FEEDLOT PERFORMANCE AND IMMUNOLOGY OF BEEF STEERS FED A LOW

VITAMIN A DIET AND SELECTED FOR ADH1C GENOTYPE

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ABSTRACT

Prior nutrigenetic studies on the interaction between limiting vitamin A (VA) and the *ADH1Cc.-64T>C* SNP have shown that *TT* animals with reduced VA intake have improved intramuscular fat (IMF) in the *longissimus thoracis* muscle in beef cattle. The intent of this study was to determine whether this marker-assisted management (MAM) strategy would be effective at a commercial level, and whether there would be any immune function ramifications from limiting dietary VA. This occurred in two separate experimental groups, the first being a smaller immunology population, and the second in a large-scale commercial feedlot.

Crossbred steers (n = 18), black in colour, were selected from a prior feeding trial so that all combinations of *ADH1C* genotype (*TT*, *CT*, and *CC*) and VA level (25% or 75% of NRC, 1996 recommendations) were equally represented. Blood cell count analysis, peripheral blood mononuclear cell (PBMC) proliferation and stimulation assays, and quantitative reverse transcription polymerase chain reaction (qRT-PCR) examination of cytokine expression were conducted and compared. Vitamin A did not affect any of the measured parameters, and any significant ($P < 0.05$) pen and genotype effects did not equate to clinically ill animals.

The second study population included 2000 mixed breed beef steers, separated into 40 feedlot pens. Genotype at *ADH1C* (*TT* or *CT*), VA level (50% or 100% of NRC, 1996 recommendations), and implant status (IMS; implanted or non-implanted, IMP or NI respectively) were all equally represented with 5 pens of each possible combination of variables. Production and carcass data were collected, and the expected VA X *ADH1C* interaction effect was not observed. An IMS X *ADH1C* interaction effect was observed

with average daily gain (ADG; P=0.03), and IMS alone had significant effects on dry matter intake (DMI; P<0.01), total days-on-feed (P<0.01), USDA yield grade (P<0.01), marbling score (P<0.01), rib-eye area (REA; P=0.01), and backfat thickness (P<0.01). Overall, IMP animals finished on fewer days-on-feed with higher ADG, DMI, and REA and lower yield grades, marbling scores, and backfat thickness. No other interaction or main effects were observed, suggesting that the *ADH1C* X VA nutrigenetic MAM strategy is not effective at a feedlot level at this time.

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LIST OF ABBREVIATIONS

ADG	Average daily gain
ADH	Alcohol dehydrogenase
ADH1C	Alcohol dehydrogenase 1 C
BANDS	Band neutrophil cells
BVDV	Bovine viral diarrhea virus
C/EBP	CAAT/enhancer binding protein
cDNA	Complementary deoxyribonucleic acid
CFIA	Canadian Food Inspection Agency
Ct	Cycle threshold
DM	Dry matter
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
FAT	Backfat thickness
FIB	Fibrinogen
G:F	Gain to feed ratio
HCW	Hot carcass weight
ICAM	Intercellular adhesion molecule
IFN	Interferon
IMF	Intramuscular fat
IMP	Implanted
IMS	Implant status

IL	Interleukin
IU	International unit
kg	Kilogram
LT	Longissimus thoracis
MAM	Marker-assisted management
µl	Microliter
ml	Milliliter
mm	Millimeter
mRNA	Messenger ribonucleic acid
MUFA	Monounsaturated fatty acid
NI	Non-implanted
NRC	National Research Council
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RA	Retinoic acid
RAL	Retinaldehyde
RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RBC	Red blood cell
REA	Rib-eye area
RFLP	Restriction fragment length polymorphism

RNA	Ribonucleic acid
ROL	Retinol
RXR	Retinoid X receptor
SAS	Statistical analysis system
SEGS	Segmented neutrophil cells
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
sq cm	Square centimeter
STIM	Stimulation index value
TBA	Trenbolone acetate
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TOTDOF	Total days-on-feed
TOTPROT	Total protein
USDA	United States Department of Agriculture
VA	Vitamin A
VGMARB	Vision grade marbling score
VGUSYLD	Vision grade USDA yield score
WBC	White blood cell
WBSF	Warner-Bratzler shear force

1. INTRODUCTION

Marbling in beef is important to both producers and consumers. Premiums are offered on grading scales for carcasses that score above a certain level of marbling (DiCostanzo and Dahlen, 2000). Marbling is an important determining factor in the food purchasing decisions of beef consumers, as they tend to search for juiciness, flavour, and an overall enjoyable taste experience (Wheeler *et al.*, 1994). The widespread use of growth promotants in North America has however, shifted the quality of beef in the opposite direction of consumer demand, while increasing overall lean meat yield (King *et al.*, 2012), which is economically beneficial. This shift has caused an increase in research surrounding improving marbling through the reduction of dietary vitamin A (VA), with mixed results.

Altering management strategies based on genotype to improve efficiency is referred to as marker-assisted management (MAM). A proposed MAM strategy is feeding limiting levels of VA to *TT* animals at the *alcohol dehydrogenase 1 C* (*ADH1Cc.-64T>C*) single nucleotide polymorphism (SNP) to improve marbling in beef cattle (Ward *et al.*, 2012). Steers that are *TT* could be targeted for carcass quality, taking advantage of premiums, while *CT* and *CC* steers would be sold on live weight or hot carcass weight. This strategy takes advantage of the role of VA and its retinoids in the process of adipogenesis. The *ADH1Cc.-64T>C* SNP was thought to remove a transcription factor binding site, decreasing its expression, and decreasing the level of intramuscular fat (IMF) in animals with the mutation when VA was limited (Ward et al., 2012).

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Limiting dietary VA for improvements in marbling, and therefore potential profits, comes with the additional responsibility of ensuring that animal welfare is not hindered by possible deficiency. Symptoms ranging from decreased average daily gain to death can all be attributed to VA deficiency (Wolbach and Howe, 1925; Smith *et al.,* 1987; NRC, 1996). Vitamin A and its retinoids play important roles in vision, reproduction, and immune function, alongside their roles in adipogenesis. Specifically, failures of immune function due to VA deficiency could be catastrophic in a feedlot setting given that animals enter the feedlot with varying levels of stored VA, and tend to face several periods of stress and disease challenge throughout their time there.

The goal of this research was to determine whether feeding an intermediate level of VA (50% of NRC recommendations) in finishing rations to *TT* animals at the *ADH1Cc.-64T>C* SNP in a commercial feedlot setting would improve marbling. Observing the effect of hormonal implants on the *ADH1C* X VA MAM strategy, as well as classifying any signs of immune function deficiency were additional objectives.

2. LITERATURE REVIEW

2.1. Vitamin A

Vitamin A (VA) is a fat-soluble vitamin, and is ingested primarily in the form of β-carotenoid in feedlot rations (Eroglu *et al*., 2012). This form of pro-vitamin A is easily converted to retinol (ROL; Figure 2.1.1.) within the body (Deming and Erdman, 1999). While the form of VA found in animal sources (retinyl esters) can be transformed through hydrolyzation to ROL in the lumen of the intestine (Blomhoff *et al.*, 1990; Harrison, 2005), carotenoids can either remain intact or be transformed to ROL through a cleaving process (Harrison, 2005). In the cytosol of enterocytes, alcohol dehydrogenase (ADH) is the enzyme responsible for converting ROL to retinaldehyde (RAL). The oxidization process from RAL to retinoic acid (RA) is completed by the enzyme retinaldehyde dehydrogenase (RALDH; Duester, 2000). While RALDH only completes its forward reaction, ADH is able to convert from one retinoid to another in the forward and reverse directions (Boleda *et al.*, 1993).

The liver acts as a primary depot for VA within the body, and when carotene intake is in excess, VA can also be stored in fat cells. When dietary VA is limiting, the mobilization of stored VA from the fat and liver can occur, delaying the occurrence of VA deficiency (McDowell, 1989). Stored liver VA varies in a coordinated way with fluctuations in VA intake (Bryant *et al*., 2010; Ward *et al*., 2012; Pickworth *et al*., 2012). Retinol-Binding-Protein-4 is able to bind ROL and mobilize it from the liver and into the circulatory system (Quadro *et al.*, 2004; Blomhoff, 1990). The concentration of VA in the blood depends on both VA intake and liver stores (McDowell, 1989).
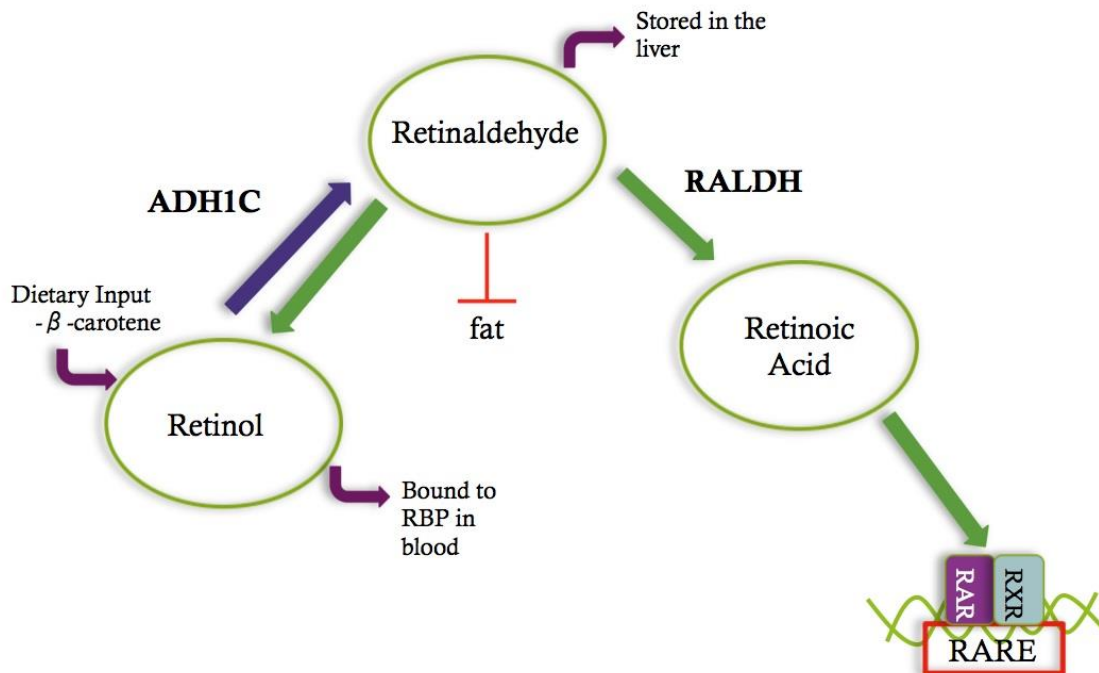
Figure 2.1.1. Diagaram depicting the relevant steps of the vitamin A pathway that involve the alcohol dehydrogenase 1C (ADH1C) enzyme. (RBP = Retinol binding protein; RALDH = Retinaldehyde dehydrogenase enzyme; RXR = Retinoid X nuclear receptor; RAR = Retinoic acid nuclear receptor; RARE = Retinoic acid response element transcription factor)

2.1.1. The Importance of Vitamin A

Vitamin A is important for the proper functioning of a wide spectrum of bodily functions, including: vision, bone and tooth development, reproduction, epithelial development, immune function, cell division and differentiation, adipogenesis, and maintaining moisture in mucous membranes, skin, and eyes (Goodman, 1984; Dawson, 2000; Anderson and Young, 2008). These effects of VA often occur by means of changes in gene expression. Humans living in developed nations tend to be more concerned with VA toxicity than being deficient, likely due to the often sufficient levels of VA in a typical daily diet combined with high concentrations of VA in some multivitamin supplements. Early symptoms of VA toxicity are loss of appetite, headache, nausea, and itchiness,

while more severe acute toxicity symptoms include dizziness, blurred vision, growth impairment, and birth defects. Night blindness and over-keratinized skin are early symptoms of VA deficiency, while long-term effects include: reduced immune function, delayed tooth and bone development, birth defects, seizures, and mortality.

The concern for VA levels in cattle diets tends to revolve around avoiding deficiency, as toxicity would be difficult to achieve, and the symptoms of deficiency would be quite costly to the producer and would have animal welfare repercussions. Early symptoms of VA deficiency in cattle often include inefficiencies in production traits, followed by weakness, ataxia, blindness, xerophthalmia, convulsions, syncope, and reproductive issues (Radostitis *et al.*, 2000). Appetite, coat condition, presence of night blindness, swelling of the joints, drooling, and general ill behaviour were all indicators used by Kruk *et al.* (2008) to assess their study animals for VA deficiency. Spears (2000) stated that there was a clear link between VA deficiency, infectious disease occurrence, and mortality. The effect of VA deficiency on immune function is therefore a large concern for cattle producers. Consequently, feedlot rations often surpass the required 2,200 IU/kg dry matter (NRC, 1996), to eliminate the concern of clinical VA deficiency altogether.

2.1.2. The Effect of Vitamin A on Immune Function

Over-feeding of VA to feedlot cattle is common but may have a negative impact on carcass quality. As a result, reduced VA supplementation may be a strategy that can be implemented in the future to improve marbling. However, the following 21 immune functions have been reported to be affected by retinoids: keratinization; hematopoiesis;

apoptosis; *ICAM-1* expression; mucin, immunoglobulin, TNF-α, TGF-β, phospholipase A$_2$, IFN-γ, IL-1, IL-2, IL-3, and IL-4 production; neutrophil, natural killer cell, monocyte/macrophage, Langerhans cell, T lymphocyte, cytotoxic T lymphocyte, and B lymphocyte function (Semba, 1998). Clearly, retinoids have a vast array of effects on immune responses, encompassing physical barrier properties, innate, and acquired immune responses. Deciphering between the effects of VA deficiency on immunology and the effect of disease on VA deficiency is not an easy task. The relationship between the two is quite cyclic in that VA deficiency lessens the immune system's ability to defend against infectious disease, and infectious disease can greatly reduce the uptake and absorption of VA, while also increasing its excretion (Stephensen, 2001). Before reduced VA supplementation is implemented, the effect of this strategy on markers of immune function and animal health should be evaluated.

The first lines of defense in mammalian immunity are physical barriers and innate immunity. Vitamin A deficiency primarily affects physical barriers through an overall reduction in mucosal immunity due to the loss of goblet cells and reduction of mucin and mucus production in the respiratory, gastrointestinal, and urogenital tracts (Stephensen, 2001). This decrease in mucin and mucus production is harmful for three reasons: 1) mucus is important because it allows for the trapping and expelling of invaders; 2) mucus also provides a protective barrier for underlying epithelial cells; and 3) mucosal epithelia are less capable of regenerating (Stephensen, 2001). Once epithelial cells on the mucosal surface are exposed, squamous metaplasia, necrosis, and inflammation can occur (Stephensen, 2001), all of which are risk factors for the systemic spread of pathogens. Immune deficiencies reported in animals lacking VA lead to an increased risk of invasive

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disease and mortality, even if improved availability of VA through dietary supplementation may reverse physical barrier damage (Stephensen, 2001).

The recognition of pathogen-associated molecular patterns by pattern recognition receptors such as Toll-like receptors is central to the function of the innate immune system (Medzhitov and Janeway, 1997). Neutrophils are incredibly efficient at phagocytosing and destroying extracellular infectious agents and the available evidence shows that their killing ability is diminished in VA deficient animals (Semba, 1998; Stephensen, 2001). This is likely due to the fact that the VA nuclear receptor retinoic acid receptor (RAR) regulates the expression of genes involved in neutrophil development (Stephensen, 2001). Macrophages also phagocytose, but function as antigen presenting cells as well as destroying the foreign infectious agent. They present antigens on their major histocompatibility complex II molecules, which can then be recognized by T helper cells to drive cell- or antibody-mediated immune responses in the presence of the appropriate co-stimulatory signals (Mosmann and Coffman, 1989; Stephensen, 2001). Macrophages are also affected by retinoids in that they have a reduced phagocytic ability in VA-deficient animals (Semba, 1999; Stephensen, 2001). All cells that do not display normal cell signals (non-self, often virus-infected or tumor cells) are recognized by natural killer cells, which then destroy them (Stephensen, 2001). In VA deficient animals, both natural killer cell numbers and killing ability are diminished (Semba, 1998; Stephensen, 2001).

