCTCTACA	60	758
Exon 5	AGGCGAAGGTGGTGAGAG	AGCCAAGCAAAGGCCAGT	60	500
Exon 6	CACCATCTTCCAGAGCAAGAC	GTAGGACAAGTGCTCCATCG	59	600
Exon 7 & 8	CTTTGTGGAAGGCGTTTACC	AGAGGAAGGAGAGGCTGGAG	60	765
GPX3				
5' UTR & Exon 1	AGCAGATGACACCTCAGACG	CAGGTCTCCAGCAGATGAAA	59	358
Exon 2	AGCACTCACCAGCTTGTTCA	AGGGGATCAGACCTCAGGTTA	59	555
Exon 3	TCCAGGTTGGACTCCATAGG	TCCTGGTACCTCTTCCACTTG	59	496
Exon 4	GCAGCGGTTATTGAGCAGTT	GCCCCTAGGGTCTACAAAGC	60	495
Exon 5	GGGACAAGCTTAGGGTCTCA	GCCCATGTGCAGAGCAGTA	60	587
IGFBP6				
5' UTR & Exon 1	TACGACTGCTCTGGGAGGAC	TGCCAGAGATAGGGAGAAGG	60	486
Exon 2 & 3	ACTCTAAGCCCCATCACTGC	AATCTAACCCCTCCCACAGC	59	953
Exon 4 & 3' UTR	CTCTGGTGGGTGGAGAAGAG	AGCCGACACCAACAATCTTT	59	462
SPARC				
SPARC_E1	AAAGACTCGTTAGCACCAAACT	CTGGAATCTCCTCCCAATGA	59	484
SPARC_E2	CTATACAAAGCGCGGTGCTC	GAGGTTGCTGGGAGAGTCTG	60	470
SPARC_E3	CATAGAGTTCTGGGCCCTTG	GGCAGTTTGTCCTTCACAGC	60	466
SPARC_E4	CTGGCCCTGACCTCAGAC	CTCTCTGGCTGGGTCTCTTG	60	491
SPARC_E5	GCTGCTCAGAGACCAGCAG	GATCCCTGACGCTCAGACAG	60	480
SPARC_E6	GGGGATGTGCCTTTGTACTT	GGATGTGGGCTTACATTTGG	60	500
SPARC_E7	TTTTTCCTTGCCACTCTGCT	CTCTCTTGTCGCAGCCCTTA	60	549
SPARC_E8	AGGGCCCTTTAGACATTATGA	CAGGGAGAGCAGACAGGAGT	58	499
SPARC_E9	CACTGCTTTGGGAACTGACA	GGCAGAACAACAAACCATCC	60	370
ADFP				
ADFP_E1_2	TGGAGAATTAATCTGGGAATCTTG	AGCTCAGGGCGTAAGGCTA		500

ADFP_E3	TGGCAAGGATCTCTTTTCTGA	CAAATAAGTACTGCCTGTTTGTGG	348
ADFP_E4	AAGGGCCAGATTCATCCTTT	AAGGGCCAGATTCATCCTTT	500
ADFP_E5	GCAGATAGGAGCCATCTTGG	TTGGCCCATTTTTCTACTGG	475
ADFP_E6	TGTAAGTATTGATGATGGCTGGTT	AACAAGCAACGTGTGTATCCTG	375
ADFP_E7	TCCACATGTGTGGTGGACAT	TTTTTCCAGCCAGGTAAGTAACA	686

APPENDIX B

Table B. Amino acid sequence alignment of exon 1 in ANPEP for Bos taurus (reference and population 1 alternate allele sequence) compared to other species. Orange box = tag SNP, Yellow box = locations of SNPs in cattle, Purple box = differences from cattle, red box = missing/spaces, Green text = non-polar, Red text = polar, Blue text= basic, Purple text = acidic.

