ASSOCIATION ANALYSIS OF *MC1R*, *MC4R* AND *AGRP* IN BEEF CATTLE

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By

Kim Lauren McLean

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ABSTRACT

Three interrelated genes postulated to affect economically important traits related to growth and/or carcass quality of beef cattle were chosen to characterize and perform association analyses for this study. Melanocortin 1 receptor (*MC1R*), melanocortin 4 receptor (*MC4R*) and agouti related protein (*AGRP*) play an integral role in the appetite pathway and in fat deposition.

We genotyped 328 crossbred steers of various coat colours that were purchased at weaning and fed until slaughter for the previously published alleles E^D, E^+ and *e*. The E^+ allele was present at five percent in this population and therefore was not included in further analysis. Black cattle of E^D/E^D or E^D/e genotype had increased backfat (P<0.05) and required significantly fewer days (15-25) (P<0.01) on feed to reach a target fat level for slaughter compared to the red cattle. Red cattle of *e/e* genotype were found to have significantly larger *longissimus dorsi* (*l. dorsi*) area, shipping weight and hot carcass weight. Subsequent analysis revealed that the differences were comparable whether black versus red coat colour or *MC1R* genotype was used as the criteria for the group of cattle.

MC4R sequence was obtained from 20 random crossbred steers. In addition to several previously published polymorphisms, a novel Ser330Asn polymorphism was detected. A population of 382 crossbred Canadian steers and 985 crossbred American steers was genotyped for this Ser330Asn polymorphism. A minor allele frequency of 0.01 was observed in the Canadian and 0.02 in the American steer populations. No homozygous g.989AA cattle were detected. In the Canadian population, heterozygous steers had increased grade fat (*P=0.036*) and decreased lean meat yield (*P=0.032*). Similarly in the American population, steers of the g.989GA genotype had increased backfat (*P=0.031*) and less desirable yield grades (*P=0.022*,) but also lower ribeye area measurements *(P=0.031)*. These results suggest that genotyping for the Ser330Asn polymorphism may lead to increased quality of carcasses either through lean meat production or backfat measurements, depending on the goal of the beef operation.

Sequence data obtained from 38 *Bos taurus* beef cattle, 4 Holsteins and 4 *Bos indicus* cattle revealed six polymorphisms in the *AGRP* gene. No polymorphisms that altered amino acids were detected in *Bos taurus* cattle. Genotyping of 382 crossbred beef

steers was performed for two polymorphisms, an intronic deletion (g.439_440delTC) and a base pair substitution in exon 4 that did not alter an amino acid (g.715G>A). An ANOVA analysis, using PROC Mixed, was performed for both polymorphisms on several growth and carcass traits. No significant differences were observed.

Polymorphisms in *MC1R* and *MC4R* could be used as genetic tests which may be beneficial for beef producers in North America. The significant differences observed in this study in relation to cattle growth and fat deposition would represent savings for producers when used for sorting feedlot cattle or in selection of breeding cattle.

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

α-MSH	α-melanocyte stimulating hormone
β-AA	β-adrenergic receptor agonists
β-MSH	β -melanocyte stimulating hormone
° C	degrees celsius
μl	microliter
ACTH	adrenocorticotropin hormone
ADG	average daily gain
AGRP	agouti related protein
ASIP	agouti signalling protein
bp	base pair
BTA	*Bos taurus* autosome
cAMP	cyclic adenosine monophosphate
CBRH	Canadian beef reference herd
ddH$_2$O	doubled distilled water
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
E$_2$	estradiol
EDTA	ethylene diaminetetra acetic acid
HCW	hot carcass weight
HPLC	high performance liquid chromatography
IGF2	insulin-like growth factor 2
l. dorsi	longissimus dorsi
LDA	longissimus dorsi area
LS	least squares
MC1R	melanocortin one receptor
MC2R	melanocortin two receptor
MC3R	melanocortin three receptor
MC4R	melanocortin four receptor
MC5R	melanocortin five receptor
MgCl$_2$	magnesium chloride

mM   millimolar

mRNA   messenger ribonucleic acid

MSTN   myostatin

ng   nanogram

PB   pelleted barley diet

PCR   polymerase chain reaction

pm   picomole

PMCH   promelanin concentrating hormone

POMC   pro-opiomelanocortin

QTL   quantitative trait loci

RB   rolled barley diet

REA   ribeye area

RFLP   restriction fragment length polymorphism

RNA   ribonucleic acid

SEM   standard error of the mean

SNP (s)   single nucleotide polymorphism

TBA   trenbelone acetate

USBF   ultrasound backfat

USLD   ultrasound longissimus dorsi area

UTR   untranslated region of a gene

UV   ultraviolet

WBSF   Warner-Bratzler shear force

ZH   zilpaterol hydrochloride

1.0 GENERAL INTRODUCTION

Melanocortin 1 Receptor (*MC1R*), Melanocortin 4 Receptor (*MC4R*) and Agouti Related Protein (*AGRP*) play an integral role in the appetite pathway. Any genetic variants found through this analysis should affect multiple traits of importance in beef cattle production. These genes are interrelated and therefore a study that addressed all three in beef cattle was warranted.

MC1R is considered to be the main gene controlling production of eumelanin, black coat colour, in response to α-melanocyte stimulating hormone (α-MSH) or phaeomelanin, red coat colour, when agouti signalling protein is bound to MC1R (Jackson 1993). α-MSH causes appetite suppression when bound to MC4R. We hypothesized that α-MSH would bind to MC1R in black cattle more than red cattle, which could result in less α-MSH binding to MC4R leading to less appetite suppression. The mutant MC1R does not bind α-MSH and therefore red cattle would have a larger amount of α-MSH to bind to MC4R, suppressing appetite more than black cattle.

MC4R binds α-MSH and reduces feed and energy intake in several species of animals (Benoit et al. 2000; Vaisse et al. 2000). This has led to several studies of the *MC4R* gene in humans (Farooqi et al. 2003) and some livestock animals looking for associations with weight gain and/or fat depositions (Kim et al. 2000a; 2000b; Buchanan et al. 2005). This gene has not completely been characterized in cattle.

AGRP is a potent antagonist to this melanocortin appetite pathway. Specifically targeting melanocortin 3 receptor and MC4R, AGRP binds to MC4R blocking the ligand binding of α-MSH (Ollmann et al. 1997; Graham et al. 1997). This then stimulates the appetite and decreases energy expenditure of an individual (Korner et al. 2000). Knockout studies in rats (Shutter et al. 1997) and polymorphisms found in the human *AGRP* have demonstrated that increased expression of AGRP results in severe obesity, hypertension and an increased risk for type two diabetes (Schwartz et al. 1996; Argyropoulos et al. 2002). *AGRP* has not been previously characterized in cattle.

A population of 382 crossbred steers all fed the same diet and slaughtered within a two week period were used as the initial study population to assess associations with polymorphisms detected in these three genes of the appetite pathway. This allowed potential interactions of mutations to be assessed. It was hoped a commercial test to improve beef production could be a result of this study.

2.0 LITERATURE REVIEW

2.1 Cattle growth

2.1.1 Growth curves

Owens et al. (1995) describes growth as an accretion of protein, bone and adipose tissue. Growth is often described as an increase in mass which includes both an increase in the number of cells, hyperplasia, and an increase in the size of existing cells, hypertrophy (Owens et al. 1993). Prenatal growth occurs through hyperplasia with some muscle hyperplasia occurring postnatally (Di Marco et al. 1987). Postnatally, growth primarily occurs through hypertrophy as satellite cells are incorporated into the muscle or adipose tissue (Owens et al. 1993). As the animal grows a sigmoid growth curve results when age is plotted against animal weight (Owens et al. 1993). Figure 2.1 illustrates four distinct stages of growth in the animal (postnatally) including a prepubertal, self-accelerating, post-pubertal and a self- inhibiting phase (Owens et al. 1993).