T and B cells of the acquired immune system are very much interconnected, where by a change in function of one cell type is likely to affect the other. During VA deficiency, antibody-mediated immune responses tend to be more significantly affected

(Stephensen, 2001). Vitamin A deficient animals have been shown to demonstrate reduced lysing ability of cytotoxic T cells, but T helper 2 cells are the most affected, as their functioning is significantly impaired (Semba, 1998; Stephensen, 2001). These larger-scale effects of VA levels on lymphocytes may be due to the fact that retinoids are present within every lymphocyte subset in humans (Semba, 1998). Interestingly, the T helper 1 cell response is unaltered or even improved by VA deficiency in humans (Stephensen, 2001). It is hypothesized that this is due to a shift from a T_H2 to a T_H1 response. This shift is characterized by a decrease in IL-4, IL-5, and IL-10 cytokines and an increase in IFN-γ and IL-12 cytokine release (Semba, 1998).

These effects of VA deficiency on immune function are incredibly relevant to the feedlot industry, where animals face high stress environments and disease challenge frequently. Stress has been shown to be immunosuppressive, depressing T cell responses, natural killer cell function, and IL-2 production (Tizard, 2000). Bovine viral diarrhea virus (BVDV) is an example of a condition that could be exacerbated by the negative effect of VA deficiency on immune function. It is common in feedlots, resulting in large annual losses in production and profit. The cytokine IL-1 is critical in both innate and acquired immunity, and its inhibition during a non-cytopathic BVDV infection has been suggested (Peterhans *et al.,* 2003). In addition to reduced cytokine and co-stimulatory signaling of IL-1, BVDV infection may also lead to irritation of the gut epithelium, and therefore the VA deficiency effects previously discussed could be devastating to affected feedlot populations.

The appearance of VA deficiency symptoms was obvious in a group of extensively grazed beef cattle during one year on poor pasture. The effects were most

prominent in newborn calves, even when their dams did not show signs of clinical deficiency (Hill *et al.*, 2009). Over 40% of the calves born in this herd died, with the majority being stillbirth, in addition to a high incidence of prenatal loss. Hill *et al.* (2009) concluded that VA deficiency likely played a large role in the deaths of these animals as their serum and liver VA levels were deficient or undetectable. Hepatic storage of VA is known to buffer extended periods of poor feed quality and the period of deprivation is often not long enough for deficiency symptoms to occur (Radostitis *et al.*, 2000). This is an extreme example of VA deficiency within a beef cattle herd, and outlines the difference in buffering capacity between dam and calf on poor quality forage.

In theory, all of the effects of VA deficiency on immune function would be quite harmful with respect to the acquired immune system and its antibody-mediated responses, but in reality this is not always the case. Jee *et al.* (2013) discovered that antibody titers did not significantly differ between calves fed 3300 IU VA/kg DM versus 1100 IU VA/kg DM per day after receiving multiple vaccinations. The antibody response to an ovalbumin vaccine was not affected by VA restriction in a study by Gorocica-Buenfil *et al.* (2014) where animals were fed either no supplemental VA or the recommended 2200 IU/kg DM supplement for 216 days. The immune response of newborn Holstein calves fed 0, 5000, 10 000, and 20 000 IU VA/day also did not differ over a 56 day period (Hidiroglou and Markham, 1996). These findings are important as they suggest that a fairly severe level of VA deficiency may be necessary before biologically significant alterations in immune responses take place.

Immune responses in vertebrates are quite difficult to discuss separately, as physical barrier function, innate, and adaptive immune responses are all linked. In

addition to that complexity, VA deficiency can be the result of insufficient dietary intake, improper absorption, excess excretion, due to prolonged illness, or any combination of these factors. This could be of significant concern for production, as animals entering a feedlot have been on varying qualities of pasture or feed, will face multiple disease challenges or may be chronically ill, and will generally be under stress multiple times during their stay. Although reaching a level of VA deficiency in many animals is likely difficult to achieve, once at that level, this vicious cycle of dampening the immune response and disease causing further deficiency could be difficult to break without intensive and likely expensive treatment.

2.1.2.1. Cytokine Gene Expression Methods of Analyzing Immune Function

Quantitative Polymerase Chain Reaction (qPCR) is based around the process of gene amplification that is accomplished through typical PCR, with the added ability to determine the amount of amplification occurring, or the original amount of genetic material in the sample. In Reverse Transcription Polymerase Chain Reaction (RT-PCR), RNA is reverse-transcribed to cDNA to be used for PCR, which therefore demonstrates the amplification of genes that are actively transcribed in a specific cell/tissue of interest. Additionally, the ability to monitor amplification during a PCR run is possible through Real-Time PCR (Ginzinger, 2002; Bustin *et al.*, 2009). Any combinations of these methods are commonly used to analyze gene expression. Cytokine gene expression, disease susceptibility polymorphism genotyping, and viral load determination can all be assessed through the use of qRT-PCR, making it a very useful tool in many fields, including immunology.

The analysis of qRT-PCR data can occur by two general methods, absolute quantification and relative quantification. Both methods depend on the generation of an amplification curve. This curve demonstrates the required number of PCR cycles that would create enough fluorescence to surpass the pre-determined threshold level or value (Ct; Giulietti *et al.*, 2001). The Ct and the original amount of nucleic acid in the starting material are therefore inversely proportional, and the doubling of the amount of product at every cycle is the basic assumption of PCR (VanGuilder *et al.*, 2008).

The creation of a standard curve for the gene of interest is required for the absolute quantification qRT-PCR method, where each point on the curve would be of known copy number. Comparisons can then be made against this curve to determine the specific number of nucleic acid copies in the tested sample through its experimentally obtained Ct value. Not all genes of interest are accessible in known concentrations; therefore the creation of a standard curve may not always be possible (Giulietti *et al.*, 2001).

Relative quantification requires the use of a housekeeping gene (ie. *Beta-actin*, *GAPDH*, ribosomal proteins) that functions as an external control. Housekeeping genes must only be selected if their expression is unaltered by the experimental treatments (Giulietti *et al.*, 2001). The Ct values of the gene of interest as well as the housekeeping gene are then compared in the following formula:

$\Delta\Delta$Ct = ΔCt (test) – ΔCt (control);

where ΔCt (test) = Ct *gene of interest* (test) – Ct *housekeeping gene* (test),

and ΔCt (control) = Ct *gene of interest* (control) – Ct *housekeeping gene* (control).

An additional correction of $2^{-\Delta\Delta Ct}$ must then be used to determine the relative quantity

value if relative quantification comparisons are required. If the amount of product doubles after the completion of each amplification sample, this correction is the only value that is statistically correct when making comparisons between relative quantification values (VanGuilder *et al.*, 2008). The efficiency, accuracy, and precision of today's qRT-PCR (Ginzinger, 2002) along with its multiple options for chemistry and analysis method allow for it to be useful for diverse applications in many different disciplines.

More specifically, the application of RT-PCR in assessing cytokine gene expression is commonly used to determine and explain the level of immune responsiveness in cattle. When infected with *Mycobacterium bovis* (Thacker *et al.*, 2007) or *Cooperia punctata* (Bricarello *et al.*, 2008), cytokine gene expression was studied as a marker for the immune function of Holstein and Nelore cattle, respectively. Expression of *IL-2*, *IL-4*, *IL-5*, *IL-10*, *IFN-γ*, and *TNF-α* were analyzed by means of RT-PCR to study immune competence and inflammation precursors in Holstein cattle inoculated with differing lines of *S. aureus* (Luby, 2010). Additionally, RT-PCR can be used to determine the effect of vitamins on cytokine expression. Li-Weber *et al.* (2002) studied the effects of vitamin E on human peripheral blood T cells by means of RT-PCR and concluded that vitamin E suppresses *IL-4* transcription. Vitamin A is also known to influence gene expression, which has multiple downstream effects.

2.1.3. The Role of Vitamin A in Gene Expression and Adipogenesis

As mentioned previously, VA plays a role in many systemic bodily functions. Its effect on adipogenesis is intrinsically linked to how VA alters gene expression. The RA and

RAL retinoids are able to act in opposing manners on the expression of genes involved in adipogenesis through their interactions with nuclear receptor proteins. The retinoic acid receptor (RAR) and retinoid X receptor (RXR) are both able to bind to RA, which then allows them to form either homo- (with the same receptor type) or hetero- (with the other receptor type) dimers (Heyman *et al*., 1992; Repa *et al*., 1993; Petkovich, 2001; Desvergne, 2007). Within the promoter region of many genes lie retinoic acid response elements (RAREs) that these RAR/RXR homo- or hetero-dimers bind to (Heyman *et al*., 1992; Zhang *et al*., 1992), potentially altering the expression of that gene. Up-regulation of transcription is the common outcome of RA binding to RAR/RXR dimers that then bind to RARE (Heyman *et al*., 1992; Zouizenkova *et al*., 2007), while RAL is thought to do the opposite through acting as a substrate in competition with RA. Other nuclear receptors, including peroxisome proliferator-activated receptors, vitamin D receptors, thyroid hormone receptors, and liver X receptors, are also able to form heterodimers with RXR (Bugge *et al.*, 1992; Zhang *et al.*, 1992; Bardot *et al.*, 1993; DiRenzo *et al.*, 1997; Desvergne, 2007; Ziouzenkova *et al*., 2007), which may also affect gene expression.

The intricate maturation process of pre-adipocytes to adipocytes is termed adipogenesis, which can be altered by VA. Essentially, multipotent stem cells can be influenced by the presence or absence of VA, to become pre-adipocytes through hyperplasia (Harper and Pethick, 2004). When applied to embryonic stem cells, RA is able to cause large amounts of adipogenesis (Gregoire et al., 1998), and the inhibition of fat deposition is possible with sufficient levels of RAL (Ziouzenkova *et al.*, 2007) or ROL (Kawada *et al.*, 1996). This stimulation process by RA may be effected by the interaction of the retinoid receptors RAR and RXR (Mizoguchi *et al.*, 2014) with the

RARE complex, which alters gene expression, and therefore up- or down-regulates the expression of genes key to adipogenesis (Zhang *et al.*, 1992).

2.1.4. Implementation of Vitamin A Restriction in Beef Cattle

The recommendation for dietary VA for feedlot cattle, as determined by the National Research Council (NRC; 1996), is 2200 IU/kg dry matter. This recommendation however, is often surpassed, sometimes doubled or tripled in today's feedlot management systems. The over-feeding of VA at these levels is not typically a concern for hypervitaminosis, but with knowledge of RAL impeding fat deposition (Ziouzenkova *et al.*, 2007; Kawada *et al.*, 1996), and all-trans retinoic acid inhibiting adipocyte differentiation (Ohyama *et al.*, 1998; Kawada *et al.*, 2000), research has been conducted to determine the potential benefits of limiting VA in feedlot diets.

Wagyu steers have been shown to have an increase in marbling with a decreased intake of beta-carotene (Oka *et al.*, 1998) and when blood levels of VA were low (Adachi *et al.*, 1999). Similar findings have also shown in beef breeds in North America. Kruk *et al.* (2008) found that Angus cattle, unsupplemented for VA, had no symptoms of deficiency, but did have: 35% higher IMF (P < 0.0026); numerically higher US marbling scores (P = 0.094); 33% higher seam fat (P < 0.05); lower VA concentrations in extrahepatic tissues and fat; and lower melting points of the subcutaneous fat than their supplemented counterparts. The lower melting point of this fat suggests a softer, more unsaturated fatty acid-rich (Siebert *et al.*, 2000) desirable fat. A significant negative correlation between serum ROL and marbling scores was also found (Kruk *et al.*, 2008),

demonstrating that this association between VA and marbling does not solely occur in Wagyu cattle.

Angus crossbred steers fed an unsupplemented diet for VA had significantly improved quality grades and tended ($P = 0.06$) to have increased ether extracted IMF (Pickworth *et al.*, 2012a). Results also demonstrated significant interaction effects of VA and vitamin D supplementation on backfat thickness and USDA Yield grade, where both backfat thickness and yield grades were decreased with VA and vitamin D supplementation. These traits are related however, and there was no interaction effect on IMF, therefore VA is likely the main contributor of the two to fat deposition, at least with respect to quality grade.

Studies have also found evidence to refute these associations. Bryant *et al.* (2010) found no difference ($P > 0.10$) in marbling score, hot carcass weight (HCW), *longissimus thoracis* (LT) muscle area, and 12th-rib fat thickness among all VA supplementation treatment levels (0, 1103, 2205, 4410, and 8820 IU/kg DM) for a group of yearling black feedlot steers. Gorocica-Buenfil *et al.* (2008) also found no effect ($P > 0.10$) of VA restriction in Angus-based steers on ADG, DMI, G:F, HCW, 12th-rib fat, yield grade, marbling score, and IMF measures. Animals that received zero supplemental VA did have reduced serum ($P < 0.01$) and liver ($P < 0.01$) ROL levels when compared to animals supplemented at the NRC (1996) recommendations, suggesting that the restriction was able to reduce stored and circulating VA within the body (Gorocica-Buenfil *et al.*, 2008). However, monounsaturated fatty acids (MUFAs) were observed to increase ($P = 0.03$) in unsupplemented animals, while saturated fat levels decreased ($P = 0.03$). This finding was in agreement with the results on fatty acid content by Kruk *et al.*

(2008), and suggested a potential effect of VA restriction on desaturase enzyme activity

(P = 0.01).

These mixed results are typical in the VA restriction literature and could, in part, be due to the timing and duration of restriction. Pickworth *et al.* (2012b) found that both serum and hepatic VA in Angus crossbred steers were significantly highest at 56 days after cattle were weaned and arrived in the feedlot, for all levels of VA supplementation. The hepatic VA stores were reduced, and remained at low levels when animals remained unsupplemented. While growth traits and USDA yield grades were not affected by VA restriction, IMF in the (LT) muscle increased in unsupplemented animals. It has been suggested (Pickworth *et al.*, 2012b; Kruk *et al.*, 2008) that the level of restriction is not the sole factor in improving fat deposition, and that the duration of said restriction may be equally important. With proper timing and implementation, feedlot limitation of VA has the potential to increase quality grades, IMF, or MUFA content, in turn possibly increasing profits. However, the quality of pasture that animals are raised on or backgrounded on must be considered, as unsupplementing VA in the finishing ration may cause severe losses with respect to VA deficiency.

2.1.5. Nutrigenetics of *ADH1C* and Vitamin A

The way in which food components affect gene expression is nutrigenomics, while nutrigenetics refers to the effect certain genetic mutations have on this process. Prior work on the interaction between limiting dietary VA and a mutation in the *ADH1C* gene has been performed in black coloured beef steers (Ward *et al.*, 2012; Krone *et al.*, 2016) with encouraging results with respect to producing better-marbled beef. Ward *et al.*

(2012) discovered a mutation in the promoter region of *ADH1C* (*ADH1C c.-64T>C*) that, when changed from a thymine to a cytosine base at position 64 upstream of the start codon, removes a binding site for the transcription factor CCAAT/enhancer-binding protein α (C/EBPα). The *ADH1C* gene produces the protein that oxidizes retinol (ROL) to RAL, which can be further oxidized to RA by RALDH (Duester, 2000). Napoli (1996) found that RALDH activity increased threefold when VA was deficient.