	1				5					10					15					20					25				
Bos taurus (ref)	M	A	K	G	F	Y	I	S	K	A	L	G	I	L	A	I	L	L	G	V	A	A	V	A	T	I	I	A	L
Bos taurus (CFL1)	M	A	K	G	F	Y	I	S	K	A	L	G	I	L	A	I	L	L	G	V	A	A	V	A	T	I	I	A	L
Bos indicus	M	A	K	G	F	Y	I	S	K	A	L	G	I	L	A	I	L	L	G	V	A	A	V	A	T	I	I	A	L
Bison bison	M	A	K	G	F	Y	I	S	K	A	L	G	I	L	A	I	L	L	G	V	A	A	V	A	T	I	I	A	L
Ovis aries	M	A	K	G	F	Y	I	S	K	A	L	G	I	L	A	I	L	L	G	V	A	A	V	A	T	I	I	A	L
Capra hircus	M	A	K	G	F	Y	I	S	K	A	L	G	I	L	A	I	L	L	G	V	A	A	V	A	T	I	I	A	L
Odocoileus virginianus	M	A	K	G	F	F	I	S	K	A	L	G	I	L	A	I	L	L	G	V	A	A	V	A	T	I	I	A	L
Sus scrofa	M	A	K	G	F	Y	I	S	K	A	L	G	I	L	G	I	L	L	G	V	A	A	V	A	T	I	I	A	L
Homo sapiens	M	A	K	G	F	Y	I	S	K	S	L	G	I	L	G	I	L	L	G	V	A	A	V	C	T	I	I	A	L
Mus musculus	M	A	K	G	F	Y	I	S	K	T	L	G	I	L	G	I	L	L	G	V	A	A	V	C	T	I	I	A	L
Oryctolagus cuniculus	M	A	K	G	F	Y	I	S	K	S	L	G	I	L	G	I	L	L	G	V	A	A	L	C	T	I	V	A	L
Canis lupus familiaris	M	A	K	G	F	Y	I	S	K	A	L	G	I	L	A	I	V	L	G	I	A	A	V	S	T	I	I	A	L

	30					35					40					45					50					55			
Bos taurus (ref)	S	V	V	Y	A	Q	E	K	N	K	N	A	E	R	G	T	A		A	P	T	S	P	T					
Bos taurus (CFL1)	S	V	V	Y	A	Q	E	K	N	K	N	A	E	R	G	T	A		A	P	T	S	P	T					
Bos indicus	S	V	V	Y	A	Q	E	K	N	K	N	A	E	R	G	T	A		A	P	T	S	P	T					
Bison bison	S	V	V	Y	A	Q	E	K	N	K	N	A	E	R	G	T	A		A	P	T	S	P	T					
Ovis aries	S	V	V	Y	A	Q	E	K	N	K	S	G	P	A	S	P	P		G	P	M	S	P	T					
Capra hircus	S	V	V	Y	A	Q	E	K	N	K	N	A	E	C	G	T	S			P	T	S	P	T	S	A	T	S	P
Odocoileus virginianus	S	V	V	Y	A	Q	E	K	N	K	N	A	E	C	G	T	A		A	P	T	S	P	T					
Sus scrofa	S	V	V	Y	A	Q	E	K	N	K	N	A	E	H	V	P	Q		A	P	T	S	P	T					
Homo sapiens	S	V	V	Y	S	Q	E	K	N	K	N	A	N	S	S	P	V		A	S	T	T	P	S	A			S	A
Mus musculus	S	V	V	Y	A	Q	E	K	N	R	N	A	E	N	S	A	T		A	P	T	L	P	G	S		T	S	A
Oryctolagus cuniculus	S	V	V	Y	A	Q	E	K	N	K	N	T	S	Q	S	P	S	M	A	P	S	N	P	T		A	T	S	
Canis lupus familiaris	S	V	V	Y	A	Q	E	K	N	K	N	A	E	S	S	P	V		S	S	P	V	S	S	P	V	S	S	P