This sigmoid growth curve is present in every animal, however the length of time to reach maturity and complete the individual growth curve, will vary. Figure 2.2 illustrates two example animals, one animal with a slow growth rate and one with a faster rate of growth and also illustrates tissue growth which occurs in the same order no matter what rate of growth ensues (Owens et al. 1993). The initial growth of the animal, with the highest percentage of body weight change, occurs primarily in bone and protein tissue and finishes with the majority of tissue deposition being adipose, whether this is intermuscular, body cavity or intramuscular adipose deposition. Intramuscular adipose is the last tissue to be deposited on the individual carcass and is energetically expensive to deposit in large amounts (Owens et al. 1993; Owens et al. 1995).
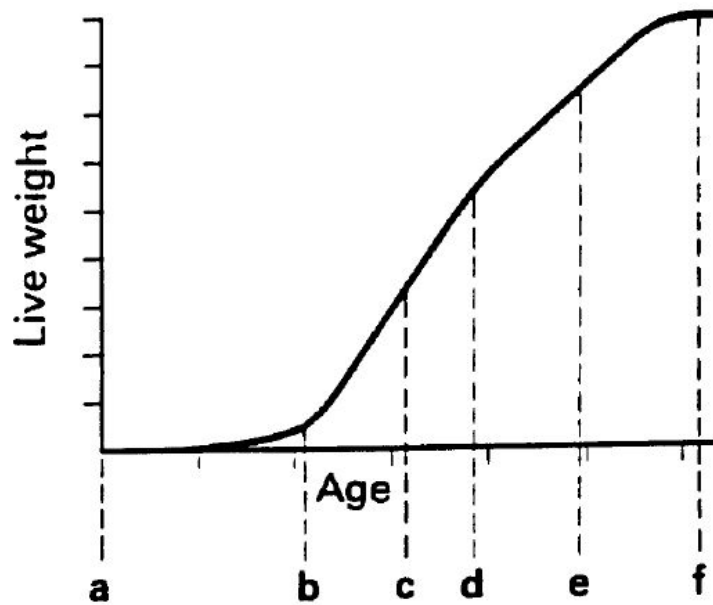
Figure 2.1 A sigmoid growth curve of a lamb, illustrating the rate of growth in mass as the animal matures. The points indicated include a, conception; b, birth; c, self-accelerating phase; d, inflection point often caused by puberty; e, self retardation phase; f, maturity. Taken from Owens et al. (1993) with permission
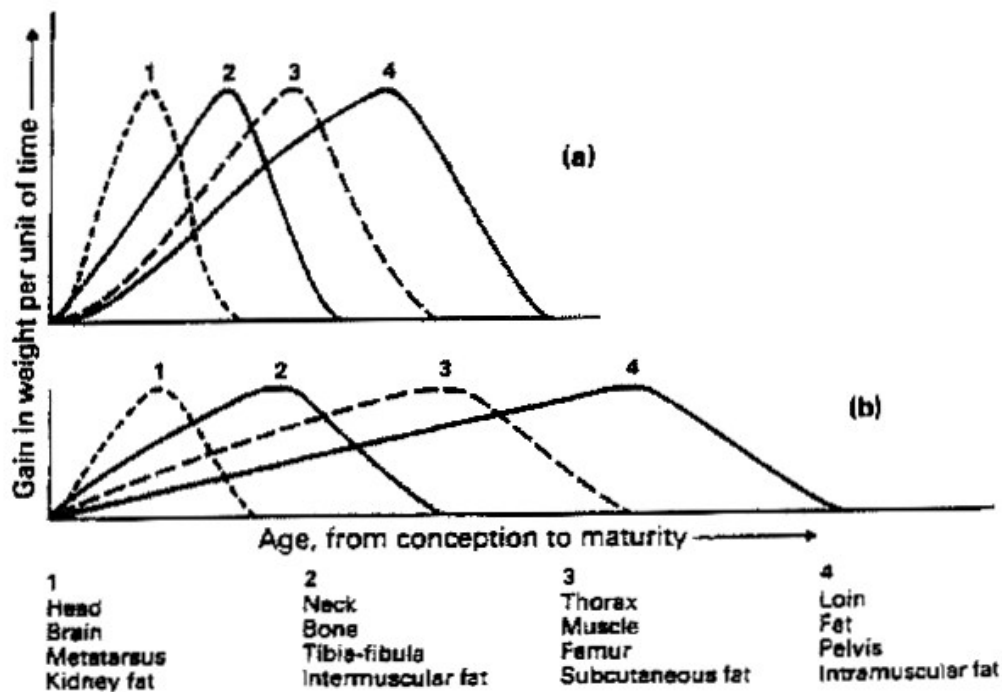
Figure 2.2 Growth rates of several tissues throughout the ruminant animal fed to achieve two different growth rates, a, rapid growth and b, slow growth. Taken from Owens et al. 1993 with permission

2.1.2 Factors that modify rate of growth

The length of time each animal takes to reach point four (Figure 2.2), the state in which the loin increases in size and rate of intramuscular fat deposition increases can be modified by several factors. Some of these factors include mature body size, nutrition, hormonal status, environment and genetics (Owens et al. 1993; 1995). Several studies have analyzed the effect of nutrient restriction in order to retard growth for a period of time and in turn increase mature size, as fat deposition is held to a minimum for this period of restriction (Drouillard et al. 1991; Coleman et al. 1993; Block et al. 2001). Coleman et al. (1993) determined that by restricting energy intake through the growth period of early-maturing cattle, such as British type steers, carcass weights would be increased and still have adequate fat deposition. The larger framed, later maturing cattle did not need to have this nutritional restriction as mature body weight was appropriate for a leaner carcass of adequate size (Coleman et al. 1993). Block et al. 2001 reported that a

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longer period of backgrounding did result in heavier slaughter weights in both medium and large framed cattle compared to a short introductory backgrounding period. Drouillard et al. (1991) cautions that in order to see the compensatory gains in a finishing feedlot it is more beneficial to restrict energy intake rather than protein intake as mature body weight was shown to decrease with significant protein restriction.

Sandelin et al. (2002) when studying the effect of two different types of feed, Bermuda grass or endophyte infected fescue, noticed a production difference, not only with breed type but also with different feed stuffs within each breed. Sandelin et al. (2002) suggested that it was necessary to match feed sources with different breed types of animals as forage environment significantly impacted cattle of similar breeds. Such results have been indicative of a nutrigenetic effect when feeding cattle.

Arkadianos et al. (2007) stated the theory behind nutrigenetics is to personalize an individual diet, taking into consideration genetic variation for the individual. Ordovas and Mooser (2004) describe a gene-diet interaction as one where the diet modulates the effect of a genetic variant on the phenotype of the individual or alternatively one where the diet's affects on a particular phenotype is modified by a genetic variant. Ordovas (2008) continues to state that nutrition is the most important environmental factor to modify genetic expression and subsequent phenotype. Nutrigenetics has the potential in the cattle feeding industry to modify feed efficiency and carcass composition in animals of different genotypes.

The hormonal status of the animal can affect finishing ability by partitioning growth into different proportions of bone, muscle and fat based on hormones present in the animal throughout their growth period. For instance, heifers have been known to reach a mature size at a lighter weight, with more adipose tissue present on the carcass, when compared to steers or bulls with more testosterone present, who partition growth towards more lean muscle growth and reaching a heavier mature size with less adipose tissue (Hassen et al. 1999, Casas et al. 2009). Commercial companies have used this knowledge to their advantage, developing steroidal implants and different β-adrenergic receptor agonists (β-AA) to increase myogenesis and decrease energy being expended towards adipogenesis (Johnson et al. 1996; Johnson and Chung 2007). These hormonal modifiers alter the hormonal status of the animal aiming to increase feed efficiency and

lean muscle tissue growth, increasing productivity and profitability of the operation (Foutz et al. 1997).

A combination trenbelone acetate (TBA) and estradiol (E_2) implant was shown to result in increased carcass weight of 40 to 60 lbs with an increased longissimus area and no effect on overall carcass fat (Johnson et al. 1996). By implanting steers or heifers in a feedlot, it is possible to shift their growth curve and increase the amount of lean muscle tissue accretion before the animal reaches point four (Figure 2.2), the final phase of growth where adipose tissue begins to accrete. Once implant hormone levels are depleted, the animal is able to focus on adipogenesis and increase adipose deposition, particularly intramuscularly (Johnson and Chung 2007).

Bruns et al. (2005) analyzed the time of implantation of a combination TBA/E_2 implant on overall meat quality and production in feedlot steers. Two time periods of implanting, day 1 at 309 kg and at day 57 at 385 kgs were evaluated for their effect on carcass growth and quality. The expected increases in average daily gain (ADG), gain to feed ratio, hot carcass weight (HCW), dressing percentage and longissimus muscle size were observed in this study. Significant differences were observed for those implanted, however day of implantation was not significantly different at the trial finish (Bruns et al. 2005). The animals implanted earlier did gain more quickly than the later implanted or control steers, however the same end point was attained with either implantation strategy (Bruns et al. 2005). A key point in this timing analysis showed that although there was no significant difference in subcutaneous back fat there was a significant decrease in marbling scores of the early implanted steers compared to the controls and delayed implantation strategy (Bruns et al. 2005). By altering timing of implant administration it was possible to modify intramuscular fat deposition and in turn percentage of carcasses making higher quality grades. This suggests that a later implanting strategy will increase profitability of the carcass, while still achieving similar gains as early implanted cattle (Bruns et al. 2005).

A new β-AA feed additive, zilpaterol hydrochloride (ZH), has recently been evaluated for use in feedlot production (Elam et al. 2009). ZH was reported in multiple studies to have increased gain to feed ratio, increased ADG, HCW, longissimus muscle size and dressing percentage (Elam et al. 2009). Elam et al. (2009) also reported that fat

deposition was limited in cattle fed ZH, with decreased fat over the twelfth rib and marbling scores. Leheska et al. (2009) reported subprimal cutability and decreased trim fat was observed in a similar study in which steers and heifers were fed ZH. ZH did result in increased Warner-Bratzler shear force (WBSF) values by 24 % in heifers and 22% in bulls and decreased sensory evaluations for juiciness, flavour intensity and beef flavour scores (Leheska et al. 2009). Leheska et al. (2009) reports that the implications ZH has on sensory evaluations are not significant enough to affect consumer acceptance and supported the use of ZH as a repartitioning agent to increase protein accretion in beef cattle.

Genetics has been correlated with mature size, adipose deposition and overall carcass composition in beef cattle. The broad term genetics has traditionally been used as a way of distinguishing between different cattle frame size and types. Often breed type, British or Continental, has been used as a way to associate frame size and in turn final body weight with cattle. For instance, British cattle, Angus, Hereford, Shorthorn and Galloway have been thought to finish at much lighter weights as fat deposition is more prevalent in these breeds of cattle (Nadarajah et al. 1984; Urick et al. 1991; Block et al. 2001; Sandelin et al. 2002). Continental breeds, such as Simmental, Charolais, Gelbvieh and Maine Anjou, have been assumed to be larger framed with heavier mature body weights and less adipose tissue deposition (Nadarajah et al. 1984; Urick et al. 1991; Block et al. 2001). Casas et al. (2009) reported that crossbred cattle with Angus grandsires had heavier carcass weights, grew faster, with increased marbling scores and the highest percentage of USDA choice carcasses when compared to cattle with Hereford, Beefmaster, Brangus, Bonsmara or Romosinuano as grandsires.

With the advancements of molecular genetics, genetics could assume a different role in modifying the growth curves of beef cattle. By knowing the molecular makeup of cattle, breed is irrelevant and specific feedlot tools can be tailored to modify the growth curves of cattle improving feedlot efficiency. Kononoff et al. (2005) suggested that it is possible to match leptin genotype, a gene known to increase carcass fat (Buchanan et al. 2002), to feeding protocols to improve feed efficiencies in finishing programs. Kononoff et al. (2005) also suggested that knowing the leptin genotypes of cattle makes it possible to target different end-points with these groups of cattle, increasing suitability for certain

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markets and overall profitability for the feedlot. Other genetic tools are available for feedlot operators to manipulate growth. An 11 bp deletion in Myostatin (*MSTN*), has been associated with increased HCW and longissimus area while having no impact on fat deposition (Gill et al. 2008). Several genetic tests have been discovered which hold the potential to modify growth in feedlot steers including polymorphisms in insulin-like growth factor 2 (*IGF2*) (Goodall and Schmutz 2007) which results in an increased longissimus muscle area or pro-melanin concentrating hormone (*PMCH*) affecting carcass fat in feedlot steers (Helgeson and Schmutz 2008).