The *ADH1Cc.-64T>C* SNP has been found to affect gene expression (Ward *et al.*, 2012) and levels of ADH1C protein in liver tissue (Krone *et al.*, 2016). Less ADH1C would impact the level of RAL, ultimately affecting the level of RA generated. As mentioned previously, the expression of numerous genes in the adipogenesis pathway can be altered by RA and/or RAL, which act as ligands for RXR and RAR receptors that interact with one another as well as with the RARE to influence gene expression (Ziouzenkova *et al.*, 2007). This typical up- or down-regulation of adipogenesis genes may be affected by the reduction in *ADH1C* expression when C/EBPα is removed with the *C* allele. This is because *TT* animals were found to have 22.9% higher levels of IMF than *CC* animals, when both sets of animals were unsupplemented for VA (Ward *et al.*, 2012). Unsupplemented *TT* animals had 24.4% higher IMF than other *TT* animals receiving the recommended 2200 IU/kg DMI VA levels (NRC, 1996; Ward *et al.*, 2012). The nutrigenetic interaction between the *ADH1C* SNP and level of VA on IMF could be useful in a MAM strategy for feedlots where animals could be sorted based on genotype and *TT* animals could be fed limiting VA levels in order to market them on a marbling grid. However, the appropriate level of dietary VA, in order to avoid the complications of VA deficiency, still had to be determined.

Krone *et al.* (2016) set out to establish an appropriate level of VA inclusion for feedlots that in combination with the *ADH1C* SNP would improve consistency in carcass quality. They fed limiting amounts of VA (25, 50, and 75% of NRC, 1996 recommendations in a monthly oral bolus) in finishing rations of black feedlot steers. The expected effect of increased marbling for animals with the *TT* genotype was only significant ($P < 0.05$) at the 75% of NRC (1996) level. This may have been due to the lack of variation in IMF levels at the 25% and 50% VA diets, as these animals consistently finished well above the Canada AAA baseline (Krone *et al.*, 2016). The animals studied by Ward *et al.* (2012) were also fed VA deficient diets through the backgrounding period, while Krone *et al.* (2016) fed their study animals a normal backgrounding ration that included 1.7 times the NRC (1996) recommendation of VA due to available feedstuffs being high in VA that year. Krone *et al.* (2016) confirmed that *TT* animals had significantly ($P = 0.02$) higher ADH1C protein levels in liver tissue than *CT* and *CC* animals, and that there was significantly ($P = 0.03$) higher ADH1C protein levels in all animals at the end of test compared to concentrations in the liver at the start of test. This confirmed that the removal of the C/EBPα in animals with a *C* allele does in fact reduce the expression of the *ADH1C* gene, resulting in less ADH1C protein available to convert ROL to RAL. Nutritional background and the genetics of the animal are important factors impacting the growth potential of beef cattle (Platter *et al*., 2003; Bruns *et al*., 2005), and therefore combining the two in MAM strategies for feedlot use could offer great benefits for producers. These strategies however, would need to work in harmony with growth promotants as they are commonly used in feedlots today.

2.2. Effects of Hormonal Implant Use in Beef Cattle Production

Hormonal implants are commonly used in commercially raised beef animals, in fact, 22 different hormonal implants are listed on the CFIA website as being approved for use. Trenbolone and zeranol preparations are the two types of exogenous preparations that are approved, and several endogenous preparations such as progesterone and estradiol are also approved in Canada (CFIA, 2014). The recommended implant site for all hormonal implants is the ear, and animals may be implanted several times throughout their lives, however implants in animals intended for veal meat are not permitted (CFIA, 2014).

The purpose of these implants is to improve production and carcass traits with the overall end-goal of an increased return on investment for producers. A meta-analysis reviewing the effects of implants on beef cattle found significant increases in average daily gain (ADG) for implanted heifers and in ADG, DMI, and gain to feed ratio in implanted steers (Wileman *et al.*, 2009). The study by Wileman *et al.* (2009) also estimated a decreased cost of production of $77 per implanted steer when compared to non-implanted steers. This reduction became more drastic, at $349 per implanted steer, when compared to organically raised steers (Wileman *et al.*, 2009).

Trenbolone acetate (TBA) is an exogenous preparation used in many beef cattle implants as a synthetic form of testosterone. It is often combined with some level of estradiol, which is a highly potent anabolic steroid, and these combination implants tend to be highly effective in improving production traits. Foutz *et al.* (1997) found that steers given an estrogen plus TBA steroid implant gained faster, and had improved feed efficiency than control or estrogen plus progesterone implanted animals. Steers receiving implants with TBA all had significantly larger LT areas, and a trend for lower marbling

scores and yield grades. A trend was also observed between increased shear force values for implanted steers versus controls (Foutz *et al.*, 1997).

The number of implants also plays a role in production characteristics and carcass traits. Platter *et al.* (2003) found that implanted animals had significantly lower marbling scores, increased ADG from weaning to harvest, and increased LT muscle area compared to non-implanted groups. A panel of consumers rated steaks from non-implanted animals as more desirable for overall eating experience than steaks with 2, 3, 4, or 5 implants throughout their lives, regardless of the timing of these implants (Platter *et al.*, 2003). Interestingly, Platter *et al.* (2003) also found that among the implanted animals, those with 2 lifetime implants had higher marbling scores than those from more aggressive implant strategies (4 and 5 lifetime implants).

Overall, hormone implants can improve yield and ADG, but may also greatly reduce quality in the form of marbling and eating experience – especially when aggressive implanting strategies are used. A negative public opinion regarding the use of hormonal implants in beef production is growing, and organic foods are increasingly trendy, suggesting a large market for hormone-free and organically raised beef. Due to these factors, Platter *et al.* (2003) suggest that producers decide on a customized implant strategy based on marketing intentions.

2.3. Beef Cattle Markets and Consumer Preferences

Beef consumers today are often able to distinguish between tenderness (Boleman *et al.*, 1997; Shackleford *et al.*, 2001) and marbling levels in steak. These consumers are also willing to pay premiums for tender (Boleman *et al.*, 1997; Shackleford *et al.*, 2001) and

more marbled beef (Killinger *et al.*, 2004). However, the consistency of beef quality has long been a significant problem according to those purchasing these products (Savell *et al.*, 1987). There are also problems with grading differences, whereby depending on the cut of the meat, consumers often cannot tell the difference between a Low Choice and High Select (or Low Canada AAA and High Canada AA) or between Low Select or High Select (or Low Canada AA and High Canada AA; Neely *et al.*, 1998). However, in the study by Neely *et al.* (1998), consumers were able to differentiate between High Choice (or High Canada AAA) steaks from the other grades in top loin and top round cuts, but not in top sirloin. This means that the difference between a high AA steak and one grading low AAA may be smaller than between low AAA and high AAA grades, yet premiums are often given for beef consistently grading AAA or higher (DiCostanzo and Dahlen, 2000). This may leave consumers confused about their perception of the AAA grade if they happen to purchase a steak on the low end, giving them more of an AA eating experience. There is a clear interplay between the quality grading of beef and consumer purchasing decisions based upon their eating experiences. This interplay should have a major influence on the beef market for profits to increase.

A study by Platter *et al.* (2005) found that consumers were more likely to prefer steaks with shear force measurements associated with increased tenderness, as well as higher quality graded (the equivalent of mid-Canada AAA or higher) beef. Aversion from buying tougher steaks (Warner-Bratzler shear force: WBSF; measure of > 3.9 kg), and those of lower quality grade (mid-AA or lower) were also observed. The researchers predicted that a minimum quality grade of mid-Canada AA and a maximum WBSF measurement of 3.9 kg were the baselines above which most beef consumers would

attempt to buy a steak. In a bidding scenario, consumers offered to pay on average

$0.89/kg more for steaks graded in the upper half of Canada AAA, and $2.47/kg more for

Prime steaks (Platter *et al.*, 2005). With respect to tenderness, a decrease in consumer-

valued price of $1.02/kg was predicted for every 1 kg increase in WBSF measurement

(Platter *et al.*, 2005).

Froehlich *et al.* (2009) also found that consumers were willing to pay more for

Canada AAA steaks, and those guaranteed to be tender. They suggest that by providing

beef of guaranteed higher eating quality to knowledgeable consumers, through labeling or

branding, the industry could increase the value of beef in this country. Therefore, both the

Platter *et al.* (2005) and Froehlich *et al.* (2009) studies show that consumer demand, as

well as value for the product can be bolstered by improvement upon the quality of beef

being produced and offered in the marketplace.

The marketing and pricing of cattle is variable between producers, markets, and

over time. They can be priced on a live weight, dressed weight, or grid or formula pricing

system, and often alliances or agreements are made between feedlots and packing plants.

These agreements involve decisions with respect to base price and premiums or discounts

received for certain carcass traits (DiCostanzo and Dahlen, 2000).

Cattle can be marketed towards many different consumer bases, as distinctive

purchaser groups have different preferences (i.e. well-marbled, lean, hormone-free,

organically raised; Feuz, 1999). The end result of marketing beef could vary greatly

depending on pricing system and time. By developing grids based on the market to be

targeted (based on buyer preferences), and through the production of cattle to better fit

those grids, increased profits could be made as consumer experiences improve (Feuz,

1999). Developing an effective farm-to-retail supply chain has been suggested (Froehlich *et al.*, 2009) with marketing strategies to brand a product benefiting all parties involved.

2.4. Marker-Assisted Management Strategies

Marker-assisted management (MAM) is the strategy of altering the way an animal is managed (i.e. raised/fed/bred) once the animal's genetic information has been taken into consideration. This change in management is aimed towards increasing efficiency and/or the quality of the final product through improving upon a trait that is affected by specific genes. This MAM strategy is an offshoot of marker-assisted selection, which is the strategy of changing how animals are bred based on genetic information in order to improve upon a trait in future generations. Management strategies that may be altered include: the sorting of pens based on genotype to either feed different diets or for differing amounts of time; the use of implants or B-agonists in certain animals; or targeting certain animals for different markets to improve profits (Van Eenennaam and Drake, 2012).

Genetic variations between individuals that are associated with a difference in a phenotypic trait of interest are called DNA markers (Van Eenennaam *et al.*, 2007). Some traits, referred to as simple traits, are more easily explained and predicted as they are controlled by only one gene with an associated marker allele. Multiple genes and the environment control other traits, known as complex traits, as they are often associated with multiple marker alleles. These complex traits are more difficult to predict and explain, but are the category under which many beef cattle production and carcass traits fall (Thompson *et al.*, 2014).

A MAM strategy currently employed commercially uses a single nucleotide polymorphism (SNP) in the leptin gene (Buchanan *et al.*, 2002). This *LEPc.73C>T* SNP is used to select animals by genotype to sort and manage them differently, as *TT* animals achieve a higher degree of marbling in the final carcass earlier than *CC* animals (Buchanan *et al.,* 2007). This MAM strategy can significantly increase the profit for producers, both through breeding and management decisions. Testing for this leptin SNP is currently marketed for feedlot use by Quantum Genetics (Saskatoon, SK, Canada). The effect of the SNP is somewhat lessened when Zilpaterol Hydrochloride is fed (Kononoff *et al.*, 2013), therefore this type of MAM may not be ideal in all feedlot management strategies (Van Eenennaam and Drake, 2012).

Van Eenennam and Drake (2012) suggest that the greatest value of DNA testing in MAM lies in the breeding sector, but it may be more feasible at the feedlot level as the cost of testing lessens with increased numbers. The authors determine that so long as good accuracy exists for the tested markers at predicting valuable feedlot traits, and that incentives are offered up the beef production chain to have animals tested, MAM could be a valuable strategy in feedlots. Consistently well-marbled carcasses may receive premiums, and in previous studies (Ward *et al.*, 2012; Krone *et al.*, 2016) the *TT ADH1Cc.-64T>C* genotype has been shown to increase IMF when VA is limited in finishing rations – giving it great potential as a feedlot MAM strategy to increase profits.

3. HYPOTHESES

Previous research suggests that beef cattle marbling could be improved by restricting dietary VA and that the *ADH1C* X VA MAM strategy improves IMF when *TT* animals are fed limited VA. Growth promotants are known to improve production and yield characteristics, while hindering fat traits. Clinically relevant VA deficiency symptoms often only occur after a severe limitation of dietary VA in beef cattle. Given these previous findings, my hypotheses were as follows:

1) Animals with lower VA levels in their diet will have increased marbling scores. In this case, animals fed the test level of VA (50% of NRC) will show improved fat characteristics when compared to animals consuming the 100% NRC level (2200 IU/kg; NRC 1996).

2) Animals with the *TT* genotype at *ADH1Cc.-64T>C* will show increased marbling scores when compared to *CT* animals, when VA is fed at 50% of NRC.

3) Implanted animals will show reduced fat traits (including marbling scores) when compared to non-implanted animals.

4) The highest amount of marbling will be seen in *TT* animals, fed 50% NRC, that were non-implanted. The second highest will be *TT*, 50% NRC animals, with implants. This would prove the efficiency of using the *ADH1C* SNP in MAM practices at a commercial level.

5) Animals with reduced 25% and 75% NRC levels of dietary VA will not show any form of diagnosable indicators of decreased immune function or symptoms of VA deficiency.

4. A FEEDLOT TRIAL REDUCING VITAMIN A IN COMBINATION WITH ADH1C GENOTYPE TO IMPROVE INTRAMUSCULAR FAT

4.1. Abstract

A previously discovered variant in the *ADH1C* gene has been shown to improve intramuscular fat in beef cattle when dietary vitamin A (VA) was limiting. This *ADH1Cc.-64T>C* single nucleotide polymorphism (SNP) has the potential, through a marker-assisted management (MAM) strategy, to increase profits for producers, and therefore it needed to be tested at a commercial level. Two thousand mixed breed beef steers were separated into 40 feedlot pens depending on genotype (*TT* or *CT*). Half of the pens were implanted, the other half were not. Treatments of 50 or 100% levels of the NRC (1996) recommendation of dietary VA were randomly assigned so an equal number of treatment combinations were achieved. Steers were sent to slaughter when the pen average weight reached 612 kilograms, and production and carcass data were collected. The expected VA X *ADH1C* effect was not observed for any trait in either the implanted (IMP) or non-implanted (NI) animals, however implant status did significantly affect Dry Matter Intake (IMP = 8.55. ± 0.113 kg; NI = 7.87. ± 0.113 kg; $P < 0.01$), Total Days-on-Feed (IMP = 164.40 ± 2.782 days; NI = 210.45 ± 2.782 days; $P < 0.01$), USDA Yield Grade (IMP = 2.40 ± 0.046; NI = 2.77 ± 0.046; $P < 0.01$), Marbling Score (IMP = 391.90 ± 4.193; NI = 454.90 ± 4.193; $P < 0.01$), Rib-Eye Area (IMP = 84.97 ± 1.135; NI = 80.65 ± 1.135 cm^2; $P = 0.01$), and Backfat Thickness (IMP = 8.07 ± 0.281 mm; NI = 10.01 ± 0.281 mm; $P < 0.01$). Average Daily Gain was affected by implant status in combination with *ADH1C* genotype, however the genotype effect may be a type I error. No other main effects of VA or *ADH1C* genotype were observed, suggesting along with a lack in their

interaction effects, that this MAM strategy is likely not viable at a commercial level. The
findings related to implant status effects support previous literature. The significant
effects of the hormonal implants also continue to emphasize their profound benefits in
improvements in production traits and yield, but also their deficits, such as reductions in
marbling scores and other fat traits.

Implications

The marker-assisted management strategy utilizing *ADH1C* and limiting vitamin A in
finishing rations is not yet ready for application at a commercial level. The expected
effects on fat traits were not observed. This may have been due to insufficient time on
the 50% level of recommended vitamin A in the diet. In this study, hormone implants had
a strong effect on production and carcass traits, suggesting major benefits, but also some
drawbacks to their use. With premiums being possible for consistently well-marbled beef,
the negative effects of implants on fat traits should be considered.