		60					65					70					75					80					85			
Bos taurus (ref)		G	P						T	T	T	S	A	T	T	L	D	Q	S	K	P	W	N	R	Y	R	L	P	T
Bos taurus (CFL1)		G	P						T	T	T	S	A	T	T	L	D	Q	S	K	P	W	N	R	Y	R	L	P	T
Bos indicus		G	P						T	T	T	S	A	T	T	L	D	Q	S	K	P	W	N	R	Y	R	L	P	T
Bison bison		G	P						T	T	T	S	A	T	T	L	D	Q	S	K	P	W	N	R	Y	R	L	P	T
Ovis aries		G	P	T			S	P	T	T	T	S	A	T	T	V	D	Q	S	K	P	W	N	R	Y	R	L	P	T
Capra hircus	T	G	P	T			S	P	T	T	T	S	A	T	T	V	D	Q	S	K	P	W	N	R	Y	R	L	P	T
Odocoileus virginianus		G	P						T	T	T	S	A	T	T	V	D	Q	S	K	P	W	N	R	Y	R	L	P	T
Sus scrofa		I							T	T	T	A	A	I	T	L	D	Q	S	K	P	W	N	R	Y	R	L	P	T
Homo sapiens	T			T	N	P	A					S	A	T	T	L	D	Q	S	K	A	W	N	R	Y	R	L	P	N
Mus musculus	T			T	A				T	T	T	P	A			V	D	E	S	K	P	W	N	Q	Y	R	L	P	K
Oryctolagus cuniculus							S	P					A	T	T	L	D	Q	N	L	P	W	N	R	Y	R	L	P	K
Canis lupus familiaris	V	S	P	T	N	P	S	P	T	T		A	A	T	T	L	A	Q	S	K	P	W	N	H	Y	R	L	P	K

			90					95					100					105					110					115	
Bos taurus (ref)	T	L	L	P	D	S	Y	R	V	T	L	R	P	Y	L	T	P	N	N	N	G	L	Y	I	F	T	G	S	S
Bos taurus (CFL1)	T	L	L	P	D	S	Y	R	V	T	L	R	P	Y	L	T	P	N	N	N	G	L	Y	I	F	T	G	S	S
Bos indicus	T	L	L	P	D	S	Y	R	V	T	L	R	P	Y	L	T	P	N	N	N	G	L	Y	I	F	T	G	S	S
Bison bison	T	L	L	P	D	S	Y	R	V	T	L	R	P	Y	L	T	P	N	N	N	G	L	Y	I	F	T	G	S	S
Ovis aries	T	L	L	P	D	S	Y	R	V	T	L	R	P	Y	L	T	L	D	K	N	G	L	Y	I	F	T	G	S	S
Capra hircus	T	L	L	P	D	S	Y	R	V	T	L	R	P	Y	L	T	L	D	K	N	G	L	Y	I	F	T	G	S	S
Odocoileus virginianus	T	L	L	P	D	S	Y	R	V	T	L	R	P	Y	L	T	P	N	E	N	G	L	Y	I	F	T	G	S	S
Sus scrofa	T	L	L	P	D	S	Y	N	V	T	L	R	P	Y	L	T	P	N	A	D	G	L	Y	I	F	K	G	K	S
Homo sapiens	T	L	K	P	D	S	Y	R	V	T	L	R	P	Y	L	T	P	N	D	R	G	L	Y	V	F	K	G	S	S
Mus musculus	T	L	I	P	D	S	Y	R	V	I	L	R	P	Y	L	T	P	N	N	Q	G	L	Y	I	F	Q	G	N	S
Oryctolagus cuniculus	T	L	I	P	D	S	Y	N	V	V	L	R	P	Y	L	S	P	N	S	Q	G	L	Y	I	F	T	G	S	S
Canis lupus familiaris	T	L	I	P	S	S	Y	N	V	T	L	R	P	Y	L	T	P	N	S	N	G	L	Y	T	F	K	G	S	S