2.1.3 Economics of growth

In livestock production, animals are fed until a point of maturity in which protein accretion has ceased and an adequate adipose tissue has accumulated on the carcass. Economics plays a significant role in determining when cattle are shipped and what is deemed the optimal point for profitability between protein accretion and fat deposition. Marketing protocols will impact where the optimal point in this growth curve is economically ideal. By implementing growth modification strategies, producers will be able to optimize profitability of their operations.

Producer preference and production situation will ultimately determine where and when their cattle can be marketed. The cow-calf producer's number one concern will be to wean a calf and one with the highest weight possible, as these producers are paid per pound when calves are sold to finishers. The backgrounding and finishing sector has the most to gain through implementation of growth modification strategies. By understanding growth physiology and what factors can be utilized for manipulating growth, it is possible to target cattle for specific markets or finishing dates (Block et al. 2001; Kononoff et al. 2005).

Carcass composition is also altered by manipulating growth. Depending on markets and ease of selling, producers will aim for carcasses of different compositions. If it is advantageous for producers to market to a value-based grid at different slaughter facilities a carcass showing adequate muscle size and more marbling is desirable. Grading schemes in Canada evaluate carcass for degree of muscling, maturity, carcass fat

cover and intramuscular fat or marbling (Canadian Beef Grading Agency 2009). Marbling is the highest rewarded premium in value-added programs as it is the major contributor to determining quality grade in both Canada and the United States (United States Department of Agriculture 1997; Canadian Beef Grading Agency 2009). If selling on a live weight or dressed hot carcass weight basis, the producer will aim for a heavier, leaner carcass as dressing percentage will be increased with a higher percentage of lean muscle. Deciding early on in the feeding period where the producer plans to market the cattle will be highly beneficial as production practices can be altered as early as purchasing of the cattle to meet marketing strategies.

2.2. Appetite pathway

The focus of this study was to analyze the effect of melanocortin 1 receptor (*MC1R*), melanocortin 4 receptor (*MC4R*) and agouti related protein (*AGRP)* in the appetite pathway of cattle. We anticipated that the role of α-MSH (Figure 2.3) in both the appetite and pigmentation pathways will lead to significant differences in growth and carcass qualities.

$$\alpha\text{-MSH}$$

ASIP → MC1R ← α-MSH → MC4R ← AGRP

MC1R → Pigment → e/e , E^D/E^D

MC4R → Appetite → − Red , + Black?
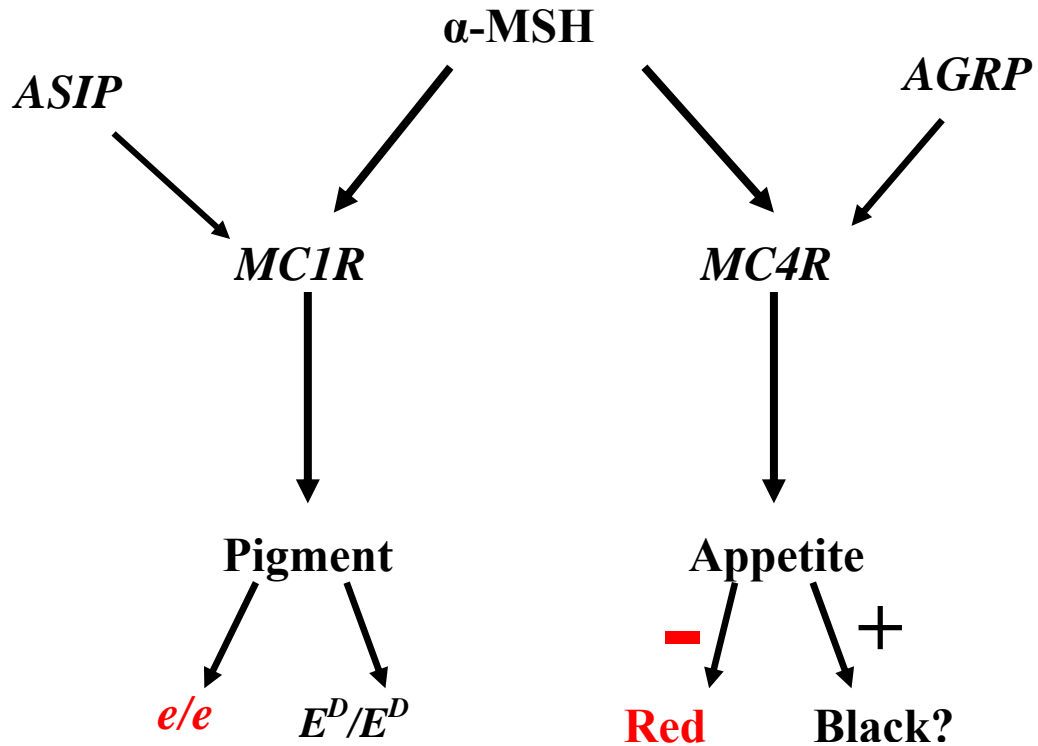
Figure 2.3 The role of *MC1R*, *MC4R* and *AGRP* in the appetite pathway

2.3 Melanocortin 1 receptor *(MC1R)*

2.3.1 MC1R gene structure and function

MC1R is one of the five members of the melanocortin family, a seven transmembrane G protein coupled receptor responsible for coat and hair pigmentation of mammals (Jackson 1993). MC1R is expressed at the surface of all melanocytes. Normal MC1R binds two different peptides. When α-MSH is bound to MC1R, a eumelanin or black coat colour is produced (Jackson 1993). Agouti signalling protein (ASIP) acts to block the binding of α-MSH and when bound to MC1R results in a phaeomelanin or red/yellow coat colour (Bultman et al. 1992; Jackson 1993).

MC1R is a gene consisting of one exon, 317 amino acids long in humans (Garcia-Borron et al. 2005). Amino acid alignment shows that there is high homology between

the cattle MC1R sequence and sheep (96%), horse (83%), pig (81%), dog (81%), human (80%), mouse (74%), and chicken (59%) (Figure 2.4).

Scioth et al. (1998) determined that the N-terminus loop (Figure 2.5), the first 27 amino acids, could be removed with no effect on ligand binding as the signal sequence had low homology to this region of the MC1R. The seven transmembrane structures of all melanocortins makes this family of receptors unique. There is relatively high conservation both between species and between the melanocortin receptors (Cone et al. 1996). All of the melanocortin receptors have transmembrane loops, both extracellular and intracellular (Garcia-Borron et al. 2005). The extracellular loops of the MC1R are quite small (Figure 2.5) with the third extracellular loop being highly conserved across the melanocortin family (Gracia-Borron et al. 2005). This particular region is also high in cysteine and proline residues, perhaps indicating that this is crucial for specialized functions (Holst and Schwartz 2003). The intracellular loops of MC1R are often a binding site for the G proteins necessary for signalling and processing (Strader et al. 1994).

```
Cattle    MPALGSQRRL LGSLNCTPPA TLPFTLAPNR T:::GPQCLE VSIPDGLFLS
Sheep     --V------- ---------- ---L------ -:::------ ----------
Horse     --LQ-P---- -----S-L-- -PYLG-TT-Q -:::E-P--- ----------
Pig       --V--PE--- -ASLSSA--- APRLG--A-Q -NQT------ ----------
Dog       -SGQ-P---- -----G-S-- -PH-E--A-Q -:::--R--- ----D-----
Human     -AVQ------ -----S--T- IPQLG--A-Q -:::-AR--- ---S------
Mouse     -STQEP-KS- -----SNATS H-GLATNQSE PW:::::--Y ----------
Chicken -SM-APL-L- REPW-ASEGN QSNATAGAGG AW:::::CQG LD--NE---T

Cattle    LGLVSLVENV LVVAAIAKNR NLHSPMYYFI CCLAVSDLLV SVSNVLETAV
Sheep     ---------- ---------- ---------- ----M----- ----------
Horse     ---------- ---T------ ---------- ---------- -M-----M-I
Pig       ---------- ---------- --------V ---------- ----------
Dog       -----V---- ---------- ---------- G--------- --S-------
Human     ---------A ----T----- -------C-- ----L----- -G--------
Mouse     ---------- ---I--T--- ---------- ----L---M- ---I----TI
Chicken --------L ------L--- -----T---- -------M-- ----LAKTLF

Cattle    MPLLEAGVLA TQAAVVQQLD NVIDVLICGS MVSSLCFLGA IAVDRYISIF
Sheep     -L-------- -R-------- -------SS ---------- ----------
Horse     LL-------- -Q-S-L---- -I-------- ---------S ----------
Pig       LL-----A-- A--------- --M------- ---------- ------V---
Dog       -L-VA--A-- A--------- DI-------- ---------- ------L---
Human     IL-----A-V AR---L---- -----IT-S- -L-------- ----------
Mouse     IL-----I-V ARV-L----- -L-------- ---------I --I-------
Chicken -L-M-H---V IR-SI-RHM- ----M---S- V----S---V -------T--

Cattle    YALRYHSVVT LPRAWRIIAA IWVASILTSL LFITYYNHKV ILLCLVGLFI
Sheep     ---------- ---------- ---------V -S------TV V------F--
Horse     -------IMM ---V--A-V- ---V-V-S-T -F-A----TA V-----TF-V
Pig       -------I-- ----G-A--- --AG-V-S-T ---A--H-TA V--G--SF-V
Dog       -------I-- -Q----A-S- -----V-S-T ---A----TA V-----SF-V
Human     -------I-- ----R-AV-- -----VVF-T ---A--D-VA V-----VF-L
Mouse     -------I-- ----R-AVVG --MV--VS-T ------K-TA V-----TF-L
Chicken -------IM- -Q--VVTM-S V-L--TVS-T VL----RNNA -----I-F-L

Cattle    AMLALMAVLY VHMLARACQH ARGIARLQKR QRPIHQGFGL KGAANLTILL
Sheep     ---------- ---------- ---------- ---------- ----T-----
Horse     ---V------ ---------- -------H-- -H-------- ----T-----
Pig       ---------- ---------- G-H----H-T -H-TR--C-- ----T-----
Dog       ---V------ ---L------ -------H-- -HFIP----- ----T-----
Human     ---V------ ---------- -Q-----H-- ---V------ --VT-----
Mouse     -------I-- A--FT----- -Q---Q-H-- R-S-R---C- ----T-----
Chicken F--V--L--- I--F-L-RH- V-S-SSQQ-Q PTIYRTS*S- GK-VT-----
```