4.2. Introduction

The competitive beef cattle industry is constantly looking for ways to produce more
valuable beef in an efficient manner. Marker-assisted management (MAM) is a strategy
used to achieve this. It is a process by which genotypic information of an animal is used
to facilitate management decisions in order to improve the end product. Prior nutrigenetic
studies have shown that a single nucleotide polymorphism (SNP) in the *alcohol
dehydrogenase 1C* (*ADH1C*) gene, *ADH1Cc.-64T>C,* when combined with limiting
dietary vitamin A (VA), significantly increases intramuscular fat (IMF) deposition in beef

steers (Ward *et al.*, 2012; Krone *et al.*, 2016). Separating *TT* animals at this SNP, and feeding them reduced dietary VA has been suggested as a possible MAM strategy to improve marbling for feedlots moving forward, however testing at a commercial level was still required.

Adipogenesis is one of the many bodily functions in which VA plays an important role. Ingested in a carotenoid form, VA is stored primarily in the liver. When mobilized from the liver, now retinol (ROL) binds to retinol binding proteins to circulate within the blood (Blomhoff, 1990; Quadro *et al.*, 2003). The enzyme ADH1C converts ROL to retinaldehyde (RAL) and this reaction can also function in the opposing direction. Another enzyme, RALDH, can then further oxidize RAL to retinoic acid (RA; Duester, 2000). Retinaldehyde is known to have a negative impact on fat deposition (Ziouzenkova *et al.*, 2007), while RA can bind to the retinoic acid receptor (RAR) and the retinoid X receptor (RXR), which then are able to dimerize and interact with retinoic acid response elements (RAREs) within genes involved in apdiogenesis to affect their expression (Heyman *et al.*, 1992; Zhang *et al.*, 1992; Ziouzenkova *et al.*, 2007). This altering of expression primarily encourages fat deposition, which leads to an opposing effect of RAL and RA on adipogenesis.

The SNP discovered in *ADH1C* by Ward *et al.* (2012) is thought to remove a CCAAT/enhancer binding protein α binding site when the *C* allele is present, which leads to decreased *ADH1C* expression in *CC* compared to *TT* animals. This decrease in expression of the *ADH1C* gene (Ward *et al.*, 2012) and the subsequent decrease in the amount of ADH1C protein present in the liver (Krone *et al.*, 2016) are thought to account for a decreased conversion of ROL to RAL. This decreased conversion would also

decrease the amount of RA produced that would normally have a positive impact on marbling.

A dietary inclusion level of 2200 IU/kg dry matter (DM) per day is recommended by the National Research Council (NRC, 1996) as the requirement for VA in feedlot rations. This level is often surpassed in feedlots, with no negative health effects; however, VA in excess impedes marbling as RAL decreases fat deposition (Kawada *et al.*, 2000; Ziouzenkova *et al*., 2007). Limiting dietary VA in order to improve marbling has been researched extensively, with mixed results. Significant improvements in fat traits were observed in some research where VA was limited (Oka *et al.*, 1998; Wang *et al.*, 2007; Kruk *et al.*, 2008; Gibb *et al.*, 2011; Pickworth *et al.*, 2012a), while others (Gorocica-Buenfil *et al.*, 2008; Bryant *et al.*, 2010) found no significant effects of altering the dietary level. Through grid marketing programs, consistently high marbling grades can lead to premiums being paid to producers (DiCostanzo and Dahlen, 2000), therefore the study of VA restriction leading to improved fat traits is highly relevant to today's beef market.

It is common practice in North America to use hormonal implants or other growth promotants in commercial beef production. While having a primary purpose of improving lean meat yield and decreasing finishing time in feedlots, hormone implants have been shown to significantly: increase average daily gain (ADG), dry matter intake (DMI), and rib-eye area (REA); and improve gain to feed ratios in feedlot steers (Foutz *et al.*, 1997; Wileman *et al.*, 2009). Foutz *et al.* (1997) also found that implants tended to be associated with decreased marbling scores, yield grades (however decreased yield grades signify improved yield with a score of 1 being optimal), and increased shear force values.

The objective of this study was to determine whether feeding an intermediate

level of VA to animals *TT* at the *ADH1Cc.-64T>C* SNP in a commercial feedlot setting

would improve marbling. We hypothesized that the impact of hormone implants would

negatively impact marbling, and that animals *CT* at *ADH1Cc.-64T>C* as well as animals

fed the NRC recommended level of VA would have lower marbling scores.

4.3. Material and methods

All animals were cared for according to the *Guide to the Care and Use of Experimental*

Animals (Canadian Council on Animal Care, 1993) guidelines.

Animals

Mixed breed beef steers (N = 2960) were purchased at auction (averaging 274 kg) and

transported to Cattleland Feedyards Limited (CFL). All animals were fed a commercially

equivalent step-up diet upon arrival, with the diet being stepped-up every 5-7 days. They

were held for a maximum of one month on a 73.11% barley (DM) diet until enough

animals were accrued and genotyped. Once sorted (averaging 340 kg), the cattle were

moved to an 86.80% barley finishing diet (Table 4.3.1.), where they were held until

slaughter. Supplementation of VA was consistent for all pens at 30 000 IU/head/day

before sorting as per typical feedlot protocol. The barley silage averaged 13 472 ± 1580

IU VA/kg DM. Based on an average dry matter intake of 8 kg/head/day, all pens received

8500 IU VA/head/day from the basal ration. Pens assigned the 100% NRC diet received

an additional 9290 IU/head of VA supplement mixed in the feed, which equaled the 2200

IU/kg DM recommended by NRC (1996). The 50% NRC pens received only the VA in

the basal diet, which equaled 1062.5 IU VA/kg DM (Table 4.3.1.). Representative silage samples were sent to DSM Nutritional Products Inc. (Ayr, ON) for β-carotene analysis using AOAC official method 974.29 and the DSM Nutritional Products Green Book (2004) as in Gibb *et al.* (2011). Beta-carotene was converted to VA using a conversion factor of 400 (NRC, 1970). Vitamin A supplement was adjusted accordingly. All pens received Rumensin® Premix with microtracer (Elanco, Guelph, ON) at 33.00 ppm, Tylan 100 premix (Elanco, Guelph, ON) at 11.00 ppm, and 454.00 mg/head BioPowerLA Micro cell (Lallemond Animal Nutrition, Montreal, QC), as well as 2870 IU/head vitamin D and 150 IU/head vitamin E. Animals were sent for slaughter once the average weight of each pen reached approximately 612 kg. Sick animals were pulled from pens and treated on site in the feedlot clinic on up to three occasions before being removed from the study. Numbers of animals that were removed from their pens for treatment, pulled from the study, railed, or died were totaled for each pen and were analyzed statistically as a proportion of animals per pen.

Table 4.3.1. *Diet composition and nutrient analysis of low and high vitamin A (VA) finishing rations on a dry-matter basis*

	Low VA	High VA
Diet composition, % DM		
Rolled barley	86.80	86.80
MICRO	0.66	0.66
CFY DFS C05430	2.01	2.01
Molasses	2.68	2.68
Barley silage	7.85	7.85
Nutrient analysis, % DM		
Total digestible nutrients	81.50	81.50
Crude protein	13.00	12.90
Acid detergent fiber	8.60	8.50
Neutral detergent fiber	15.40	16.10
Ca	0.53	0.60
P	0.42	0.41
Dietary VA (IU/kg DM)	1062.5	2223.9

VA = Vitamin A; DM = Dry Matter; IU = International Units

Genotyping and Sorting into Treatment Groups

Using the ear tissue tagging system and DNA extraction method at Quantum Genetix (Saskatoon, SK), DNA from each animal was obtained. A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) test for the *ADH1C* SNP (Ward *et al.*, 2012; Krone *et al.*, 2016) was then run, and 2000 animals were randomly selected and sorted at CFL into pens of 50 animals each, based on genotype (either *TT* or *CT*; Figure 4.3.1.). Half of the pens were implanted (IMP) with Component TE100 (Elanco,

Guelph, ON) at sorting, and again at 70 days after the first implant. Pens were assigned to

either 50% or 100% of NRC VA requirement, therefore there were 5 pens each of: *CT*

genotype, IMP, 50% VA; *CT*, IMP, 100%; *CT*, non-implanted (NI), 50%; *CT*, NI, 100%;

TT, IMP, 50%; *TT*, IMP, 100%; *TT*, NI, 50%; and *TT*, NI, 100%. Vitamin A supplement

was mixed in with the daily ration and hence pen was the statistical unit.

The 2000 cattle selected for the trial were also genotyped at the *MC1R* SNP

(Klungland *et al.*, 1995) to determine coat colour, as black or not black. Previous studies

researching this nutrigenetic interaction between the *ADH1C* genotype and VA level

involved only black animals while this trial included steers of any colour.
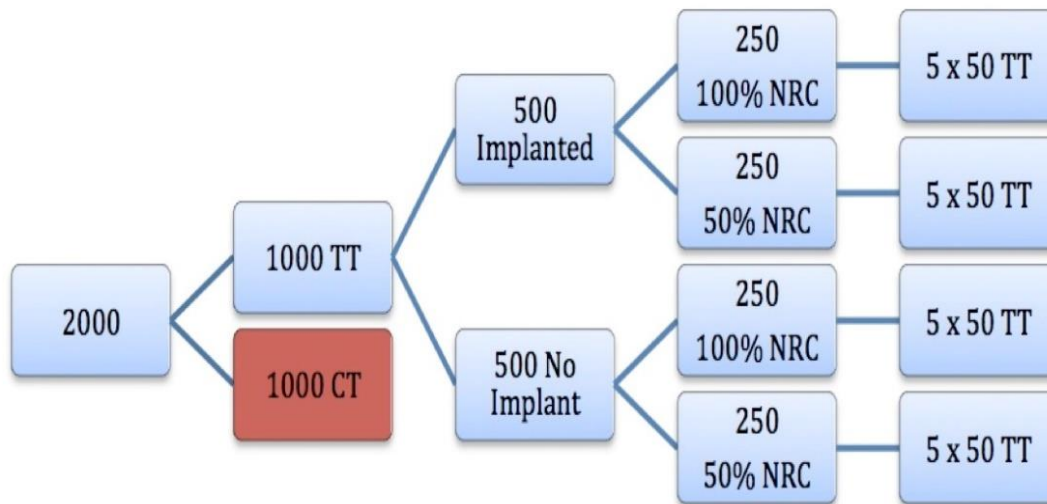
Figure 4.3.1. Diagram demonstrating how steers were sorted into different treatment pens based on *ADH1Cc.-64T>C* genotype. Implants and vitamin A treatments (percentage of NRC, 1996 recommendations) were then also applied accordingly. The red cell depicts the group of animals that would follow the same pattern as shown for the *TT* animals, but would have the *CT* genotype. The end result is 40 pens in total, made up of an equal number (n = 5) of every treatment combination.

Production Data

Steers were weighed at the time of sorting, at 70 days post-sorting when implanted animals were re-implanted, and at shipping. Average daily gain (ADG, kg per day) values were calculated as the difference in weights divided by the number of days between the two measurements. Dry matter intake (DMI) data was obtained from CFL, where the cattle were provided feed *ad libitum* intake once daily, and the amount of feed provided per pen was recorded daily. Bi-weekly ration samples were collected, and DMI values were confirmed by a nutritionist. Total days-on-feed (TOTDOF) is the number of days from start of trial until slaughter.

Carcass Data

Carcass data (USDA Yield Grade, VGUSYLD, scale from 1.0-5.9; Marbling Score, VGMARB, scale from 200-900; Rib-eye Area, REA, square centimeters; Fat Thickness, FAT, mm) was obtained for each animal from JBS Food Canada (Brooks, AB) where the animals were slaughtered. A VBG 2000 e+v Technology GmbH vision camera grading system was used to obtain the VGUSYLD, VGMARB, REA, and FAT data.

Statistical Analysis

The mixed model procedure of SAS 9.4 (SAS Version 9.4; SAS Institute, Inc., Cary, NC, USA) was used to analyze data for the two genotypes (*TT* or *CT*), by two VA treatments (50% or 100%), by two implant status treatments (IMS or NI; IMS) factorial design. The experimental unit in this study was the pen. 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 $0.05 > P \leq 0.10$.

4.4. Results and discussion

Animals

There were no significant differences between treatment of sick animals or mortality with *ADH1C* genotype, VA, or IMS (data not shown). There were also no interaction effects between these variables on animal health. As VA also impacts immune function, this measure was important to assess any negative effect that this MAM strategy could have had on animal health.

Genotyping

Genotyping at the *ADH1Cc.-64T>C* SNP was conducted on 2960 British crossbred beef steers to select 2000 animals for the feeding trial. The minor allele (*C*) frequency in this population was 0.26, compared to 0.3 in the Ward *et al.* (2012) study. Genotyping the entire population of steers only yielded 970 *CT* animals, therefore the *CT* pens had to be supplemented with 30 *CC* animals. These two genotypes do not differ significantly with respect to carcass or production data (Ward *et al.*, 2012; Krone *et al.*, 2016). The minor allele (*C*) frequency is 0.3 (Ward *et al.*, 2012), which made it difficult to select for a large number of *CC* animals. Since there is no significant difference between *CC* and *CT*, they were not included in the study as a separate genotype variable. The following number of animals finished the trial: 978 *TT* and 974 *CT,* including the 30 *CC*. Genotyping at the

MC1R SNP in the NI animals led to allele frequencies of $T = 0.80$ and $C = 0.20$, where 276 were black and 639 were not.

Production Data

The only significant interaction effect observed was that between *ADH1C* and IMS on ADG ($P = 0.03$; Figure 4.4.1.). In implanted steers, *CT* animals had a significantly increased ADG (3.92%) over *TT*, while both were higher (28.98% and 24.12% respectively) than NI animals. An increase in ADG would be of benefit to producers as it would mean they could finish their animals sooner, save on production costs, and reduce the risks of keeping animals in the feedlot longer than necessary. The increase observed between IMP and NI animals was expected, as the use of implants increases lean meat yield while decreasing the feeding period. The significant difference between *CT* and *TT* genotypes within IMP animals was unexpected, and may in fact be coincidental. The significant difference in ADG has not been previously reported (Ward *et al.*, 2012; Krone *et al.*, 2016), but prior nutrigenetic work on the *ADH1C* and VA interaction used only NI animals. In these earlier studies by Ward *et al.* (2012) and Krone *et al.* (2016), the leaner of the two genotypes had in fact been the *CT* animals, so it may be possible for *CT* animals, when implanted, to also have a higher ADG. More research would be necessary before much weight is placed on its applicable significance.
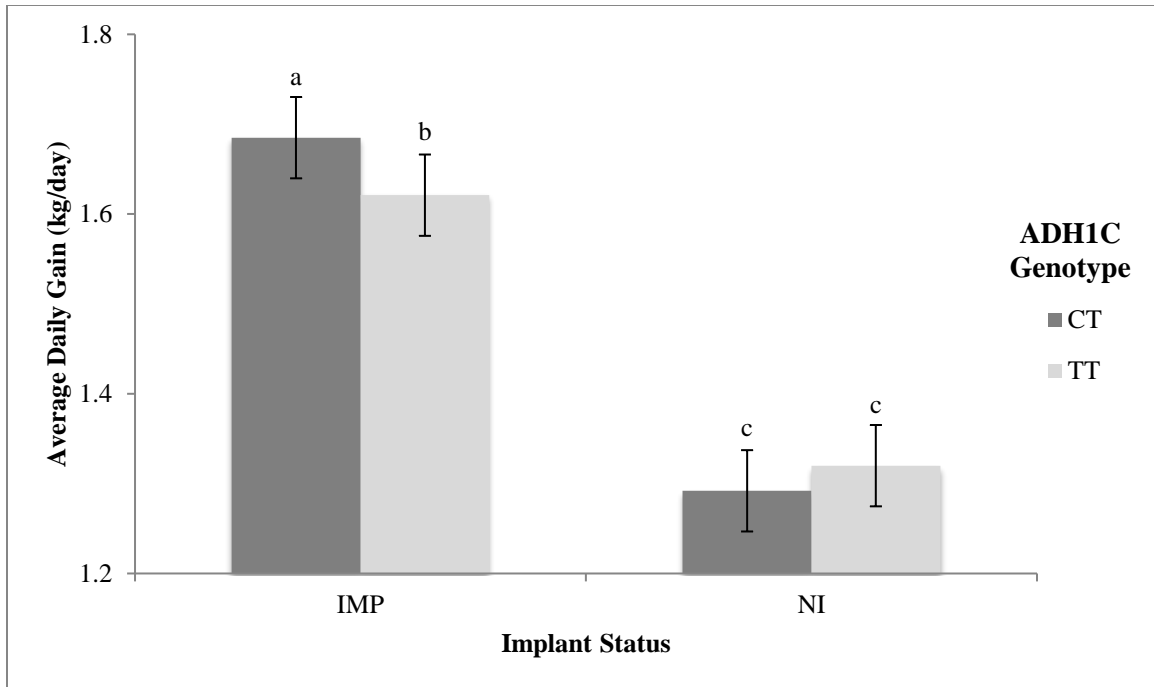
Figure 4.4.1. A column graph representing the significant interaction effect of *ADH1Cc.-64T>C* genotype and implant status (IMP = implanted; NI = non-implanted) on Average Daily Gain. Each column symbolizes the mean of 10 pens. Differing letters on the figures represent significantly different values. Significance is considered as $P \leq 0.05$.