				120					125					130					135					140					145
Bos taurus (ref)	T	V	R	F	T	C	K	E	P	T	D	V	I	I	I	H	S	K	K	L	N	Y	T	Q	H	S	G	H	L
Bos taurus (CFL1)	T	V	R	F	T	C	K	E	P	T	D	V	I	I	I	H	S	K	K	L	N	Y	T	Q	H	S	G	H	L
Bos indicus	T	V	R	F	T	C	K	E	P	T	D	V	I	I	I	H	S	K	K	L	N	Y	T	Q	H	S	G	H	L
Bison bison	T	V	R	F	T	C	K	E	P	T	D	V	I	I	I	H	S	K	K	L	N	Y	T	Q	H	S	G	H	L
Ovis aries	T	V	R	F	A	C	K	E	S	T	D	V	I	I	I	H	S	K	K	L	N	Y	T	T	T	N	G	H	L
Capra hircus	T	V	R	F	A	C	K	E	S	T	D	V	I	I	I	H	S	K	K	L	N	Y	T	T	T	N	G	H	L
Odocoileus virginianus	A	V	R	F	T	C	K	E	S	T	D	V	I	I	I	H	S	K	K	L	N	Y	T	S	R	D	G	H	L
Sus scrofa	I	V	R	F	I	C	Q	E	P	T	D	V	I	I	I	H	S	K	K	L	N	Y	T	T	[redacted]	Q	G	H	M
Homo sapiens	T	V	R	F	T	C	K	E	A	T	D	V	I	I	I	H	S	K	K	L	N	Y	T	L	S	Q	G	H	R
Mus musculus	T	V	R	F	T	C	N	Q	T	T	D	V	I	I	I	H	S	K	K	L	N	Y	T	L	K	G	N	H	R
Oryctolagus cuniculus	T	V	R	F	T	C	Q	E	A	T	N	V	I	I	I	H	S	K	K	L	N	Y	T	I	T	Q	G	H	P
Canis lupus familiaris	T	V	R	F	T	C	K	E	S	T	S	M	I	I	I	H	S	K	K	L	N	Y	T	N	I	Q	G	Q	R

					150					155					160					165					170				
Bos taurus (ref)	A	A	L	K	G	V	G	D	T	Q	A	P	E	I	D	R	T	E	L	V	L	L	T	E	Y	L	V	V	H
Bos taurus (CFL1)	A	V	L	R	G	V	G	D	T	Q	A	P	E	I	D	R	T	E	L	V	L	L	T	E	Y	L	V	V	H
Bos indicus	A	V	L	K/R	G	V	G	D	T	Q	A	P	E	I	D	R	T	E	L	V	L	L	T	E	Y	L	V	V	H
Bison bison	A	A	L	T	G	V	G	D	T	Q	A	P	E	I	D	R	T	E	L	V	L	L	T	E	Y	L	V	V	H
Ovis aries	V	V	L	R	G	V	G	G	A	Q	A	P	E	I	D	R	T	E	L	V	L	L	T	E	Y	L	V	V	H
Capra hircus	V	V	L	R	G	V	G	G	A	Q	A	P	E	I	D	R	T	E	L	V	L	L	T	E	Y	L	V	V	H
Odocoileus virginianus	V	A	L	T	G	V	G	D	A	Q	A	P	I	I	D	R	T	E	L	V	L	I	T	E	Y	L	V	V	H
Sus scrofa	V	V	L	R	G	V	G	D	S	Q	V	P	E	I	D	R	T	E	L	V	E	L	T	E	Y	L	V	V	H
Homo sapiens	V	V	L	R	G	V	G	G	S	Q	P	P	D	I	D	K	T	E	L	V	E	P	T	E	Y	L	V	V	H
Mus musculus	V	V	L	R	T	L	D	G	T	P	A	P	N	I	D	K	T	E	L	V	E	R	T	E	Y	L	V	V	H
Oryctolagus cuniculus	V	V	L	R	G	V	G	G	S	Q	P	P	A	I	A	S	T	E	L	V	E	L	T	E	Y	L	V	V	H
Canis lupus familiaris	V	A	L	R	G	V	G	G	S	Q	A	P	A	I	D	R	T	E	L	V	E	V	T	E	Y	L	V	V	H