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```
Cattle    GVFFLCWGPF FLHLSLIVLC PQHPTCGCIF KNFNLFLALI ICNAIVDPLI
Sheep     ---------- ---------- ---------- ---------- ----------
Horse     ---------- ------LI-- --------V- ---K---T-- L-S-------
Pig       ---L---A-- ------V--- --------V- --V------V ---S------
Dog       -I-------- ------?--- ----I---V- Q------T-I ---SII--F-
Human     -I-------- ----T----- -E-------- ---------- -----I----
Mouse     -I-------- ----L----- ------S--- -------L-- VLSST-----
Chicken   ----I----- -F--I---T- -TN-F-T-F- SY-----I-- ---SV-----


Cattle    YAFRSQELRK TLQEVLQCSW
Sheep     ---------- ----------
Horse     ---------- ------L---
Pig       ---------- ----------
Dog       --------?- -----VL---
Human     ---H-----R --K---T---
Mouse     ---------M --K---L---
Chicken   ---------R --R--VL---
```

Figure 2.4 Amino acid alignment of the *MC1R* gene across various species of animals. Sequences used for the *AGRP* align include Cattle (*Bos taurus* NP_776533), Pig (*Sus scrofa* NP_001008690), Human (*Homo sapiens* NP_002377), Mouse (*Mus susculus* NP_032585), Dog (*Canus lupus familiaris* NP_001014304), Chicken (*Gallus gallus* NP_001026633), Sheep (*Ovis aries* CAA74298), Horse (*Equus caballus* NP_001108006). : represents no amino acid
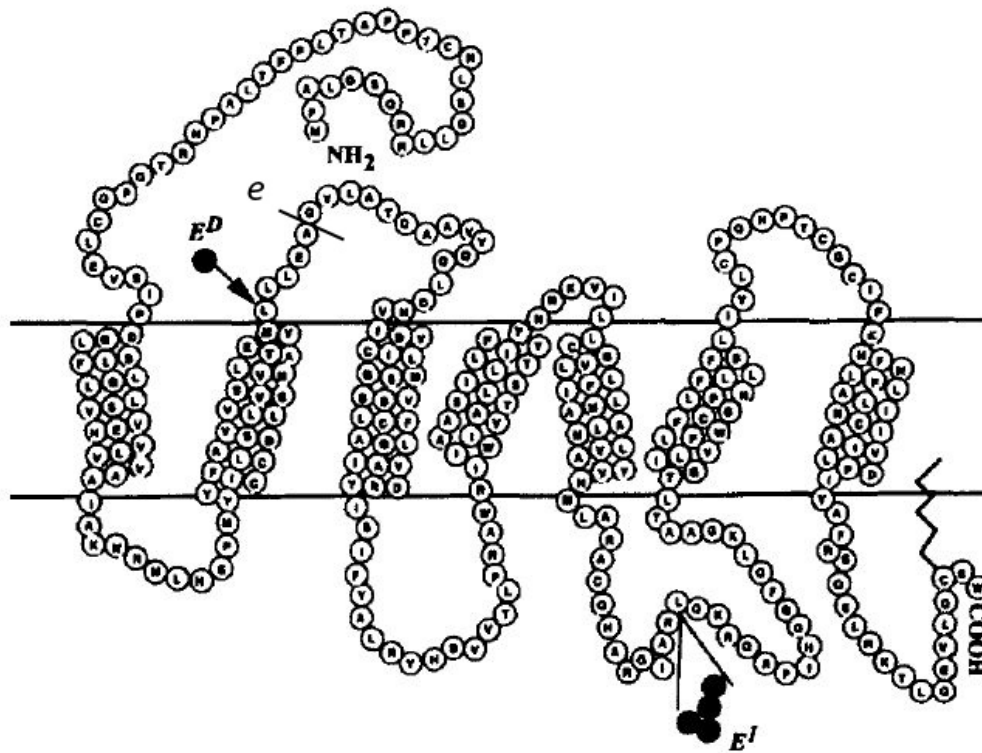
Figure 2.5 Representation of the *Bos taurus MC1R* with known polymorphisms affecting cattle highlighted. Modified from Rouzaud et al. (2000). The E^D allele, which causes black coat color, is the result of a Leu99Pro amino acid change; the recessive red, *e* allele results in a frameshift mutation at amino acid 103 (depicted by the diagonal line) causing a premature stop codon at amino acid 155; E^I represents the four amino acid insertion found by Rouzaud et al. (2000).

There are several polymorphisms in these intracellular loops that result in partial or complete loss of function of the MC1R in humans (Garcia-Borron et al. 2005). *MC1R* is highly polymorphic in humans and responsible for many pigmentation differences in other mammals. One region of the MC1R which is considered crucial for signalling and efficiency of the receptor is the C-terminus (Garcia-Borron et al. 2005). The red hair phenotypes in several species, such as humans (Beaumont et al. 2008), dogs (Newton et

al. 2000) and cattle (Klungland et al. 1995) are caused by mutations that cause a loss of function due to premature termination of the protein. This indicates that the c-terminus is important for a functional MC1R and eumelanin pigment production.

2.3.2 MC1R agonists

2.3.2.1 α-Melanocyte stimulating hormone (α-MSH)

Through the use of prohormone convertases (PCs), post translational cleavage of pro-opiomelanocortin (*POMC)* yields α-, β-, γ-MSH and adrenocorticotropin hormone (ACTH) (Pritchard et al. 2002). α-MSH is an agonist for all of the melanocortin pathways (Cone et al. 1996). In addition to the MC1R, moderate to high affinities of binding of α-MSH are observed in *MC3R*, *MC4R*, *MC5R* (Mountjoy et al. 1997). *MC2R* preferentially binds ACTH, however it is still receptive to α-MSH (Cone et al. 1996).

α-MSH consists of thirteen amino acids and is a 32 kilodalton peptide expressed throughout the central nervous system and surrounding tissues (Pritchard et al. 2002; Irani and Haskell-Luevano 2005). α-MSH is physiologically important and necessary for many bodily functions. The melanocortin genes activate adenyl cyclase, in turn stimulating cyclic AMP. They are responsible for almost all of the actions of α-MSH throughout the body (Busca and Ballotti 2000; Garcia-Borron et al. 2005). When α-MSH is bound to MC1R a cascade is stimulated in which adenyl cyclase, cyclic AMP and protein kinase A activate tyrosinase which is a necessity for eumelanin pigmentation production (Cone et al. 1993).

Thue and Buchanan (2003) found a silent, c.288C>T polymorphism in the coding region of POMC. Buchanan et al. (2005) found an association with this c.288C>T SNP in a population of 256 crossbred beef steers with hot carcass weight. Deobald (2009) found that this SNP also significantly increased carcass ribeye area and hot carcass weight and decreased grade and average fat when a T allele was present. Deobald (2009) found a 12 bp deletion which caused four amino acids to be removed, however no frameshift occurred. This deletion was found to be associated with decreased carcass ribeye area (Deobald 2009).

2.3.2.2 Agouti signalling protein (*ASIP*)

ASIP is a 131 amino acid peptide which is approximately 15,000 daltons in size with a cysteine-rich region near the C-terminus (Bultman et al. 1992). Bultman et al. (1992) reported that this protein has a putative signal peptide of 31 amino acids, with several phosphorylation sites and a basic domain in the middle. The cysteine-rich region was found to fit into the classification of an inhibitor cystine knot which is often recognized by a circle formed with two disulfide bonds and the peptide segments that connect (McNulty et al. 2001). ASIP is one of two peptides that is thought to modulate G-protein coupled receptors with such a knot. It is thought that this knot is necessary to help contact and bind ASIP to MC1R (McNulty et al. 2005).

The ASIP protein acts as a competitive antagonist to α-MSH at MC1R (Lu et al. 1994). When agouti is bound to MC1R, eumelanin cannot be produced, as activation of adenyl cyclase and subsequent production of cAMP is not possible (Abdel-Malek et al. 2001). A yellow, phaeomelanin, coat colour is the resulting phenotype in mice when ASIP is bound to MC1R (Lu et al. 1994). No mutations in the cattle *ASIP* coding gene have been reported. A promoter mutation was reported to cause brindle coat colour in Normande cattle (Girardot et al. 2006).

2.3.3 *MC1R* polymorphisms affecting pigmentation

2.3.3.1 Humans

Several studies have focused on the role of *MC1R* in pigmentation of human skin and hair colour. Several of the over 60 variants detected in the human *MC1R* sequence have been implicated with causing the red hair colour phenotype (Sturm et al. 2003) This phenotype includes red hair, freckling, fair skin and a significant increased risk of skin cancer (Garcia-Borron et al. 2005; Beaumont et al. 2008). Four main polymorphisms have been thought to have a stronger effect on red hair colour. These include p.D84E, p.R151C, p.R160W, p.D294H (Sturm et al. 2003). Sturm et al. (2003) also concluded that some polymorphisms should be classed as weaker red hair colour alleles, including p.V60L, p.V92M, and p.R163Q.

Garcia-Borron et al. (2005) proposed that the red hair colour alleles are the result of partial loss of function mutations that have varying responses and efficiency when α-MSH is bound to MC1R. A significant amount of research has focused on *MC1R* as a target gene to study for skin cancer, and increased susceptibility to melanomas. Valverde et al. (1996) found that people with one or two *MC1R* polymorphisms compared to wildtype individuals had an estimated risk of 3.9 for getting melanoma. Similarly, Palmer et al. (2000) and Kennedy et al. (2001) suggested that individuals that carry a red hair colour allele have a four to five fold increase in the chance of developing melanoma.

2.3.3.2 Domestic animals

Werth et al. (1996) mapped *MC1R*, the extension locus, to *Bos taurus* chromosome 18. Three common alleles have been reported in *Bos taurus* cattle that affect coat colour. These include the E^D allele or Leu99Pro mutation, a frameshift mutation causing a premature stop codon referred to as the *e* allele and the wildtype allele E^+ (Klungland et al. 1995; Joerg et al. 1996). The dominant E^D allele produces a black, eumelanin, coat colour in cattle when α-MSH is bound to MC1R. The recessive e allele results in red or yellow, phaeomelanin, coat colour in cattle as α-MSH is not able to bind to the MC1R (Klungland et al. 1995; Joerg et al. 1996).

Additional variants in MC1R have been reported. Rouzaud et al. (2000) found a duplication of four amino acids. This duplication, E^I, was shown to exist in French cattle breeds which were predominantly black in colour with an increased frequency in the Gasconne and Aubrac breeds (Rouzaud et al. 2000). Rouzaud et al. (2000) speculates that the coat colour of these cattle breeds could be caused by this E^I allele which might affect protein stability.

Graphodatskaya et al. (2002) found five variants including the E^D and *e* alleles of Klungland et al. (1995). The alleles E^{d1} and E^{d2} were discovered in Brown Swiss cattle. The E^{d1} allele had an amino acid change Arg223Tyr (Graphodatskaya et al. 2002). The E^{d2} allele had a duplication of twelve nucleotides or four amino acids at amino acid position 218, previously described by Rouzaud et al. (2000). An additional e^f allele was found, in the 7[th] transmembrane domain of *MC1R* in a heterozygous red bull which

showed an Ile297Thr amino acid change (Graphodatskaya et al. 2002). These additional polymorphisms all had a dose-dependent effect on cAMP production with the level of α-MSH bound however no association analysis to differences in coat colour were analyzed in these Brown Swiss cattle (Graphodatskaya et al. 2002).