Two production traits, namely DMI and TOTDOF, were significantly affected by IMS (Table 4.4.1.). Implanted animals had an 8.69% increase in DMI (Figure 4.4.2.a) and required 46 fewer days on feed compared to NI animals (Table 4.4.1**.**). While a lower DMI combined with equivalent or increased ADG would be beneficial at a production level, a slight increase in DMI for IMP animals that build lean meat yield quite quickly, would be expected. The decrease in TOTDOF for IMP animals is expected as they finish faster, which highlights again that the intended benefits to be drawn from implanting animals are in fact being achieved. It is beneficial at the feedlot level to send animals to slaughter as quickly as possible because management costs, stress, and disease challenge are all major economic and animal welfare factors.

Table 4.4.1. *Production and camera grading carcass trait means separated by implant status.*

| Variable | IMS mean | | SEM | P-value |
	IMP	NI		IMS
DMI	8.55	7.87	0.113	<0.01
TOTDOF	164.40	210.45	2.782	<0.01
VGUSYLD	2.40	2.77	0.046	<0.01
VGMARB	391.90	454.90	4.193	<0.01
REA	84.97	80.65	1.135	0.01
FAT	8.07	10.01	0.281	<0.01

IMS = Implant Status; IMP = Implanted; NI = Non-implanted; DMI = Dry matter intake (kg); TOTDOF = Total days-on-feed; VGUSYLD = Vision grade USDA yield grade; VGMARB = Vision grade marbling score; REA = Rib-eye area (sq cm); FAT = Fat thickness measurement (mm)

Carcass Data

Significant main effects of IMS were observed for VGUSYLD, VGMARB, REA, and FAT (Table 4.4.1.). Implanted animals had a 15.23% decrease in VGUSYLD scores ($P < 0.01$; Figure 4.4.2.b), where a lower score is preferable, and a 5.37% increase in REA ($P = 0.01$; Figure 4.4.2.d). Beef animals with VGUSYLD scores that are too high are often discounted; while animals with larger REA are often valued higher as it is an indicator of meat yield. It would be typical of IMP animals to have increased REA and decreased VGUSYLD scores as they are implanted with growth promotants, and therefore targeted specifically towards a lean meat yield market. There is a negative correlation between REA and yield scores, while yield score is positively correlated with fat thickness (Kauffman *et al.*, 1975).
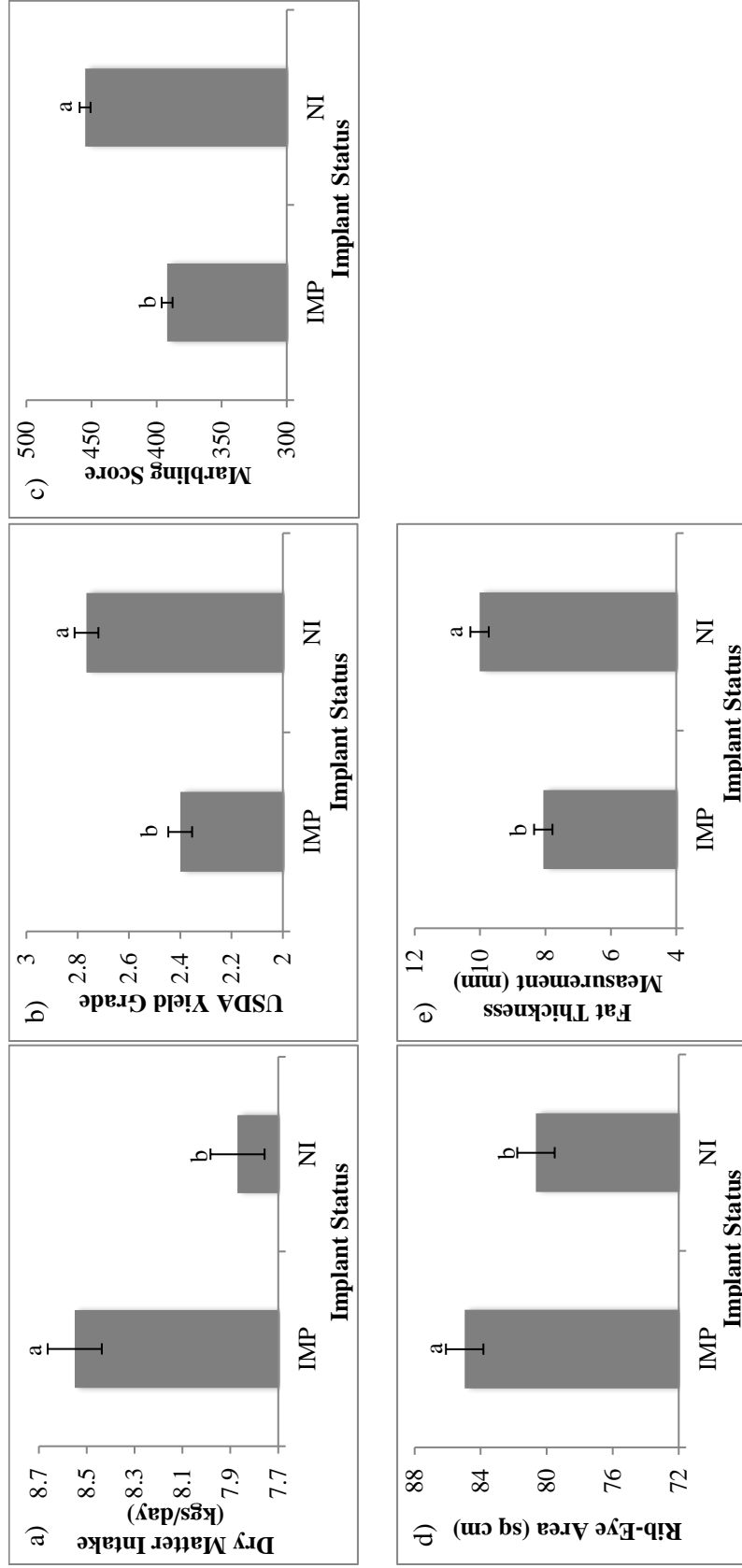
Figure 4.4.2. Column graphs representing the significant main effects of implant status (IMP = implanted; NI = non-implanted) on a) Dry matter intake, b) Vision Grade US yield grade, c) Vision Grade US yield grade, c) Vision Grade marbling score, d) Rib-eye area, and e) Average fat measurement. Each column symbolizes the mean of 20 pens. Letters on the figures represent differences in significance, where any shared letter above a column symbolizes columns that are not significantly different. Significance is considered as P ≤ 0.05.

Conversely, NI animals displayed significantly higher values for fat traits when compared to IMP steers (Table 4.4.1.). A 16.08% increase was observed for VGMARB scores (P < 0.01; Figure 4.4.2.c) and a 24.15% increase for FAT measurements (P < 0.01; Figure 4.4.2.e) of NI compared to IMP animals. Premiums are given to carcasses consistently high in marbling and of higher quality grades (DiCostanzo and Dahlen, 2000). When marketing on a marbling grid specifically, much higher profits can be achieved through consistently high IMF (Feuz, 1999). Marbling is beneficial for flavor and eating experience (Killinger *et al.*, 2004), but any increase in fat thickness is only acceptable up to a point, as too much backfat leads to increased yield grades, and discounted prices. The significant difference between IMP and NI animals for VGMARB scores is equivalent to an improvement from slight (Canada AA/USDA Select) to small (Canada AAA/USDA Choice) steak marbling grades, which would lead to increased prices for the NI steaks. There is however, a trade-off between marbling and yield, and therefore it makes sense for NI animals to be better marbled, while IMP animals are higher yielding. A reduction in total fatty acid percentage has been observed in IMP animals, although this finding seems to be due to a dilution of IMF as REA increases (Duckett *et al.*, 1999).

Table 4.4.2. *Effect of vitamin A treatment, ADH1Cc.-64T>C genotype, implant status, and their interactions on production and camera grading carcass traits. Bolded P-values indicate significance while italics indicate a trend. Significance is declared at P < 0.05, trends at P < 0.10.*

Variable	DMI (kg/day)	TOT -DOF	ADG (kg/day)	VGUS -YLD	VG -MARB	REA (sq cm)	FAT (mm)
ADH1C*IMS*VA	0.12	0.51	0.38	0.69	0.60	0.70	0.51
ADH1C*IMS	0.54	0.60	**0.03**	0.51	0.68	0.97	0.37
ADH1C*VA	0.54	0.62	0.64	0.88	0.60	0.85	0.96
IMS*VA	0.48	0.41	*0.06*	0.90	0.31	0.55	0.45
ADH1C	0.56	0.98	0.39	0.36	0.77	0.39	0.55
IMS	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.01**	**<0.01**
VA	0.26	0.80	0.40	0.92	0.84	0.94	0.86

DMI = Dry Matter Intake; TOTDOF = Total Days-On-Feed; ADG = Average Daily Gain; VGUSYLD = USDA Yield Grade; VGMARB = Marbling Score; REA = Rib-Eye Area; FAT = Fat thickness measurement; ADH1C= *ADH1Cc.-64T>C* genotype; IMS= Implant Status; VA= Vitamin A level

Effectiveness of VA X ADH1C MAM Strategy

In this nutrigenomic feedlot trial, we have primarily found that there was no *ADH1C* x VA treatment interaction effect on fat traits as previously shown by Ward *et al.* (2012) and Krone *et al.* (2016). No main effects of either *ADH1C* genotype or VA level were observed either (Table 4.4.2.). As dietary VA level has been shown to affect marbling in multiple studies (Oka *et al.*, 1998; Kruk *et al.*, 2008; Pickworth *et al.*, 2012a), we suggest that the low level of VA was either not low enough or potentially not sustained for a long enough period of time to show an effect on carcass traits. Other studies, with shorter periods of decreased VA diets (Gorocica-Buenfil *et al.*, 2008; Bryant *et al.*, 2010), have also shown no effect on carcass traits, however those with longer periods of feeding low VA in the diets (Pickworth *et al.*, 2012b) have shown significant effects. Specifically, Kruk *et al.* (2008) found that feeding unsupplemented VA rations to Angus steers for 10 months led to significant increases in ether extracted IMF, and a trend for increased

marbling scores. They suggested that if the reduced VA diets had been fed for longer, they may have achieved greater differences in marbling scores. Ward *et al.* (2012) did achieve significant differences between unsupplemented and supplemented animals for marbling scores and IMF measurements after feeding animals for just 8 months, however the first 3 months of backgrounding were on a VA deficient diet in order to deplete liver VA stores. This was a proof-of-concept approach, which is too drastic, and not feasible for implementation at the commercial level. The animals in our study were on low or high VA treatments for approximately 5.5 months for IMP and 7 months for NI. It may be likely then, that VA stores within the 50% NRC animals did not reach low enough levels to see a significant difference in carcass traits.

The delivery method of VA, along with dosage, may be another factor affecting the lack of VA effects in this trial. Both Ward *et al.* (2012) and Krone *et al.* (2016) fed a monthly dose of VA via an oral bolus, while the animals in this study were supplemented with VA through its addition to their daily rations in their feed bunks. As we could not be certain exactly how much VA each animal ingested each day, the pen of animals (to which the treatment was applied) was analyzed as the experimental unit. This daily dose in the feed as opposed to a monthly oral bolus however, may have altered the effect of limiting the animal's dietary VA.

Stress or the epigenetic effects of stressful conditions may also have played a role in how much of an effect dietary VA could have on cattle carcass traits. Curley (2004) found that more excitable temperaments in cattle led to significantly higher responsiveness to stress and higher basal glucocorticoid concentrations. Increased excitability has been significantly associated with higher serum cortisol concentrations as

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well as reduced tenderness in beef cattle (King *et al.*, 2006). Kruk *et al.* (2008) observed a drastic drop in plasma retinol in both treatment groups of animals during the final 12-day period of the trial. During these 12 days, the animals finished their time at the feedlot, were shipped 1000 km to an abattoir, and processed. The steer populations in the Ward *et al.* (2012) and Krone *et al.* (2016) studies were both transported over 750 km to a processing facility, while the animals in the current study only had to travel approximately 130 km before slaughter. The two prior *ADH1C* x VA nutrigenetic studies also included frequent handling of the animals, where they were brought through the chute at least once per month for weighing, bolusing, or sampling. The animals in this study were allocated to treatment groups, weighed once about halfway through the trial, and were otherwise not removed from their pens unless being treated for injury or disease. These differences in handling and transport may have allowed for the Ward *et al.* (2012) and Krone *et al.* (2016) steers to be more prone to VA effects on carcass traits.

Prior *ADH1C* x VA nutrigenetic studies only involved steers that were black in colour. This is because animals that are black in colour have at least one E^D allele at *MC1R*, which is associated with significant increases in backfat and average fat when compared to red animals (McLean and Schmutz, 2009). No restrictions on breed or colour were placed on the animals in the current study, and therefore we considered that the *ADH1C* x VA interaction effect may only be applicable to black, or primarily Black Angus cattle. As the animals of the prior studies were also NI, the current NI steers were genotyped and analyses incorporating *MC1R* genotype were run (data not shown). Black, NI steers did not show the expected nutrigenetic interaction effect, so this possible theory can also likely be ruled out. The effect of VA restriction on marbling and IMF has been

demonstrated in Japanese Black (Oka *et al.*, 1998) and Angus (Kruk *et al.*, 2008) or Angus crossbred (Pickworth *et al.*, 2012a; Pickworth *et al.*, 2012b) cattle. As the animals in this study were mixed breed beef cattle, breed effect may have played a role in the absence of a significant effect of VA restriction.

The steers in the current study were allocated to either be NI or IMP, while the previous nutrigenetic studies similar to this one used only NI animals. This could be a confounding factor, but further analyses (data not shown) where IMP and NI pens were separated did not show the expected interaction effect either. Additional main effects aside from IMS were also not significant in the results of our original statistical model, which would account for this, so this is also not a plausible explanation for our unexpected results.

4.5. Conclusions

The validation of the *ADH1C* x VA nutrigenetic approach in the commercial feedlot sector was the overall goal initially however; this study suggests it may not be a plausible MAM strategy at this stage. The lack of an *ADH1C* main effect was expected as none were observed by either Ward *et al.* (2012) or Krone *et al.* (2016), and Ward *et al.* (2012) concluded that limiting VA was required to also have a significant effect of the *ADH1C* genotype. The lack of significance of VA as a main effect was unexpected, however the limited length of time on decreased dietary VA may have confounded our results. Significant main effects of IMS on carcass traits VGUSYLD, VGMARB, REA, and FAT were expected, as were the effects on production traits ADG and TOTDOF due to the fact that the overall effect of hormonal implants is the building of lean meat reasonably

quickly. The effect of IMS on DMI is not as commonly reported in the literature, however it would make physiological sense that IMP animals would eat slightly more than NI animals in order to quickly build this lean yield.