	175					180					185					190				195					200				
Bos taurus (ref)	L	K	S	S	L	E	A	G	K	T	Y	E	M	E	T	T	F	Q	G	E	L	A	D	D	L	A	G	F	Y
Bos taurus (CFL1)	L	K	S	S	L	E	A	G	K	T	Y	E	M	E	T	T	F	Q	G	E	L	A	D	D	L	A	G	F	Y
Bos indicus	L	K	S	S	L	E	A	G	K	T	Y	E	M	E	T	T	F	Q	G	E	L	A	D	D	L	A	G	F	Y
Bison bison	L	K	S	S	L	E	A	G	K	T	Y	E	M	E	T	T	F	Q	G	E	L	A	D	D	L	A	G	F	Y
Ovis aries	L	K	S	P	L	E	A	G	K	M	Y	E	M	E	T	T	F	Q	G	E	L	A	D	D	L	A	G	F	Y
Capra hircus	L	K	S	P	L	E	A	G	K	M	Y	E	M	E	T	T	F	Q	G	E	L	A	D	D	L	A	G	F	Y
Odocoileus virginianus	L	K	S	S	L	E	V	G	K	M	Y	E	M	E	T	A	F	Q	G	E	L	A	D	D	L	A	G	F	Y
Sus scrofa	L	K	G	S	L	Q	P	G	H	M	Y	E	M	E	S	E	F	Q	G	E	L	A	D	D	L	A	G	F	Y
Homo sapiens	L	K	G	S	L	V	K	D	S	Q	Y	E	M	D	S	E	F	E	G	E	L	A	D	D	L	A	G	F	Y
Mus musculus	L	Q	G	S	L	V	E	G	R	Q	Y	E	M	D	S	Q	F	Q	G	E	L	A	D	D	L	A	G	F	Y
Oryctolagus cuniculus	L	Q	G	Q	L	V	A	G	S	Q	Y	E	M	D	T	Q	F	Q	G	E	L	A	D	D	L	A	G	F	Y
Canis lupus familiaris	L	R	E	P	Q	V	N	S	Q		Y	E	M	D	S	K	F	E	G	E	L	A	D	D	L	A	G	F	Y

	205					210					215
Bos taurus (ref)	R	S	E	Y	M	D	G	N	V	K	K
Bos taurus (CFL1)	R	S	E	Y	M	D	G	N	V	K	K
Bos indicus	R	S	E	Y	M	D	G	N	V	K	K
Bison bison	R	S	E	Y	M	D	G	N	V	K	K
Ovis aries	R	S	E	Y	M	D	G	N	V	K	K
Capra hircus	R	S	E	Y	M	D	G	N	V	K	K
Odocoileus virginianus	R	S	E	Y	M	D	G	N	V	K	K
Sus scrofa	R	S	E	Y	M	E	G	N	V	K	K
Homo sapiens	R	S	E	Y	M	E	G	N	V	R	K
Mus musculus	R	S	E	Y	M	E	G	D	V	K	K
Oryctolagus cuniculus	R	S	E	Y	M	E	G	N	V	R	K
Canis lupus familiaris	R	S	E	Y	T	E	N	G	V	K	K

APPENDIX C

P0471

A nutrigenomic perspective to search for gene variants that influence carcass traits of feedlot cattle

UNIVERSITY OF SASKATCHEWAN

KE Hamilton, KM Madder and FC Buchanan

College of Agriculture and Bioresources, University of Saskatchewan, Saskatoon, SK.

kelsey.hamilton@usask.ca

UNIVERSITY OF SASKATCHEWAN

Introduction

- Vitamin A (VA) has a nutrigenomic effect on intramuscular fat, so searching for variants in genes involved in fat deposition and affected by VA could allow feedlots to precision feed cattle to optimize carcass traits
- Genes previously shown to be affected by VA in bovine pre-adipocytes may also be influenced at a whole animal level[1]
- Aminopeptidase (ANPEP) is a highly conserved enzyme that has been shown to play a role in vasodilation, immune recognition, cell-cell adhesion and tumor growth[2]
- We hypothesized that a variant in one or more of our candidate genes (*ADFP*, *GPX3*, *IGFBP6*, *SPARC*, CLU, *ANPEP*) would be associated with carcass traits and would be influenced by VA

Materials & Methods

Animals:

- Discovery population (n=16): beef steers of various breeds used for sequencing all candidate genes
- Population 1 (n=988): crossbred, implanted beef steers on either 100% or 50% NRC recommended VA[3]
- Population 2 (n=708): crossbred, implanted beef steers all on 100% NRC recommended VA[3]

Carcass Evaluation:

- Canadian Beef Grading Agency grade & yield
- VBG 2000e+v Technology GmbH vison camera grading system for USDA yield grade (YIELD; 1.0-5.9), marbling score (MARB; 00-900), rib-eye area (REA; sq. in.) and backfat measurement (FAT; in.)