Sequencing of the *Sus scrofa MC1R* revealed seven *MC1R* variants, four related to distinct color phenotypes in both domestic and wild pigs (Kijas et al. 1998). Kijas et al. (1998) reported that the European wild boar was homozygous for the E^+/E^+ genotype. Sequence analysis revealed that the dominant black colour shown in the Large Black and Meishan pigs carried a mutant allele, while the Hampshire pigs carried another mutant allele, both varying from the wildtype sequence by at least one missense mutation (Kijas et al. 1998). Kijas et al. (1998) did find the Leu99Pro which Klungland reported as the cause of the dominant E^D allele in black cattle as part of the haplotype which caused black in the Meishan and Large Black breeds. Similar to cattle, there was a recessive allele (A240T) associated with the phaeomelanin red colour of the Duroc breed of pigs (Kijas et al. 1998).

Vage et al. 2003 postulated that the black pigment observed in the Norweigan Dala, Damara, Black Merino and Black Corriedale breeds of sheep was due to two polymorphisms, Met73Lys and Asp121Asn. Both these mutations have known effects on eumelanin coat colour in other species. Met73Lys has been observed in black chickens (Takeuchi et al. 1996) and the Asp121Asn mutation was implicated in the black hide colour of pigs (Kijas et al. 1998).

Marklund et al. (1996) reported the chestnut *e* allele of horses was the result of a Ser83Phe mutation. Rieder et al. (2001) sequenced DNA from horses of varying phenotypes in horses and did not find any dominant black, gain of function polymorphisms. Instead, black in horses is caused by a recessive mutation in ASIP. Wagner and Reissmann (2000) sequenced 60 horses from the Black Forest breed which were all chestnut in colour. In addition to the *e* allele another sequence variant was found, an Asp84Asn polymorphism which was termed, e^a (Wagner and Reissmann 2000). This variant however could not be related to any specific shade or pattern of the chestnut horses genotyped.

Research in chickens has determined that black plumage is due to *MC1R* polymorphisms (Takeuchi et al. 1996; Kerje et al. 2003). Animals such as Alaskan foxes have a eumelanin coat due to a C123R mutation (Vage et al. 1997).

Previous research involving coat colour and animal production has included analyzing the effect of temperature and absorption of solar radiation of cattle of various phenotypes in warm climates. Darker red calves were capable of absorbing heat more efficiently than lighter coloured cattle and showed an increase of approximately 10 to 20 pounds per year (Schleger 1962). Finch (1985) reported differences between *Bos taurus* and *Bos indicus* cattle species and their ability to handle heat through perspiration. Significantly lower weight gains were noticed in cattle of brown or black colour when compared to white cattle (Finch 1986). Supporting data from Becerril (1993; 1994) showed that Holstein cattle of primarily white coat colour are less sensitive to heat than primarily black cattle. Milk yield, reproduction and body temperature were found to be significantly different due to lower percentage of white area in the Holstein (Becerril 1993; 1994). Hansen and Arechiga (1999) suggested that coat colour and length of coat could possibly affect pregnancy rates of dairy cattle dependent on the percentage of white in the coat of the cow. West (2003) indicated that selection of coat colour may be necessary when in a warm climate, however no production differences were evident in his study of southeastern United States dairy cattle.

Other genes in the colour pathway may impact production traits. Gratten et al. (2008) reported a significant association in dark and light Soay sheep with body size and weight when studying the tyrosinase-related protein 1 (*TYRP1*) gene. These dark sheep c.869GG and c.869GT were significantly heavier at birth than the c.869TT light sheep (Gratten et al. 2008).

2.4 Melanocortin 4 receptor *(MC4R)*

2.4.1 *MC4R* gene structure and expression

MC4R is part of the central melanocortin pathway located in the hypothalamus, an area of the brain in which appetite is regulated (Gantz et al. 1993). *MC4R,* like the other four melanocortin receptors, is a 7 transmembrane G protein coupled receptor, consisting

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of a 332 amino acid protein in one exon (Figure 2.6) (Gantz et al. 1993; Mountjoy et al. 1994; Scioth et al. 2003).   Agonists β- and α-MSH and antagonist agouti related protein (AGRP) regulate a very complex feedback system releasing anorexigenic or orexigenic signals at the *MC4R* (Irani and Haskell-Luevano 2005). Through the binding of α-MSH, satiety signals are released throughout the body by the central nervous system, regulating energy homeostasis and lowering the level of energy that an individual desires or requires (Benoit et al. 2000, Vaisse et al. 2000).
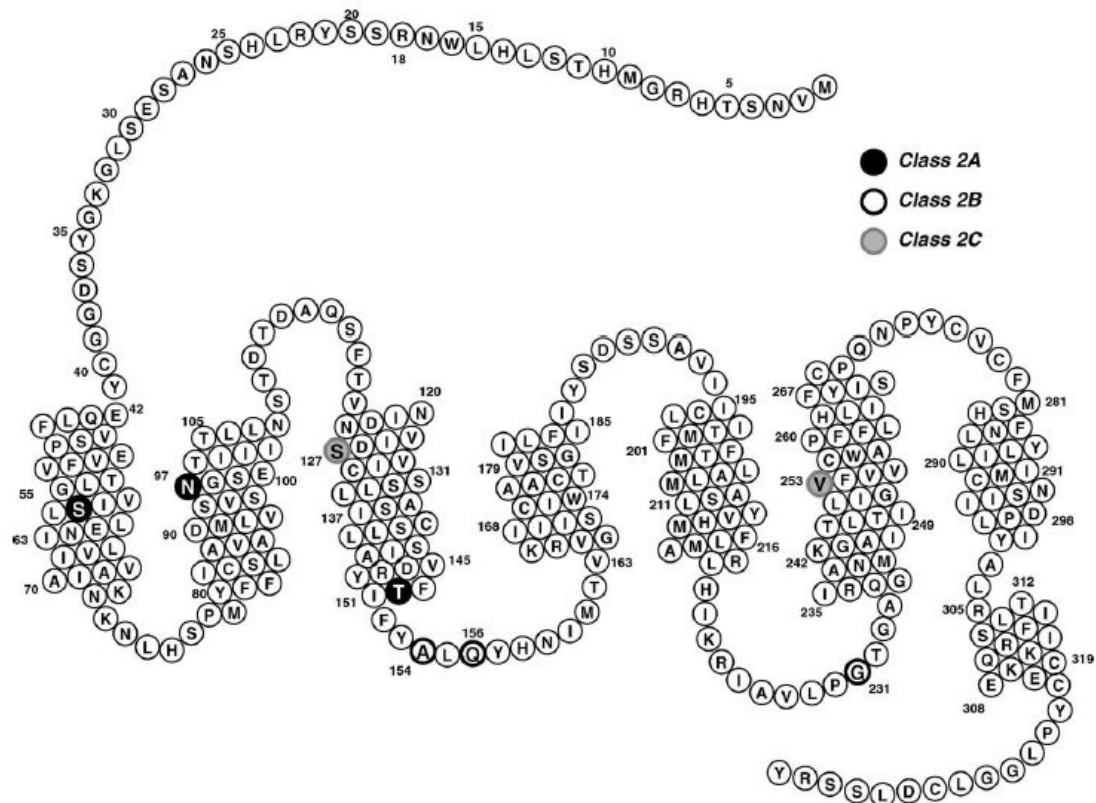
Figure 2.6 Representation of the human *MC4R*. Taken from Govaerts et al. (2005) with permission. Class 2a mutations affect all possible agonists; Class 2b mutations do not affect α-MSH activity however this class does affect basal activity of the receptor; Class 2c mutations affect α-MSH activity

Huszar et al. (1997) were the first to link *MC4R* and obesity. Huszar et al. (1997) was able to demonstrate that *MC4R* knockout mice were hyperglycemic, hyperinsulinemic and hyperphagic. Both male and female mice were heavier and had

significantly increased linear growth while consuming more food in comparison to control mice. Through complex regulation of agonist and antagonists, *MC4R* has been considered one of the key components contributing to obesity in humans (Vaisse et al. 2000; Farooqi et al. 2003).

Increased leptin hormone production in adipose cells promotes leptin signaling decreasing energy intake (Pritchard et al. 2002; Trevaskis and Butler 2005). This decrease in energy intake stimulated by increased leptin hormone production results in increased POMC levels (Gantz et al. 1993; Ollmann et al. 1997; Beckers et al. 2005). Increased POMC levels result in more α-MSH bound to *MC4R*. Agonist activity at the *MC4R* results in the body sending satiety signals to decrease energy intake (Huszar et al. 1997; Pritchard et al. 2002).

2.4.2 *MC4R* agonists

2.4.2.1 α-Melanocyte stimulating hormone (α-MSH)

Originally, the only agonist that researchers believed to effect *MC4R* activity was α-MSH. α-MSH demonstrated the highest activation of cAMP in the first studies conducted on *MC4R* and was always considered the most important agonist from then on for *MC4R* (Mountjoy et al. 1994; Harrold and Williams 2006). Questions began to surface, as α-MSH levels do not elevate in relation to nutritional status which alters *MC4R* and *AGRP* levels. Considerable debate arose as to whether the main agonist of *MC4R* is α-MSH or β-MSH (Harrold and Williams 2006).

β-MSH is a 22 amino acid protein product from the c-terminus of *POMC* (Irani and Haskell-Luevano 2005). Harrold and Williams (2006) found that β-MSH had very high affinities for human *MC4R*.   β-MSH was able to activate the *MC4R* and during restricted feed intake, levels of β-MSH elevated.

2.4.2.2 Agouti related protein (AGRP)

Discovery of the antagonistic effect of ASIP on *MC1R* and *MC4R* lead to discovery of the inverse agonist *AGRP*, another 132 amino acid protein with high homology to *ASIP* and exhibiting similar antagonistic functions (Ollmann et al. 1997). The antagonistic actions of AGRP counter balance the activity of α-MSH at *MC4R*. AGRP is a potent orexigenic peptide responsible for increasing food intake in individuals (Pritchard and White 2005).

α-MSH, β-MSH and AGRP are crucial to energy homeostasis. Through the introduction of the agonists, α-MSH and potentially β-MSH a satiety signal is released when bound to MC4R (Govaerts et al. 2005; Harrold and Williams 2006). Opposing antagonists of MC4R or MC1R, AGRP and ASIP, release an orexigenic signal stimulating food intake (Govaerts et al. 2005; Pritchard and White 2005). No AGRP association analysis has been published in cattle.

2.4.3 *MC4R* polymorphisms affecting obesity and appetite

2.4.3.1 Humans

Obesity research involving humans has lead to the realization that *MC4R* is one of the main contributors to today's obese population (Farooqi et al. 2003). The *MC4R* sequence is highly polymorphic with many variants detected through sequencing. Many different polymorphisms in the *MC4R* sequence have been linked to an obese phenotype. These are not necessarily the same from population to population (Vaisse et al. 1998; Yeo et al. 1998; Vaisse et al. 2000; Farooqi et al. 2003; Larsen et al. 2005). In 2000, there were 15 known *MC4R* mutations (Vaisse et al. 2000) and presently there are 90 known mutations (Govaerts et al. 2005). No specific mutation has been determined to be the main cause for obesity associated with *MC4R*. *MC4R* mutations were found in 5.8% of individuals with early onset obesity studied by Farooqi et al. (2003). Necessary classifications have been developed by different research groups in order to facilitate a