5. ANALYSIS OF IMMUNE FUNCTION IN FINISHING BEEF STEERS FED VARYING LEVELS OF VITAMIN A IN COMBINATION WITH DIFFERING ADH1C GENOTYPES

5.1. Abstract

A marker-assisted management strategy has been proposed that involves the *ADH1Cc.-64T>C* single nucleotide polymorphism (SNP) combined with limiting dietary vitamin A (VA) in feedlot cattle to improve intramuscular fat and marbling traits. The degree to which dietary VA can be limited is not well understood and deficiency could lead to production and animal health consequences. Both of these consequences would result in losses for the producer. Of interest in feedlot animals are the consequences that limiting VA may have on immune function, as these animals may endure significant times of stress and disease challenge. Eighteen crossbred steers were sampled, with the *ADH1C* genotype (*TT*, *CT*, and *CC*) and VA level (25% or 75% of NRC, 1996 recommendations) equally represented. A complete blood cell count analysis, peripheral blood mononuclear cell proliferation and stimulation assay, and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis of cytokine expression was completed for each sample. The level of VA did not significantly affect any of the analyzed measures indicating immune responsiveness. A significant pen effect was observed for fibrinogen concentration in whole blood, however the values for this measure did not fall outside of the reference range. A significant genotype effect was observed on *IL-4* expression, where *CC* animals had significantly lower expression levels than *CT* and *TT* animals. As *CC* animals did not have any other observable signs of VA deficiency or impaired immunity, we assume that this finding may speak more to the relationship between retinoids (and their effector enzymes – ADH1C) and lymphocytes than to deficiency

concerns. Previous research has suggested that a severe level of VA limitation would be required to diminish immune function and our research supports this.

Implications

A mutation in the *ADH1C* gene has been shown to improve fat traits when vitamin A is limited in beef cattle. Restricting vitamin A could lead to impairment of immune responsiveness due to deficiency, so the immune effects of this management strategy needed to be tested. No significant differences were found between diets of 25% and 75% of the vitamin A recommendations. Two significant effects were observed, however they were either not biologically significant, or did not coincide with other deficiency markers. Feeding 25% of the recommended level of vitamin A did not result in immunological deficiencies in this study.

5.2. Introduction

Cattle in North American feedlots today often receive more than the required 2200 IU/kg dry matter (NRC, 1996) of vitamin A (VA). It is suggested that higher dietary levels of VA have the potential to impede fat deposition (Kawada *et al.*, 1996; Ohyama *et al.*, 1998; Kawada *et al.*, 2000; Ziouzenkova *et al.*, 2007). For beef cattle, a decrease in fat deposition or marbling potential could result in reduced profits for producers. Limiting dietary VA has therefore become a strategy attempted in research studies with results being: significantly increased intramuscular fat (IMF), a negative correlation between serum retinol (ROL) and marbling scores, and numerically increased marbling scores (Kruk *et al.*, 2008); and significantly increased quality grades and a trend towards increased IMF (Pickworth *et al.*, 2012) in Angus and Angus crossbred steers respectively.

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Wagyu cattle have shown an increase in marbling with decreased VA intake (Oka *et al.*, 1998) as well.

Nutrigenetic studies looking at a thymine to cytosine substitution in the promoter region of the *alcohol dehydrogenase 1C* gene (*ADH1C*; Ward *et al.*, 2012; Krone *et al.*, 2016) found that crossbred beef steers *TT* at the *ADH1C c.-64T>C* single nucleotide polymorphism (SNP) had significantly increased IMF when dietary VA was reduced, when compared to other genotypes and other levels of VA. This *ADH1C c.-64T>C* SNP removes a binding site for the transcription factor CCAAT/enhancer-binding protein α, and therefore reduces the expression of the ADH1C protein (Ward *et al.*, 2012). The ADH1C protein is responsible for the oxidization of ROL to retinaldehyde (RAL), which can be oxidized by RALDH to retinoic acid (RA; Duester, 2000). The activity of the ADH enzyme is known to increase threefold when VA is deficient (Napoli, 1996), likely depleting RAL if it is limiting. Retinaldehyde is known to inhibit fat deposition (Ziouzenkova *et al.*, 2007), and RA is thought to stimulate fat deposition through its binding to retinoic acid response elements in the promoter regions of genes (Heyman *et al.*, 1992; Gregoire et al., 1998). Having a *TT* genotype at *ADH1C* (normal levels of the circulating ADH1C protein) would allow for enough ROL to be converted to RAL for ADH to then convert it to RA. This conversion would then improve IMF deposition both from depleting the available RAL, and increasing levels of RA (Ward *et al.*, 2012). At a feedlot level, increases in IMF, and consistent improvements in marbling scores could mean increased profits for producers.

Increasing profits by limiting dietary VA in feedlot cattle, depending on genotype, could be an effective marker-assisted management (MAM) strategy. Vitamin A also

plays a significant role in the immune system, which is a system of critical importance for these animals during times of repeated stress and disease challenge. Sending feedlot animals into VA deficiency could be disastrous. Inefficiencies in production traits, weakness, and reproductive issues tend to be the earlier symptoms of the deficiency, while ataxia, blindness, xerophthalmia, convulsions, and syncope may occur at later stages (Hill *et al.*, 2009). As VA deficiency decreases the efficacy of immune function, and disease can reduce VA uptake and absorption, as well as increasing its excretion (Stephensen, 2001), this cyclic relationship could greatly harm feedlot animal welfare.

While improving profits for producers through a MAM strategy using the *ADH1Cc.-64T>C* SNP and limiting dietary VA is an economically beneficial endeavor, the wellbeing of the animals must be proven before this technology could be implemented at a commercial level. Should VA deficiency occur in a commercial feedlot, increased disease and mortality in feedlot animals could reduce profits substantially. In this study, we aimed to determine any adverse immunological effects that may arise in feedlot cattle, *TT*, *CT*, or *CC* at the *ADH1C c.-64T>C* SNP, fed 25 or 75% of the NRC (1996) recommended level of VA. We used clinical indicators for immune responsiveness from complete blood cell counts, peripheral blood mononuclear cell (PBMC) stimulation indexes (STIM), and real-time polymerase chain reaction (RT-PCR) results for the expression of important cytokines (*IL-2*, *IL-4*, *IL-5*, *IL-10*, and *IFN-γ*) in order to determine possible immune function deficiencies. We hypothesized that animals with reduced VA supplementation up to 25% of NRC (1996) recommendation would not show any form of diagnosable decreased immune function or symptoms of VA deficiency.

5.3. Material and methods

All animals were cared for according to the *Guide to the Care and Use of Experimental Animals* (Canadian Council on Animal Care, 1993) guidelines. All experimental work involving animals was approved by the University of Saskatchewan Animal Care Committee.

Animals

Three animals of each *ADH1C* genotype (TT, CT, and CC) and VA level (25% and 75% NRC) were randomly selected, across genotype and VA level, from two intensive pens from a trial that occurred in Fall 2012-Summer 2013 (Krone *et al.*, 2016) at the University of Saskatchewan's Beef Cattle Research Facility. Initially, 450 black crossbred beef steers were genotyped at the *ADH1C* SNP locus through the use of a tail hair sample, DNA extraction procedure, and a PCR-RFLP test (see Krone *et al.*, 2016). These 450 animals were then randomly selected from to reach a study population consisting of 45, 45, and 27 animals of TT, CT, and CC genotypes respectively, that were fed either 25%, 50%, or 75% of NRC recommendations for VA. These VA levels were achieved using a monthly oral gelatin bolus (Torpac Inc., Fairfield, NJ, USA) filled with sugar and the appropriate monthly dose of VA in the form of retinyl palmitate (1,000,000 IU/g, Adisseo, Antony, France). Daily fed rations were unsupplemented for VA, and their naturally occurring level of VA was used to calculate the appropriate inclusion level of VA in the monthly boluses (as in Krone *et al.*, 2016). All treatment information and disease events were recorded over the duration of the study.

Blood Cell Counts

Blood samples (24 ml) were drawn from the subset of 18 animals from the jugular vein into vacutainers containing EDTA as an anticoagulant. These samples were taken two weeks before slaughter, and a 6 ml portion of the blood drawn from each animal was sent to Prairie Diagnostic Services (Saskatoon, SK) for complete blood cell count analysis. Of particular interest were the white blood cell (WBC; x 10^9/L), segmented cell (SEGS; x 10^9/L), banded cell (BANDS; x 10^9/L), red blood cell (RBC; x 10^{12}/L), total protein (TOTPROT; g/L), and fibrinogen (FIB; g/L) counts.

Peripheral Blood Mononuclear Cell Isolation

Another 18 ml portion of the blood sample from each animal was used for PBMC purification. Samples were separated by centrifugation at 2500 rpm (1350 g) for 20 minutes at room temperature; buffy coats were then removed and suspended in 8 ml of PBSA containing 2.7 mM EDTA (PBSA/EDTA). Solutions were each transferred to Ficoll gradients (GE Healthcare, Uppsala, Sweden) and centrifuged at 3000 rpm (1940 g) for 20 minutes at room temperature. Peripheral blood mononuclear cells were removed from the interface between Ficoll and PBSA/EDTA, suspended in 8 ml of the PBSA/EDTA solution, and centrifuged at 1200 rpm (311 g) for 10 minutes at 4°C. Supernatant was removed, cells were resuspended in 10 ml PBSA/EDTA solution, and the centrifugation at 1200 rpm as well as the resuspension steps were repeated once more. The suspension was centrifuged one last time at 1000 rpm for 10 minutes at 4°C, and supernatant was removed. The PBMCs were resuspended in 4 ml Minimum Essential Media (MEM, Invitrogen Canada Inc., Burlington, ON) which contained 10% fetal

bovine serum, 50 μM 2-mercaptoethanol, 1% sodium pyruvate, 1% non-essential amino acids and 1% HEPES, with 50 μg/ml gentamycin added (MEM-plus). A Coulter counter was used to determine cell counts.

PBMC Stimulation and Proliferation Assay

A concentration of 3.5×10^6 cells/ml PBMC suspension was achieved by resuspending purified PBMCs in MEM-plus. A 100 μl volume of this suspension was added in duplicate to the wells of a 96 well tissue culture plate (Corning Inc., Corning, NY, USA). Cells were incubated at 37°C in 5% CO_2 for 72 hours where one sample from each duplicate pair was incubated in the presence of 1 μg/ml concanavalin A (ConA; Sigma-Aldrich, Oakville, Ontario, Canada). Twenty microliters of a 0.4 μCi/ml solution of tritiated thymidine (American Radiolabeled Chemicals, St. Louis, MO) was added to each well and plates were then incubated for an additional 18 hours. The incorporation of tritiated thymidine into cultured PBMC cells was determined using a liquid scintillation counter (TopCount NXT, Perkin Elmer Inc., Waltham, MA, USA). A stimulation index was calculated by dividing stimulated cell counts by those from unstimulated controls.

Peripheral Blood Mononuclear Cell qRT-PCR

As above, 100μl of a 3.5×10^6 cells/ml PBMC suspension was allocated to 96 well tissue culture plates in duplicate and one well of each duplicate pair was stimulated with ConA but were separated for use for RNA isolation for qRT-PCR prior to the addition of tritiated thymidine. Extraction of RNA was achieved by using Trizol® reagent (Invitrogen Canada Inc., Burlington, ON) as described previously (Simms *et al.*, 1993).

Extracted RNA samples were resuspended in 40 µl RNase-free water and quantified using a NanoDrop Lite Spectrophotometer (Thermo Scientific, Burlington, ON).

Each isolated RNA sample had its concentration adjusted to 50 ng/µl and samples were prepared for RT-PCR using DNaseI (Invitrogen Canada Inc., Burlington, ON) as recommended by the manufacturer. Reverse-transcription PCR with 50 µM random hexamer primers and SuperScript™ II reverse transcriptase (Invitrogen Canada Inc., Burlington, ON) was used to complete reverse transcription. The PCR reaction was carried out using a T100™ Thermal Cycler (Bio-Rad Laboratories, Ltd., Mississauga, ON) according to SuperScript™ manufacturer recommendations with samples held at $25^{\circ}C$ for 10 minutes, $42^{\circ}C$ for 50 minutes, and $70^{\circ}C$ for 15 minutes.

Quantitative real-time PCR was performed using SYBR™ Green (Invitrogen Canada Inc., Burlington, ON) on a Stratagene Mx3005P qPCR system (Agilent Genomics, Santa Clara, CA, USA) according to the manufacturer's protocol. The qPCR cycles were as follows: 2 minutes at $95^{\circ}C$; 2 minutes at $95^{\circ}C$, 30 seconds at $60^{\circ}C$ (1 endpoint to take fluorescence reading), and 30 seconds at $76^{\circ}C$ with this cycle run 45 times; 30 seconds at $95^{\circ}C$; 30 seconds at $45^{\circ}C$; 30 seconds at $95^{\circ}C$; and a hold at $25^{\circ}C$ before storing at $-20^{\circ}C$. This cycle was followed in accordance with previous research featuring these primer sets (Table 5.3.1.), with the exception of an additional plateau at $81^{\circ}C$ (with 1 endpoint to take fluorescence reading instead of at $60^{\circ}C$) after each $76^{\circ}C$ step. This was to remove any fluorescence read from the primer dimer dissociation curve peak observed with the *IL-4* primer set. The qRT-PCR analysis was performed in duplicate and included the housekeeping gene *β-actin*. Specific primers for sense and anti-sense strands of *β-actin*, *IL-2*, *IL-4*, *IL-5*, *IL-10*, and *IFN-γ* genes were designed based on previous

work (Table 5.3.1.). Quantitative RT-PCR products were run on a 1% agarose gel,

visualized, sequenced, and compared to reference sequences to ensure that the

appropriate product was generated. Relative gene expression was calculated by

normalizing cytokine expression to *β-actin* expression and comparison with untreated

controls using the comparative threshold cycle ($\Delta\Delta C_t$) method. Relative expression data

was analyzed using the $2^{-(\Delta\Delta Ct)}$ method as described previously (Livak and Schmittgen,

2001).

Table 5.3.1. *Primers used to amplify cytokine and housekeeping gene mRNA transcripts. Primer sequence, product length, and reference number (or source) are reported.*

Gene	Primer direction	Primer sequence	Product length (bp)	NCBI accession number/ reference
B-actin	Forward	5'-AGGCATCCTGACCCTCAAGTA-3'	95	AY141970
	Reverse	5'-GCTCGTTGTAGAAGGTGTGGT-3'		
IL-2	Forward	5'-CCTCAACTCCTGCCACAATGTA-3'	376	*
	Reverse	5'-GTTTGCAACGAGTGCAAGAGTTA-3'		
IL-4	Forward	5'-ACGCTGAACATCCTCACAAC-3'	125	**
	Reverse	5'-CGCCTAAGCTCAATTCCAAC-3'		
IL-5	Forward	5'-TGGTGGCAGAGACCTTGACA-3'	320	*
	Reverse	5'-GAATCATCAAGTTCCCATCACCTA-3'		
IL-10	Forward	5'-GATGCCACAGGCTGAGAACC-3'	53	U11421
	Reverse	5'-GCGAGTTCACGTGCTCCTTG-3'		
IFN-γ	Forward	5'-TCCAGCGCAAAGCCATCAATGAAC-3'	105	***
	Reverse	5'-TCCGGCCTCGAAAGAGATTCTGAC-3'		

*From Coussens et al. (2004)
**From Whale et al. (2006)
*** From Luby (2010)

Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics Package version 23 (SPSS, Chicago, IL). Histograms and data screening was performed using the Explore feature, and data was notably skewed. Group comparisons for VA level (25% vs. 75%) and PEN (pen 9 vs. pen 10) were presented graphically as box plots, and differences between groups were tested with the non-parametric Mann-Whitney U test. Group comparisons for *ADH1C* genotype (*TT* vs. *CT* vs. *CC*) were presented graphically as box plots, and their differences were tested with the non-parametric Kruskal-Wallis test. Results were considered statistically significant at $P \leq 0.05$.