Sequence and Genotyping:

- Sequencher 4.9 software was used to analyze sequence
- A PCR-RFLP was designed for genotyping *ANPEPc.410G>A*, using *Bsu36I*, which cuts the *G* allele
- Subsets of animals from each population were sequenced to confirm the presence of the haplotype in exon 1 of *ANPEP*

Statistical Analysis:

- Mixed procedure of SAS 9.4[4] was used with significance set at $P \leq 0.05$
- Standard errors were adjusted using a Kenward-Roger adjustment and means were separated using Tukey's LSD

Results

- *ANPEP* was chosen as the best candidate gene based on the presence of an 11 SNP haplotype
- *ANPEPc.404G>A*; Val>Ala and *ANPEPc.410G>A*; Arg>Lys
  - Minor allele frequency: *G*=0.27
- Other SNPs in haplotype are silent

ANPEPc.57T>C	201A>G	404G>A	410G>A	483G>A	516T>C	519C>T	555C>T	606+18A>C	606+53T>C	606+123G>A
CC	GG	CC	AA	GG	AA	CC	TT	CC	CC	AA
TC	AG	TC	GA	CG	GA	TC	CT	AC	TC	GA
TT	AA	TT	GG	CC	GG	TT	CC	AA	TT	GG

Figure 1. Haplotype found in exon 1 of the *ANPEP* gene. Box outline indicates the SNP genotyped. Pale green signifies the homozygous genotype that is the same as the reference sequence. Dark green signifies the homozygous genotype that is different from the reference, and medium green signifies the heterozygous genotype.

Results

POPULATION 1: Crossbred beef steers on 50% or 100% NRC VA

Table 1. Statistical results of analysis of population 1 for ANPEPc.410G>A

	ANPEPc.410G>A			P-value
	AA	GA	GG	
YIELD	2.47^{a}±0.03	2.36^{a}±0.03	2.14^{b}±0.08	0.0001
MARB	397.2^{a}±2.7	388.6^{ab}±3.3	370.4^{b}±7.2	0.0010
FAT	8.52^{a}±0.17	8.52^{a}±0.17	7.04^{b}±0.46	0.0002

Variables (mean±SEM) in a row bearing different superscripts differ significantly (P<0.05)

Table 2. Statistical results of analysis of population 1 for VA

	VA		P-value
	H	L	
REA	13.47^{a}±0.11	13.09^{b}±0.11	0.0149
FAT	8.13^{a}±0.25	7.30^{b}±0.25	0.0178

Variables (mean±SEM) in a row bearing different superscripts differ significantly (P<0.05)

- *ANPEP* and VA interaction on REA (P<0.05)
  - Only treatment with significantly improved REA was high VA with *GG* genotype (n=77), likely a Type I error
- Results from *ANPEP* alone seem the most promising
- Lead to further genotyping of *ANPEP* in Population 2

POPULATION 2: Crossbred beef steers on 100% NRC VA

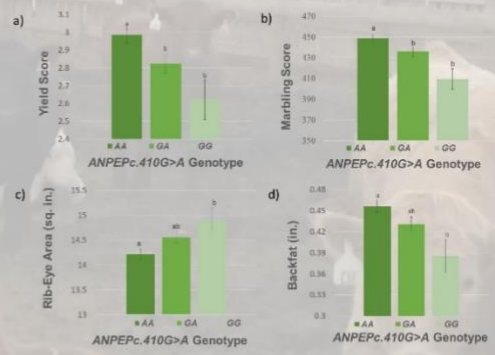

Figure 2. *ANPEPc.410G>A* genotype effect on carcass traits in crossbred feedlot steers.

Discussion & Conclusion

- The linkage disequilibrium of the 11 SNP haplotype found in *ANPEP* makes it an interesting candidate gene
- Although having a significant effect on REA and FAT, VA level does not appear to be critical to the effects of the *ANPEP* genotype on carcass traits
  - This was proven in Population 2 where only the recommended level of VA was provided
- *ANPEP* haplotype significantly affects carcass traits, but may not provide a great economic advantage when put into practice
- Further research into the functional effects of the *ANPEP* haplotype need to be completed
  - Analysis of *ANPEP* expression may provide some insight
- Due to the evolutionary conservation of the *ANPEP* gene, this haplotype may be found in other species and studies in this area could be of value

References

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