more manageable system to sort variants according to how *MC4R* functionality is affected.

Govaerts et al. (2005) proposed a classification system for *MC4R* mutations. Using intracellular retention as an indicator for the severity of obesity, class 1 mutations (Figure 2.6) cause a major loss of signaling for the *MC4R*. All of these mutations are retained within the intracellular loops. Three groups of mutations expressed at the cell membrane make up the class two mutations (Govaerts et al. 2005). Class 2a includes mutations that affect all possible agonists. Class 2b has no effect on α-MSH activity and affects basal activity of the receptor. Class 2c affects the primary activity induced by α-MSH (Govaerts et al. 2005). This classification system can be linked to the effect of these mutations on the anorexigenic energy regulation (Govaerts et al. 2005).

2.4.3.2 Livestock animals

The effect of *MC4R* on livestock was first analyzed in pigs. Kim et al. (2000a; 2000b) found a polymorphism which had a functional effect on the MC4R protein. A missense mutation causing an amino acid change from aspartic acid to asparagine at position 298 was found through the sequencing of 700 bps of porcine *MC4R* sequence. This Asp298Asn polymorphism was shown to increase backfat, growth rates and feed intakes of pigs carrying the asparagine in a study of 1800 animals with four different genetic backgrounds (Kim et al. 2000a; 2000b).

This initial research led to additional studies to determine if this polymorphism was in fact causative and more in depth studies of the role that this polymorphism plays in overall fatness and growth of the carcass. Houston et al. (2004) replicated the initial results of Kim et al. (2000b) finding a significant difference with growth rates and backfat deposition. Houston et al. (2004) also measured feed intakes and conversion rates. There were significant differences between the animals from high and low production lines for feed intakes. However no significant difference was noted for the feed conversion rate in these pigs.

Bruun et al. (2006) analyzed the effect of *MC4R* Asp298Asn genotype on backfat in various genetic backgrounds, focusing on different breeds. These data suggest that

MC4R does play a role in backfat deposition across several breeds of pigs including Hampshire, Duroc, Landrace and Yorkshire (Bruun et al. 2006). Meidtner et al. (2006) investigated the effect of this *MC4R* polymorphism on feed intake and average daily gain in F2 Mangalitsa and Pietrain pigs. This study supported the previous results by Kim et al. (2000b) as the pigs with the 298Asn allele had increased feed intake. However these animals did not appear to have increased backfat as seen previously (Meidtner et al. 2006). Meidtner et al. (2006) also found another polymorphism, Arg236His, which they suggest could be used as a potential marker for increased growth in the slower growing Pietrain pigs. Fan et al. (2009) analyzed the effect of these two polymorphisms in the pigs on cAMP production compared to the wildtype genotype. Fan et al. (2009) concluded that both the Asp298Asn and Arg236His polymorphisms did not affect cAMP signalling and therefore the mechanism of the Asp298Asn polymorphism is not clear.

The first research reported focusing on *MC4R* in beef cattle began with the sequencing of the *Bos taurus MC4R* gene by Thue et al. (2001). A Val286Leu polymorphism was detected in 20 individuals and was used to map *MC4R* to BTA 24 (Thue et al. 2001). This polymorphism resulted from a C>G substitution. The G allele frequency was 37.5%. Buchanan et al. (2005) then related this Val286Leu polymorphism to animal production, finding that there was a trend towards a difference (P< 0.085) in hot carcass weights in a study of 256 crossbred steers.

Zhang et al. (2008) further characterized the *Bos taurus MC4R* gene by sequencing the 5'UTR. Four 5'UTR polymorphisms were detected, g.-293C>G; g.-193A>T; g.-192T>G; g.-129A>G, in a population of Nanyang cattle (Zhang et al. 2008). A significant difference in the animal's body weight at six months of age was reported when analyzing the SNPs g.-293C>G and g.-129A>G which were shown to be in linkage disequlibrium (Zhang et al. 2008). This significant difference disappeared by the time the animals were twenty-four months of age. This was attributed to the difference in diet from a milk to forage base (Zhang et al. 2008).

MC4R has been implicated in many human obesity and large animal production studies which has lead to further characterization of this gene in other animal species. Zhang et al. (2006) found a polymorphism in the canine coding region that has been shown to increase body weight in Beagles. Behavioural studies in rainbow trout have

shown significant differences in time and length of feeding in fish stimulated with different MC4R antagonists suggesting that MC4R does play a significant role in appetite control of not only mammals, but also fish (Schjolden et al. 2009). Poultry scientists have also turned research focus to implementing MC4R as an indicator for body weight and carcass weights (Qiu et al. 2006; Tao et al. 2008; Sharma et al. 2008). In a North American population of elite broiler chickens, Sharma et al. (2008) found a Ser76Leu polymorphism and associated this with increased body weights in chickens heterozygous for Ser76Leu when compared to chickens homozygous for 76Leu.

2.5 Agouti related protein *(AGRP)*

2.5.1 *AGRP* gene structure and expression

With research focusing on the melanocortin pathway and its role in obesity, the *MC1R* antagonistic ligand agouti signaling protein (*ASIP*) has been considered a candidate gene for obesity. An association was made between obese, diabetic yellow mice, leading to the assumption that ASIP may be the cause of obesity as dark mice were not obese or diabetic (Ollmann et al. 1997). Through the search for the gene causing the lethal yellow obese phenotype in mice, Ollmann et al. (1997) found a protein which was almost identical to agouti signaling protein in both genomic properties and size, which was then termed Agouti Related Protein or (AGRP) or Agouti Related Transcript (ART) (Shutter et al. 1997; Graham et al. (1997).

Agouti related protein is a protein expressed primarily in the arcuate nucleus of the hypothalamus, adrenal gland and testes (Ollmann et al. 1997; Shutter et al. 1997; Graham et al. 1997; Argyropoulos et al. 2002). The expression of AGRP was noted at increased levels in diabetic obese mice (Ollmann et al. 1997; Shutter et al. 1997; Graham et al. 1997).

The preproAGRP human sequence consists of 132 amino acids in four exons which matures into a 108 amino acid protein consisting of a cysteine-rich carboxyl terminus upon cleavage from the signal sequence (Ebihara et al. 1999). This human sequence shows 25% homology to the human ASIP protein (Ebihara et al. 1999).

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Shutter et al. (1997) determined that AGRP was different from ASIP through the use of transgenic mice overexpressing the AGRP protein. These transgenic mice became hyperglycemic after ten weeks, were obese, and had increased body length but were not yellow in colour as the ASIP a^y obese mice were (Shutter et al. 1997). Ollmann et al. (1997) and Graham et al. (1997) both concluded that AGRP acts as a potent antagonist of the MC3R and MC4R regulating weight gain by preventing the signaling of α-MSH at these receptors in the hypothalamus of mice. AGRP sends an orexigenic signal to the individual, stimulating the appetite, suggesting that the animal search out food (Korner et al. 2000).

Nijenhuis (2001) evaluated the effect of amino acids 83-132 of AGRP exposed to MC4R cells in cell culture and found a reduction in adenyl cyclase activity, concluding that AGRP acts as an inverse agonist of MC4R. Fekete et al. (2002) used injection treatment of AGRP to conclude that those rats receiving supplementation of AGRP, increased their food intake, in turn gaining weight more rapidly than control rats. Based on these results, if an allele was found in cattle which had enhanced expression we should anticipate effects on food intake.

Metabolism differences were noted by Small et al. (2003) who discovered an increase in body weights and adipose deposition, when compared to controls, through the reduction of oxygen consumption (8%) in mice stimulated with AGRP treatments. Stimulated mice increased the amount of food eaten. Stimulating the mice with AGRP acts as a simulation of how animals with different genotypes may react if protein production is altered by polymorphisms. Although the 8% reduction does not appear to be large, in humans this could lead to an increase in weight from 75 kg to 112.5 kg until an optimal energy balance is obtained (Small et al. 2003). In larger animals like cattle this difference could be much more profound.