5.4. Results and discussion

Animals

Only five animals out of the total 117 were given antibiotics to treat disease events during the Krone *et al.* (2016) trial. Fat and liver biopsies had been conducted on this population and two animals were treated within days of receiving the first liver biopsy, and two animals were treated within days of receiving their second liver biopsy. One animal was treated after both liver biopsies. The need for antibiotics may have been as a result of infection from these incisions. There was no pattern in the five treated animals with respect to VA supplementation level, or genotype. Regardless, the low number of animals treated does not allow us to draw conclusions of causation of low dietary VA level or *ADH1C* genotype, but does suggest that nearly all of the animals had enough immune responsiveness to not require any treatment during the trial.

Blood Cell Counts

During a period of infection or inflammation, an increase in WBC, SEGS, BANDS, TOTPROT, and FIB counts would all be expected, while the RBC count may decrease in times of malnutrition. In VA deficient animals, natural killer cells have been shown to decrease in number (Semba, 1998; Stephensen, 2001). Cell counts for WBC, SEGS, BANDS, RBC, TOTPROT, and FIB were obtained and the band neutrophil (BANDS) counts have been excluded from further discussion as only two animals had scores that were not 0, where both scored just above the reference interval, and there were no significant statistical findings. The WBC reference interval reported (provided by PDS, Saskatoon, SK) at the time of testing was 4.51-12.1 (x 10^9 cells/L), and only one test animal fell out of that range at 13.4 (*TT*, 25% VA, Pen 10). As reference intervals constitute the 95% confidence range for animals that are examined as "normal" (ie. not ill), it is likely safe to say that the PEN effect on the WBC results (Table 5.4.1.) may be a statistical trend, but is not biologically significant, as all but one animal would have clinically been considered "normal" upon examination. The statistical significance of fibrinogen with a PEN effect (Table 5.4.1.; Figure 5.4.1.a) is also not biologically relevant, as the reference interval for fibrinogen is 1-7 g/L (personal communications with PDS, Saskatoon, SK), and our values ranged from 1-5. There may have been a slight immune challenge going through Pen 10 at the time of testing, however while these numbers may be increased, they are still within the reference interval, and therefore we consider them to be normal findings, not an indication of sickness. Five animals did fall outside of the confidence range for WBC, SEGS, BANDS, and RBC (data not shown), however only one animal (*CC*, 25% VA, Pen 10) scored outside of the confidence range for more than one measure (SEGS and BANDS). There was no consistent pattern

between genotype, VA level, or pen of these five animals, so it is believed that biologically, these are also insignificant findings.

PBMC Isolation and Stimulation

Peripheral blood mononuclear cells include all white blood cell types. During VA deficiency, there is a reduction of proper functioning of neutrophils, macrophages, and natural killer cells (Semba, 1998; Stephensen, 2001). No significant effects were observed for STIM values in our study population (Table 5.4.1.). As the STIM is calculated by dividing the number of stimulated cells by those that were unstimulated, the lack of significant results in this study would suggest that neither *ADH1C* genotype, VA level, nor PEN had an effect on the number of cells proliferating or their ability to respond to the mitogen stimulus. If immune responses had been compromised by any of these variables, we would have expected a hindrance in the ability of PBMC cells to proliferate when stimulated.

Table 5.4.1. *Effect of ADH1Cc.-64T>C genotype, pen allocation, and vitamin A treatment on markers of immune function. Bolded P-values indicate significance while italics indicate a trend. Significance is declared at P ≤ 0.05, trends at P < 0.10.*

Variable	WBC	SEGS	RBC	TOT-PROT	FIB	STIM	IL-2	IL-4	IL-5	IL-10	IFN-γ
ADH1C	0.61	0.48	0.86	0.19	0.39	0.12	0.22	**0.05**	0.57	0.39	0.63
PEN	*0.08*	0.12	0.52	0.24	**<0.01**	0.76	0.67	0.17	0.17	0.26	0.79
VA	0.67	0.67	0.86	0.49	0.22	0.55	0.96	0.87	0.67	0.38	0.65

WBC = White blood cell count; SEGS = Segmented cell count; RBC = Red blood cell count; TOTPROT = Total protein count; FIB = Fibrinogen count; STIM = Stimulation index value; IL-2 = Interleukin 2; IL-4 = Interleukin 4; IL-5 = Interleukin 5; IL-10 = Interleukin 10; IFN-γ = Interferon gamma; ADH1C = *ADH1Cc.-64T>C* genotype; PEN = Pen allocation; VA = Vitamin A level

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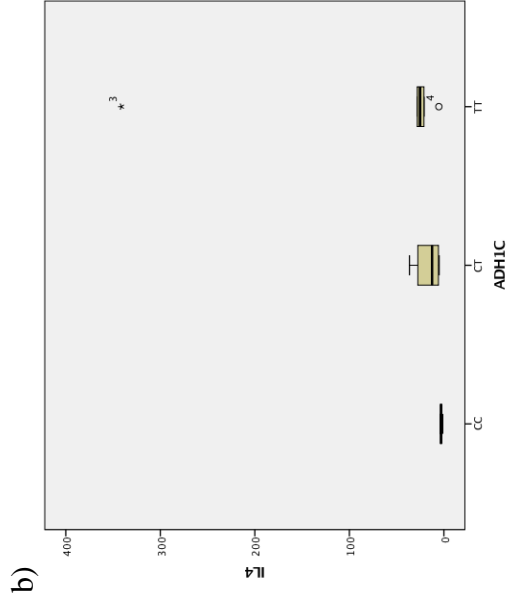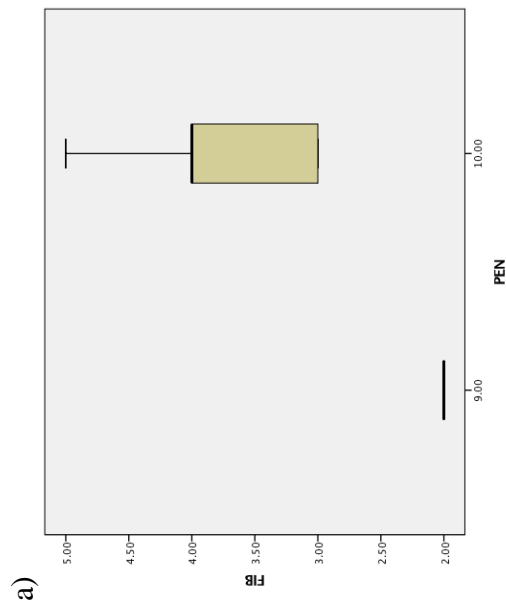
Figure 5.4.1. Box plot graphs representing the significant main effects of a) pen allocation on fibrinogen count, and b) *ADH1Cc.-64T>C* genotype on *IL-4* gene expression in fold change. In a), n = 8 for pen 9 and n = 10 for pen 10, where pen 10 values were significantly higher than those of pen 9. In b), n = 5, 4, and 6 for *CC*, *CT*, and *TT* genotypes respectively. Values for CT and TT animals were significantly higher than CC animals, but not significantly different from one another. Significance is considered as P ≤ 0.05.

PBMC Proliferation and qRT-PCR

A lack of VA appears to greatly affect the antibody-mediated immune response, with impaired function of T helper 2 cells, and to a lesser extent has an effect on the lysing ability of cytotoxic T cells of the cell-mediated immune response (Semba, 1998; Stephensen, 2001). A shift from a T helper 2 to a T helper 1 response has been hypothesized (Semba, 1998), which would lead to a decrease in IL-4, IL-5, and IL-10 and an increase in IFN-γ and IL-12 cytokines. The cytokines IL-2 and IL-10 are also thought to play essential roles in the production of $CD4^+CD25^+$ regulatory T cells (Malek et al., 2002; Zheng et al., 2004), which may be important for T cell maturation and differentiation. Expression of the cytokines *IL-2*, *IL-5*, *IL-10*, and *IFN-γ* were not significantly affected by *ADH1C* genotype, VA level, or PEN. A significant effect of *ADH1C* genotype was observed on *IL-4* expression (Table 5.4.1.; Figure 5.4.1.b), where *CT* and *TT* animals had significantly higher expression than *CC* animals. Because IL-4 is a Th2-associated cytokine and retinoids are likely important in the functioning of all lymphocytes, we can hypothesize that a reduced level of ADH1C protein present may be correlated to a decrease in *IL-4* expression, potentially due to the availability of RAL converted from ROL. Overall, the *CC* animals generally did not display clinical signs of being unwell compared to any other group, and therefore it is unlikely this is a problematic effect of genotype on expression.

5.5. Conclusions

We were concerned that feeding VA at a level of half of NRC (1996) recommendations or below could negatively affect immune function, and therefore cause the potential

ADH1C x VA MAM strategy to be detrimental to animal welfare. In previous studies

however, no difference has been observed in responses to vaccinations in calves fed 3300

IU VA/kg DM versus 1100 IU VA/kg DM per day (Jee *et al.*, 2013), or supplemented at

0 IU VA/kg DM versus 2200 IU VA/kg DM per day (Gorocica-Buenfil *et al.*, 2008). This

would suggest that a very severe level of VA limitation would likely be required to

induce deficiency to the point of clinical deficits in immune responses occurring. We

observed no effect of VA on any trait that could suggest VA deficiency or any immune

deficit complications. From our research, it seems that the *ADH1C* x VA MAM strategy

at 25% of NRC (1996) recommendations or above would likely not affect immune

function in a detrimental manner. The significant PEN effect seen in the FIB counts, and

the trend of PEN on WBC counts were, as mentioned above, not clinically significant.

The significant *ADH1C* effect on *IL-4* expression was interesting, as it may suggest a link

between the presence of the ADH1C protein and *IL-4* expression, however it also did not

equate to *CC* animals being less immunologically sound. Caution must also be taken in

interpreting these results, as the dosage of VA was orally administered monthly, and may

not be at the same level as what would be found in rations in the feed bunk. Overall, it

seems as though reducing the dietary VA in feedlot cattle to 25% of NRC (1996)

recommendations would not negatively affect immune responsiveness, however larger

scale feedlot studies should be conducted to ensure this is true at a commercial level.

6. DISCUSSION

Three main conclusions can be drawn from the two experiments of this study: there was a lack of support for a previously discovered *ADH1C* x VA MAM strategy; findings were in agreement with prior knowledge on the effects of hormone implants; and there were no significant results suggesting that limiting VA to as low as 25% of NRC (1996) recommendations would harm immune function in beef cattle.

In the trial analyzing markers of immune function, there was no significant effect of VA on any of the parameters measured. Significant pen effects were not clinically abnormal, and therefore were no cause for concern. The *ADH1C* genotype effect on *IL-4* expression, where *CC* animals had significantly lower expression, was interesting, however did not point to *CC* animals being at risk of lessened immune function. Although transcription does not always correlate to protein production, prior studies on VA limitation (Gorocica-Buenfil *et al.*, 2008; Jee *et al.*, 2013) did not identify significant differences in immune function between low and high fed levels of VA either, and suggested a severe limitation would be required to lead to these observable changes. Our research supports this, in that limiting dietary VA to as low as 25% of NRC recommendations did not display any negative effects on measured indicators of immune function.

These results should be taken with some caution however, for a few reasons. The sample size was small, although this is common for these types of PBMC studies. The animals studied were housed in a smaller feedlot, not a large-scale commercial operation, and therefore may have faced less stressors and disease challenge. Lastly, monthly oral boluses were used to dose the animals with their respective levels of VA, where a feedlot

would typically provide it in the ration. Given these limitations of the immunology portion of this study, the proportion of sick/treated/railed/condemned carcass/prematurely dead animals per pen in the commercial feedlot study was also considered. No significant differences between VA treatments that were included in the feed were observed. This suggests that a VA limitation of 50% of NRC recommendations would be safe, however more research at a commercial feedlot level would be valuable.

The commercial feedlot carcass trial resulted in only one interaction effect of *ADH1C* x IMS on ADG and main effects of IMS on DMI, TOTDOF, VGUSYLD, VGMARB, REA, and FAT. There were no other significant interaction or main effects. The lack of a significant VA x *ADH1C* effect was unexpected as Ward *et al.* (2012) and Krone *et al.* (2016) had both shown this interaction effect on fat traits in their smaller feedlot studies. There are a number of differences between the Ward *et al.* (2012) and Krone *et al.* (2016) trials and this one, which may be why we did not observe the anticipated interaction effect.

The sample size of this study was much smaller as pen was the unit. There were only 5 pens per treatment, but 36-50 steers per pen from which pen averages were calculated. The prior Ward *et al.* (2012) and Krone *et al.* (2016) VA x *ADH1C* studies used 130 and 117 steers respectively and each animal was the unit of measure. Although not statistically appropriate due to VA being applied to the bunk of the pen instead of on an animal-by-animal basis, analysis at the individual (as the unit) level still did not show the expected interaction effect (N = 1952). It was also important for the pen to be the unit in this study because profiting from grid marketing schemes requires consistently well-

marbled cattle, and therefore consistency at a pen level would be important for this MAM strategy.

This study attempted to be as commercially applicable as possible; therefore VA was fed directly, mixed in with the daily feed ration as opposed to a monthly oral bolus. Because of this, we are unsure exactly how much VA each animal consumed and how much the timing of the dose had an effect. The fact that pen was the unit of measurement should account for variations in the amount of VA consumed by each animal. With monthly oral boluses, animals have no supplemental VA all month until the one complete dose. This dosage is different from feeding VA daily in the feed, and may have resulted in lower fed VA levels in Ward *et al.* (2012) and Krone *et al.* (2016) than anticipated as a large dose all at once may have meant it was not all absorbed.

A study by Kruk *et al.* (2008) cited epigenetic effects as a potential reason for the drop in plasma retinol they observed in their animals shortly before slaughter. They stated that the stress from shipping 1000 km away from the feedlot to an abattoir, where they were processed might have reduced the VA stores of the cattle. In the Ward *et al.* (2012) and Krone *et al.* (2016) studies, the animals travelled for approximately 10 hours (over 750 km) in the middle of the summer months from the feedlot to the processing facility. Due to the feedlot and packing plant being in close proximity, the steers in this study only travelled 130 km before processing. This may account for some of the differences in results between the earlier studies and the current one. The animals in the Ward *et al.* (2012) and Krone *et al.* (2016) studies may have had a drop in VA stores within the last several days of life, and this may not have occurred in the steers of the current study. The

drop in VA may have allowed for slightly more IMF to accumulate in the cattle in earlier studies than the current one.

The *CT* and *CC* animals at the *ADH1C* SNP were analyzed separately in Ward *et al.* (2012) and Krone *et al.* (2016), while some *CT* pens in this study needed to be supplemented with *CC* animals because there were not enough *CT* animals to fill all pens. Previous studies (Ward *et al.*, 2012; Krone *et al.*, 2016) showed *CT* and *CC* animals to not be significantly different when it came to fat traits, and removing the *CC* animals for the pen averages also did not change significance in this study.

The steers used in Ward *et al.* (2012) and Krone *et al.* (2016) were all black in colour and because cattle with at least one E^D allele at *MC1R* (which makes them black) have significantly higher fat measurements than red animals (or *ee* at *MC1R*; McLean and Schmutz, 2009), they may have been more predisposed to have better IMF deposition. Again, to make this study as commercially relevant as possible, steers of all colours were used, however this may be why we did not see the same significant *ADH1C* X VA results. Non-implanted animals were therefore tested for the *MC1R* SNP, and results were analyzed in only the black animals (however this is not statistically appropriate with animal as the unit) and also by analyzing each pen as two groups per pen (by splitting the black from non-black animals). Analyses including any possible effect of *MC1R* on our results were also not significant.

Implant status was another possible confound as the Ward *et al.* (2012) and Krone *et al.* (2016) steers were all non-implanted because hormone implants are associated with reductions in IMF in cattle (Ducket *et al.*, 1999). By analyzing the IMP and NI pens separately, the *ADH1C* X VA interaction was still not significant. Given our statistical

model, any IMS effects on the *ADH1C* X VA interaction should have appeared to be significant as an IMS X *ADH1C* X VA interaction, but it was not.