2.5.2 *AGRP* polymorphisms affecting appetite

Genetic variants in *AGRP* that influenced body fat percentage and/or higher fat diet intakes have been found in humans. These polymorphisms in the human *AGRP* sequence include a promoter mutation at g.-38C>T, demonstrating differences in

promoter activity (Schwartz et al. 1996). Schwartz et al. (1996) showed an association between the CC genotype and obesity, and also type 2 diabetes.   An Ala67Thr polymorphism, located in exon 2, has been reported to be associated with late onset obesity through family studies (Argyropoulos et al. 2002). This polymorphism has also been associated with diet preference. Tracy et al. (2007) reported that heterozygous individuals chose a diet in which a smaller proportion of their diet came from fat, when compared to homozygous Ala67Ala individuals.

3.0 HYPOTHESES

Melanocortin 1 Receptor

While primarily involved in the pigmentation pathway it is postulated that *MC1R* will play a role in finishing cattle. It is thought that black cattle, E^D/E^D or E^D/e, will be heavier at an earlier age. With less circulating α-MSH to bind to MC4R in black cattle it is thought they will have larger appetites allowing them to gain and finish sooner than red cattle.

Melanocortin 4 Receptor

The role of *MC4R* in cattle production is anticipated to have an effect on weight of the cattle studied at different time points. *MC4R* and its association with differences in carcass backfat of pigs leads to the anticipation of an effect on adipose deposition in cattle. Polymorphisms found in the *MC4R* sequence of cattle are expected to be causative for differences in shipping and hot carcass weight while affecting the amount of backfat deposited in a feedlot situation. This should result in differences of time on feed when shipped either on a weight or backfat basis.

Agouti Related Protein

AGRP is primarily involved with metabolism of an individual therefore it is expected that cattle of different genotypes will have different rates of gain. Average daily gain and final weight at a common end point are the traits most likely to reflect this. Onset of obesity in humans is seen later in individuals with variant *AGRP* genotypes. Final weights of beef cattle may be affected in cattle of different *AGRP* genotypes. It has

been reported that mice will search out higher fat food to satisfy their energy balance, dependent on their *AGRP* genotype. There may be significant differences in fat deposition in cattle fed different diets, indicating a nutrigenetic effect.

4.0 ASSOCIATIONS OF MELANOCORTIN 1 RECEPTOR GENOTYPE WITH GROWTH AND CARCASS TRAITS IN BEEF CATTLE. *

McLean K.L. & Schmutz S.M.

4.1 Introduction

Four of the five major beef breeds in North America are typically red (Angus, Hereford, Simmental, and Limousin) or black (Angus). The only purebred breed which has traditionally had both red and black coat colour was Angus. In Canada, all Angus are registered as one breed. However in the United States, there are two separate breed registries. Currently some "purebred" percentage Simmental and Limousin cattle occur in black, although it is likely that the black colour exists in both breeds from introduction of black Angus cattle to these breeds in recent years in North America. Since most purebred beef cattle are red or black, the majority of crossbred beef cattle are likewise red or black, or some shade thereof. These colours are considered the result of the genotypes at *Melanocortin 1 Receptor (MC1R)* (Klungland et al. 1995; Joerg et al. 1996). *MCIR* encodes a seven transmembrane receptor which controls the development of eumelanin resulting in black or brown animals or phaeomelanin, causing cream to red animals based on the binding of α-melanocyte stimulating hormone (α -MSH); (Jackson 1993).

There have been three main functional alleles found at the *MCIR* locus determining coat colour in *Bos taurus* (Klungland et al. 1995; Joerg et al. 1996), with some additional variants reported (Vanetti et al. 1994; Rouzaud et al. 2000; Graphodatskaya et al.2000; Graphodatskaya et al. 2002). The E^D allele is due to a Leu99Pro amino acid change and considered dominant (Klungland et al. 1995). Cattle with at least one E^D allele are typically some shade of black. The recessive allele *e* is due to a frameshift mutation (Y155ter), leading to a premature stop codon (Joerg et al. 1996).

Homozygous *e/e* cattle produce predominantly phaeomelanin and are typically red, cream, or tan (Klungland et al. 1995).

The E^+ allele causes *MC1R* to be receptive to the agouti peptide produced by *ASIP* and α-MSH, therefore, eumelanin may be synthesized when α-MSH is bound, and phaeomelanin if agouti is bound instead (Chen et al. 1996). All brindle cattle studied to date had at least one E^+ allele (Unpublished data, Schmutz et al. 2008) and some breeds, such as Braunveih, are fixed at this allele, but it is relatively rare in beef cattle.

MC1R is located on cattle chromosome 18 (Werth et al. 1996). A QTL for fat thickness near the region of *MC1R* was previously identified in a pilot study (data not shown) making *MC1R* a candidate gene for cattle meat quality studies.

Although some previous studies have tried to show that different coat colours absorb solar radiation differently and thereby affect the cattle, particularly in warmer climates (Schleger 1962; Finch 1985; Finch 1986; Becerril et al. 1993; Becerril et al. 1994; Hansen & Arechiga 1999; West 2003) our hypothesis is not based on this idea. These studies focused on black versus white, not black versus red cattle.

The melanocortin genes have been implicated in many major pathways in the body, ranging from pigmentation to energy intake (Cone 2005). A reduction in appetite occurs with the binding of α-MSH to the *Melanocortin 4 Receptor (MC4R)* decreasing energy intake (Huszar et al. 1997). For this reason we hypothesized that black cattle with an $E^D/\_$ genotype would gain weight and deposit fat more readily than *e/e* animals due to more α-MSH binding to *MC1R*. Cattle of *e/e* genotype (red to cream colour) would direct more α-MSH to the *MC4R* pathway reducing overall appetite, taking longer to finish and deposit fat. This study was designed to determine if *MC1R* genotype or coat colour (black or red) affected production traits in the feedlot or carcass traits using ultrasound and blue tag measurements.

4.2 Materials and methods

4.2.1 Animals

Three hundred and twenty-eight steers of all colours including black, red, tan, and cream were purchased in Saskatchewan and assembled into 12 pens of 30 at the University of Saskatchewan Beef Research Station. Care and housing for the cattle was carried out following guidelines set by the Canadian Council for Animal Care. These cattle were purchased in small groups, at several different auction marts across Saskatchewan, over a one week period. We suggest that most of these cattle were crossbred, similar in age and reared under different management and handling practices prior to entry into the feedlot. However, neither the specific breed nor birth date of these cattle were known. All five major beef breeds in Canada were represented in this trial, determined by visual inspection of the cattle.

The cattle were given the chance to acclimatize with their environment for fourteen days prior to the official start of test. Cattle were sorted to allow for equal numbers of black to grey and red to cream coloured cattle in each pen and monitored daily to ensure the health of animals was maintained. The animals were backgrounded on the same diet for 70 days and then fed one of two finishing diet treatments for at least 71 additional days. These diets were prepared with two different processing treatments, pelleted barley (PB) (n=167) and rolled barley (RB) (n=161) (Williams et al. 2008, Appendix F). The feedlot steers were fed to reach optimal carcass weights and finish characteristics with all cattle on trial for at least 141 days, but some as long as 232 days. Ultrasound backfat (USBF) levels of 12 mm were desired before the animal was sent for slaughter. However if the steer reached a weight of 680 kg before this fat level, the animal was slaughtered. The 12 mm of backfat prerequisite was based on the Canadian beef industry grading system and consumer preference.

This feeding and slaughter design is similar to a typical Canadian feedlot which purchases weaned crossbred cattle of multiple colours and sorts them by frame and receiving weight, not colour. Normally these cattle would be slaughtered when judged to be "finished" either by eye or ultrasound backfat. Some may be held later than others in the pen, however this is not common practice.

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4.2.2 Growth and carcass Measurements

Weight, ultrasound data for longissimus dorsi (USLD) (cm^2) and backfat depth (mm) were obtained from the live steers periodically during the trial period. In addition, these data were obtained on the shipping date, which varied among individual steers. Hot carcass weight was obtained at the slaughter facility. Canadian Beef Grading Agency graders at the slaughterhouse supplied the carcass *l. dorsi* area (cm^2) and carcass grade fat and average fat measurements.