The effect of limiting VA on fat traits in beef cattle has been shown multiple times before (Oka *et al.*, 1998; Kruk *et al.*, 2008; Pickworth *et al.*, 2012a; Pickworth *et al.*, 2012b), therefore it is unexpected to not at least have significant VA main effects in the current study. Ward *et al.* (2012) did not supplement any of the steers in their study during the backgrounding period and went on to supplement either 0% or 100% NRC recommended levels of VA during finishing. This study by Ward *et al.* (2012) also observed the most convincing IMF differences between the groups of low and high supplemented animals when compared to the results of the other two phases of this project (Krone *et al.*, 2016; and the current study). This would suggest that a more drastic VA limitation, and likely for a longer period of time, would be required to observe significant differences in fat traits for this MAM strategy. At a commercial feedlot level it would likely be difficult to also limit VA in backgrounding due to many feedlots only feeding animals during finishing. It would be quite risky to implement such severe limitations at a commercial level as well because animals entering the feedlot would have varying levels of stored VA, and those on the lower end of the spectrum would be susceptible to deficiency. From a practical standpoint, it would be very difficult to more drastically limit dietary VA as silage would likely have to not be used at all, or would have to be completely deficient of VA, which is unlikely. The lowest possible level of VA for the current trial was 50% of NRC due to the amount of VA in the silage. For *TT* animals at the *ADH1C* SNP, limiting VA more severely and for a longer period of time may lead to improved fat traits, however this is likely not feasible at a commercial level.

No *ADH1C* main effects were observed in this study. This is not surprising as Ward *et al.* (2012) and Krone *et al.* (2016) also did not observe the genotype effects unless VA was limiting. Mizoguchi *et al.* (2014) however, stated that the CCAAT/enhancer binding protein α binding site is not detectable in bovine intramuscular preadipocyte cell lines. This binding site was critical in the theory by Ward *et al.* (2012) that the presence of the *C* allele would remove the binding site and cause *ADH1C* expression to be reduced in *CC* compared to *TT* animals. Ward *et al.* (2012) and Krone *et al.* (2016) showed that there was both increased expression of the *ADH1C* gene and increased concentrations of the ADH1C protein respectively in liver of *TT* when compared to *CC* animals. This would suggest that the theory by Ward *et al.* (2012) was correct, unless there is another transcription factor binding site at the same location that is altered in a similar way to CCAAT/enhancer binding protein α by the *ADH1C* SNP.

The observed *ADH1C* X IMS interaction effect on ADG is likely just a main effect of IMS as ADG does not differ by genotype in the NI pens, and the difference between genotypes in the IMP pens is very small. It is possible that *CT* animals may have increased ADG, as this interaction suggests, as they were the more lean animals of the group and may gain muscle mass ahead of fat as in the *TT* animals. This only occurred in the IMP pens however, which at this point is unexplainable. The significant effects of IMS on ADG (although in combination with genotype), DMI, TOTDOF, VGUSYLD, VGMARB, REA, and FAT are not surprising, and have been observed in prior studies (Foutz *et al.*, 1997; Platter *et al.*, 2003; Wileman *et al.*, 2009). These significant findings support the overall intended effects of hormone implants – to improve production traits and yield. Their downside however is the negative effect they have on marbling and fat

traits. Depending on which marketing system a producer intends to target, these benefits and consequences of growth promotants are important to keep in mind.

Although the results of the commercial feedlot trial were both unexpected and disappointing, the lack of significant results in the immunology population is encouraging. While the *ADH1C* x VA MAM strategy may not be effective at a feedlot level, it is likely that the NRC recommendation of 2200 IU/kg DMI is much more than is required. Limiting dietary VA may still be a useful feedlot strategy, and SNPs in other genes may influence the VA-adipogenesis pathway in a manner that could support their use in MAM.

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APPENDICES

APPENDIX A

Immune function indicator measurements by animal for a) whole blood cell counts, and b) PBMC stimulation and gene expression data

a)

Animal	ADH1C	Pen	VA (%)	WBC	SEGS	BANDS	RBC	TOT-PROT	FIB
12-025	CC	9	25	9.6	2.112	0.000	9.92	76	2
12-043	CT	9	25	5.5	0.605	0.000	8.58	81	1
12-052	TT	10	75	7.7	2.310	0.000	7.23	85	4
12-065	TT	9	25	7.2	1.872	0.000	10.30	76	2
12-066	CC	10	25	11.8	4.956	0.118	8.15	79	4
12-075	TT	10	25	13.4	2.010	0.000	8.12	83	3
12-077	TT	9	75	6.6	1.518	0.000	6.92	80	3
12-104	TT	10	25	11.3	2.034	0.113	8.80	79	4
12-172	CT	9	75	8.7	1.653	0.000	8.18	75	2
12-186	CC	10	75	7.9	1.896	0.000	8.40	78	3
12-199	CT	10	25	7.1	1.065	0.000	8.44	78	3
12-206	TT	10	75	8.8	1.584	0.000	9.15	80	5
12-210	CT	10	75	11.9	4.998	0.000	9.07	80	4
12-249	CC	9	25	11.0	2.970	0.000	7.61	78	2
12-297	CT	9	75	8.1	1.944	0.000	8.64	71	3
12-358	CC	9	75	6.4	1.280	0.000	11.1	80	2
12-428	CC	10	75	10.1	2.828	0.000	8.43	80	4
12-446	CT	10	25	7.6	2.964	0.000	8.51	73	3

b)

Animal	ADH1C	Pen	VA (%)	STIM	IL-2	IL-4	IL-5	IL-10	IFN-γ
12-025	CC	9	25	12.09	0.6113	.	7.2100	0.1550	0.8706
12-043	CT	9	25	20.33	0.0002	.	0.0001	.	.
12-052	TT	10	75	25.87	0.4090	341.3245	9.2535	0.2003	0.05567
12-065	TT	9	25	8.55	5.2598	5.3517	8629.1336	1.9656	2.6117
12-066	CC	10	25	2.75	0.6620	2.5847	552.5649	4.5631	0.3415
12-075	TT	10	25	24.02	1.2058	25.2813	13.0412	2.2191	10.1261
12-077	TT	9	75	7.68	.	0.1096	0.5946	.	0.2707
12-104	TT	10	25	6.10	1.4241	21.1121	11706.3200	1.7593	4.7076
12-172	CT	9	75	81.95	1.6760	18.6357	1930.8227	0.6666	2.1810
12-186	CC	10	75	23.54	0.3572	3.9586	6080.6085	0.8039	1.5637
12-199	CT	10	25	9.85	0.3802	6.4308	1924.1426	0.8151	0.6713
12-206	TT	10	75	4.92	1.9185	28.2465	13777.2469	1.7471	0.3711
12-210	CT	10	75	49.46	1.4794	36.3781	939.0121	2.1886	182.9112
12-249	CC	9	25	2.12	1.0830	3.0001	25.3691	1.7963	0.7605
12-297	CT	9	75	7.98	0.0005	.	0.0482	0.0148	.
12-358	CC	9	75	5.51	2.8979	3.5064	2210.2594	1.7777	7.6476
12-428	CC	10	75	6.27	0.1406	1.6245	1764.4465	2.1886	0.4506
12-446	CT	10	25	11.20	0.4414	5.0107	65.7993	0.7371	0.9828

ADH1C = *ADH1Cc.-64T>C* genotype; PEN = Pen allocation; VA = Vitamin A level as a percentage of the NRC (1996) requirement; WBC = White blood cell count; SEGS = Segmented cell count; BANDS = Band neutrophil count; RBC = Red blood cell count; TOTPROT = Total protein count; FIB = Fibrinogen count; STIM = Stimulation index value; IL-2 = Interleukin 2 gene expression fold change; IL-4 = Interleukin 4 gene expression fold change; IL-5 = Interleukin 5 gene expression fold change; IL-10 = Interleukin 10 gene expression fold change; IFN-γ = Interferon gamma gene expression fold change

APPENDIX B

Immune function indicator measurements by independent variables ADH1C genotype (a-b), pen allocation (c-d), and vitamin A level (e-f) for whole blood cell counts (a, c, e) and PBMC stimulation and gene expression data (b, d,f)

a)

ADH1C	WBC	SEM	SEGS	SEM	BANDS	SEM	RBC	SEM	TOT-PROT	SEM	FIB	SEM
TT	9.68	1.17	1.96	0.12	0.023	0.023	8.72	0.51	80.60	1.57	3.60	0.51
CT	8.83	1.08	2.67	0.87	0.000	0.000	8.55	0.19	76.50	1.55	3.00	0.41
CC	9.44	1.00	2.79	0.62	0.024	0.024	8.74	0.61	79.00	0.45	3.00	0.45

b)

ADH1C	STIM	SEM	IL-2	SEM	IL-4	SEM	IL-5	SEM	IL-10	SEM	IFN-γ	SEM
TT	13.89	4.56	2.04	0.84	84.26	64.39	6827.00	2900.63	1.58	0.36	3.57	1.84
CT	38.12	17.26	0.99	0.34	16.61	7.26	1214.94	448.34	1.10	0.36	46.69	45.41
CC	8.04	3.95	1.03	0.49	2.93	0.40	2126.65	1064.54	2.23	0.63	2.15	1.39

c)

PEN	WBC	SEM	SEGS	SEM	BANDS	SEM	RBC	SEM	TOT-PROT	SEM	FIB	SEM
9	8.33	1.01	1.94	0.36	0.000	0.000	9.30	0.83	77.25	1.11	2.13	0.21
10	9.76	0.71	2.66	0.42	0.023	0.015	8.43	0.17	79.50	1.00	3.70	0.21

d)

PEN	STIM	SEM	IL-2	SEM	IL-4	SEM	IL-5	SEM	IL-10	SEM	IFN-γ	SEM
9	24.53	19.18	2.73	0.92	7.62	3.71	3198.90	1874.04	1.55	0.30	3.30	1.50
10	16.40	4.58	0.84	0.19	47.20	32.92	3683.24	1619.91	1.72	0.39	20.22	18.10

e)

VA (%)	WBC	SEM	SEGS	SEM	BANDS	SEM	RBC	SEM	TOT-PROT	SEM	FIB	SEM
25	9.91	0.97	2.55	0.47	0.033	0.021	8.56	0.32	78.00	1.15	3.00	0.31
75	8.79	0.67	2.36	0.48	0.00	0.00	8.79	0.45	79.71	1.13	3.43	0.43

f)

VA (%)	STIM	SEM	IL-2	SEM	IL-4	SEM	IL-5	SEM	IL-10	SEM	IFN-γ	SEM
25	9.23	2.79	1.49	0.65	9.82	3.52	3273.77	18.28.93	1.98	0.48	2.89	1.34
75	28.22	10.81	1.27	0.38	61.95	46.84	3815.95	1809.13	1.37	0.30	27.88	25.86

ADH1C = *ADH1Cc.-64T>C* genotype; PEN = Pen allocation; VA = Vitamin A level as a percentage of the NRC (1996) requirement; WBC = White blood cell count; SEM = Standard error of the mean; SEGS = Segmented cell count; BANDS = Band neutrophil count; RBC = Red blood cell count; TOTPROT = Total protein count; FIB = Fibrinogen count; STIM = Stimulation index value; IL-2 = Interleukin 2 gene expression fold change; IL-4 = Interleukin 4 gene expression fold change; IL-5 = Interleukin 5 gene expression fold change; IL-10 = Interleukin 10 gene expression fold change; IFN-γ = Interferon gamma gene expression fold change

APPENDIX C

PAG 2015 Poster:

Feedlot Performance and Immunology of Beef Steers Fed a Low Vitamin A Diet and Selected for *ADH1C* Genotype

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Introduction

- Feedlots strive to minimize costs and maximize returns and Marker Assisted Management (MAM) is a way in which they could accomplish this goal, by managing cattle to improve efficiency based on genotype

- Premiums are possible for those producing consistently high-marbling carcasses

- The TT *ADH1Cc.-64T>C* genotype has been shown to increase intramuscular fat (IMF) when vitamin A (VA) is limited in finishing rations[1]

- Dietary VA is converted to retinaldehyde (RAL) by alcohol dehydrogenase 1 C (ADH1C) and RAL is further converted to retinoic acid (RA)[1,2,3]

- RA stimulates and RAL inhibits gene expression, affecting fat deposition[2,3]

- This study aimed to determine whether feeding a previously optimized VA level in a commercial feedlot setting to TT and CT *ADH1C* animals would have an effect on marbling, as well as observing the effect hormonal implants may have on this process, and identifying any negative effects on immune function

Materials and Methods

Immunology Population:

- Black Angus X steers – No implants nor growth promotants

- Fed typical commercial backgrounding and finishing rations apart from a reduction in VA during finishing

- Two treatments of vitamin A (25% and 75% of NRC recommended 2200 IU/kg DM[4]) administered via a monthly oral bolus

- Blood samples taken from 18 steers (3 of each *ADH1C* genotype and VA level combination) one month before slaughter

- Differential blood cell counts and PBMC response to mitogen assays were conducted

- Data analyzed as a 3 x 2 factorial (genotype x VA treatment) using the mixed method of SAS 9.3[6]

Production and Carcass Data Population:

Figure 1. Treatment design for the Production and Carcass Data Population. 2000 mixed breed steers were selected based on *ADH1C* genotype, were separated into pens where they were either implanted or not implanted, and received either 100% or 50% NRC of VA. There were 5 pens of each treatment combination, totaling 40 pens.

- Typical commercial backgrounding and finishing ration

- VA added to the feed (either 100% NRC recommendation or 50%) during the finishing period

- Pens sent to slaughter once pen weight average reached 1350 lbs.

- Production data from feedlot and camera grading carcass data obtained from slaughter facility

- Data analyzed as a 2 x 2 x 2 factorial (genotype x VA treatment x implant status) using the mixed method of SAS 9.3[6]

Results

Immunology Data:

- *ADH1C* x VA interactions observed for WBC counts (P=0.003) and Segmented Cell counts (P=0.010)

- No significant (P<0.05) main or interaction effects were observed for differential Banded Cell counts, RBC counts, Total Protein, or Fibrinogen

- There were also no significant main or interaction effects observed on Stimulation Indexes for the PBMC response to mitogen assays

- There were no observable clinical signs of VA deficiency in either population of steers

Figure 2. Differential WBC counts in 18 randomly selected black Angus X steers of differing ADH1C genotype and dietary VA level. Bars with differing superscripts are significantly different (P<0.05).

Production and Carcass Data:

- A significant interaction effect of *ADH1C* x implant status on Late ADG (rehandling until finishing; P=0.036) was observed

- Implant status main effects were significant for the following traits: DMI, Early ADG, Late ADG, Vision Grade US Yield, VG Marbling, VG Fat (all P<0.001), and VG REA (P=0.011)

- No other significant main or interaction effects were observed

Figure 3. Mean Average Daily Gains from rehandling until slaughter for pens (n=40) of implanted and non implanted crossbred feedlot steers with differing *ADH1C* genotypes. Bars with differing superscripts are significantly different (P<0.05).

Discussion and Conclusion

- Previous studies using non implanted black Angus X steers showed that TT steers should have increased intramuscular fat when dietary VA was reduced[1]

- There were no significant interaction effects between genotype and VA level and no main effects with either variable in this study

- Implant status had many significant main effects on both production and carcass data

- An unexpected, yet significant interaction was observed for implant status x *ADH1C* on Late ADG, where CT implanted animals had the highest ADG, followed by TT implanted, then by non implanted animals

- Significant differences were observed in the immunology population, however no consistent pattern suggested that either genotype or VA level or a combination of the two was responsible for decreased immune function

- Further investigation is required to determine why the expected VA x *ADH1C* interaction was not observed here and to study further the interaction effect on ADG

- qRT-PCR tests are being conducted to study the effects of these treatments on immune function

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