4.2.3 Isolation of DNA, PCR and PCR-RFLP

Individual animals were bled from the jugular vein. DNA was extracted from each animal using a simple blood lysis extraction (Schmutz et al. 1995).

MC1R genotyping was performed by conducting two PCR-RFLP reactions which were previously described (Klungland et al. 1995; Joerg et al. 1996). Primers P6 and P7 were used to determine if there was an *e* allele present after digestions with *Msp*1 (Klungland et al. 1995). If the animal was not *e/e,* then the primers E5 and E6 were used to distinguish E^+ from E^D after digestion with *Aci1* (Joerg et al. 1996).

The 15 µl PCR reaction included 1.5 µl 10xPCR buffer (Gibco), 0.3 µl of 10mM dNTP, 0.45 µl of 50mM $MgCl_2$, 1.0 µl of 10 pm/µl selected forward primer, 1.0 µl of 10 pm/µl reverse primer, 0.5 U *Taq* polymerase (Invitrogen), and 1.0 µl of extracted DNA ~ 50-100 ng in concentration. The cycling protocol involved 4 min denaturing at 94º C, then 38 cycles of 50 s at 94º C, 50 s annealing at 63º C (P6 and P7) or 59º C (E5 and E6), 50 s at 72º C ending with four minutes of extension time at 72º C.

4.2.4 Statistical analysis

The statistical software SAS (SAS Institute Inc., Cary, NC) was used to perform data analysis. The MIXED model of SAS was performed on the steer growth and carcass measurements with diet and genotype represented as fixed effects. Days on feed was used

as a covariate for shipping weight, hot carcass weight and l. dorsi area. Analysis for presence of an interaction between genotype and the two finishing diets was conducted using the model:

$$Y_{ijk} = \mu + \text{Genotype}_i + \text{Diet}_j + \text{Genotype x diet}_{ij} + e_{ijk}$$

Where Y_{ijk} is the dependent variable (backfat measurement, hot carcass weight, etc.); μ is the overall mean of the population; Genotype is the effect of the *MC1R* genotype on this dependent variable; Diet $_j$ represents the effect of diet on the observation; Genotype x diet$_{ij}$ is the effect of any interaction between the genotype at *MC1R* and the two differently processed diets and e$_{ijk}$ is the random error for this observation.   For comparing differences between genotypes the PDIFF option was used with a significant P-value determined at P<0.05.

Genotype was the primary focus of this analysis. It was considered a fixed effect and was analyzed to determine its effect on the different growth and carcass traits measured over the course of the trial, and whether an additive effect of the E^D allele occurred. When an interaction was present between genotype and diet, genotype effect on the various growth and carcass traits was analyzed separately within each diet.

Analysis by coat colour was conducted in a similar fashion with cattle classified as either black or red, irrelevant of shade. This analysis allowed for a simple comparison of black to grey (eumelanin) cattle with red to cream (phaeomelanin) cattle for all of the traits measured. This analysis was conducted to determine if genotyping was necessary, or if visual coat colour classification was sufficient to determine effects on the production traits measured.

4.3 Results

Frequencies of cattle of the various *MC1R* genotypes are shown in Table 4.1. The absence of E^+/E^+ cattle and relatively low numbers of cattle of either E^D/E^+ or E^+/e genotype dictated that the genotype analysis be conducted on the 295 cattle of the three main *MC1R* genotypes (E^D/E^D, E^D/e, e/e) (Appendix A, Table A.1).

Since it was determined that there was a diet x genotype interaction (P <0.05) for all growth traits measured after the first 70 days on trial, and the majority of the traits to be analyzed based on coat colour of the cattle, cattle fed each finishing diet were analyzed separately. For the majority, there was no diet x genotype interactions (P >0.05) for the carcass measures obtained, therefore the entire group was analyzed as a whole.

Table 4.1 Frequency of each *MC1R* genotype among the 328 feedlot steers

Genotype	Pelleted Diet		Rolled Diet		Total
	n	**Frequency (%)**	**n**	**Frequency (%)**	**Total**
E^D/E^D *(Black)*	40	24.0	36	22.4	76
E^D/e *(Black)*	91	54.5	87	54.0	178
E^D/E^+ *(Black)*	9	5.4	11	6.8	20
e/e (Red)	22	13.2	19	11.8	41
e/E^+ *(Red)*	5	3.0	8	5.0	13
E^+/E^+	0		0		0
Total	**167**		**161**		**328**

4.3.1 Growth traits

At the start of the feeding period, which is presumably near weaning for most cattle, there was a significant difference in weight based on genotype (Table 4.2) and coat colour (Table 4.3). Black cattle of either E^D/E^D or E^D/e genotype cattle were significantly heavier than the red cattle of *e/e* genotype at the start of backgrounding (Table 4.2 & 4.3). This difference persisted through to the end of the 70 day backgrounding period and through to the end of the period all cattle were in the feedlot together (day 141). An additive effect based on the number of E^D alleles was observed in cattle on the pelleted barley diet, but the cattle on the rolled barley diet did not show this additive effect (Table 4.2). Black cattle finished in significantly fewer days on feed than red cattle (Table 4.3).

Table 4.2 Growth traits of the 295 cattle based on MC1R genotype (LS Means ± SEM)[z]

Trait	E^D/E^D	E^D/e	e/e	P-Value
Weight, Start of Backgrounding (kg)	326.1 ± 2.7 a[y]	324.5 ± 1.8 a	310.4 ± 3.7 b	0.001
Weight, End (day 70) Of Backgrounding (kg)	415.4 ± 3.3 a	412.9 ± 2.2 a	397.3 ± 4.5 b	0.003
Day 141 Weight PB (kg)[x]	574.2 ± 5.9 a	561.2 ± 3.9 ab	547.3 ± 7.4 b	0.017
Day 141 Weight RB (kg)	570.4 ± 6.1 ab	584.6 ± 3.9 a	551.6 ± 8.6 b	0.002
Days on Feed PB	185.0 ± 4.1 a	197.1 ± 2.8 b	218.2 ± 5.3 c	<0.001
Days on Feed RB	185.8 ± 4.3 a	184.4 ± 2.8 a	212.2 ± 6.0 b	0.001

[z]LS Means ± SEM = Least squares mean ± standard error of the mean
[y]Mean values with different letters in the same row are significantly different at the P<0.05 level.
[x]Day 141 weight (kg) was the last day all cattle were in the feedlot since shipping dates varied.

Table 4.3 Growth parameters of 328 cattle analyzed by coat colour (LS Means ± SEM)[z]

Trait	Black	Red	P-Value
Start of Backgrounding Weight (kg)	325.5 ± 1.5 a[y]	315.0 ± 3.2 b	0.003
End of Backgrounding Weight (kg)	414.0 ± 1.8 a	403.5 ± 4.0 b	0.016
Day 141 Weight, Pelleted Diet (kg)[x]	564.2 ± 3.1 a	547.6 ± 6.7 b	0.026
Day 141 Weight, Rolled Diet (kg)	581.5 ± 3.3	571.3 ± 7.5	0.212
Days on Feed	188.7 ± 1.6 a	210.3 ± 3.5 b	<0.001

[z]LS Means ± SEM = Least squares mean ± standard error of the mean
[y]Mean values with different letters in the same row are significantly different at the P<0.05 level.
[x]Day 141 weight (kg) was the last day all cattle were in the feedlot since shipping dates varied.

4.3.2 Carcass measurements

Cattle with different *MC1R* genotypes displayed significant differences for some traits at finish and slaughter (Table 4.4). Likewise these same traits showed significant differences between the black and red groups (Table 4.5). Although the black cattle had significantly more backfat by day 120 of the trial than the red cattle, the l. dorsi estimates by ultrasound were not different (Table 4.5). No significant differences were found based on genotype or colour with the carcass measures: dressing percentage, meat yield, or marbling.

Table 4.4 Carcass traits of the 295 cattle based on MC1R genotype (LS Means ± SEM) [z]

Trait	E^D/E^D	E^D/e	e/e	P-Value
US Backfat Day 120, PB (mm)	6.4 ± 0.34 a[y]	5.6 ± 0.23 a	4.3 ± 0.44 b	0.001
US Backfat Day 120, RB (mm)	6.0 ± 0.36 a	6.6 ± 0.23 a	4.4 ± 0.51 b	0.001
US Longissimus Dorsi Area, Day 120 (cm^2)	86.1 ± 0.96	84.0 ± 0.63	85.6 ± 1.31	0.146
Shipping Weight, PB (kg)	614.9 ± 3.31 a	629.0 ± 2.25 b	637.5 ± 4.29 b	<0.001
Shipping Weight, RB (kg)	615.1 ± 3.42 a	618.6 ± 2.21 a	632.5 ± 4.90 b	0.013
Hot Carcass Weight (kg)	365.5 ± 1.89 a	370.6 ± 1.25 b	381.8 ± 2.58 c	<0.001
Carcass Average Fat (mm)	10.5 ± 0.28 ab	11.2 ± 0.18 a	10.3 ± 0.38 b	0.042
Carcass Longissimus Dorsi Area (cm^2)	90.7 ± 0.47 a	92.7 ± 0.31 b	98.0 ± 0.65 c	<0.001

[z]LS Means ± SEM = Least squares mean ± standard error of the mean
[y]Mean values with different letters in the same row are significantly different at the P<0.05 level.

Table 4.5 Carcass quality of the 328 cattle based on coat colour (LS means ± SEM) [z]

Trait	Black	Red	P-Value
US Backfat Day 120 (mm)	6.2 ± 0.13	4.6 ± 0.29	<0.001
US Longissimus Dorsi Area Day 120 (cm^2)	85.7 ± 1.16	84.6 ± 0.52	0.392
Shipping Weight, Pelleted Diet (kg)	623.9 ± 1.82	639.6 ± 3.97	<0.001
Shipping Weight, Rolled Diet (kg)	616.6 ± 1.74	635.4 ± 4.06	<0.001
Hot Carcass Weight, Pelleted Diet (kg)	371.3 ± 1.45	383.8 ± 3.12	<0.001
Hot Carcass Weight, Rolled Diet (kg)	365.6 ± 1.38	379.2 ± 3.20	<0.001
Carcass Average Fat, Pelleted Diet (mm)	10.7 ± 0.21	10.8 ± 0.49	0.817
Carcass Average Fat, Rolled Diet (mm)	11.4 ± 0.20	10.2 ± 0.45	0.021
Carcass Longissimus Dorsi Area, Pelleted Diet (cm^2)	92.5 ± 0.35	98.2 ± 0.77	<0.001
Carcass Longissimus Dorsi Area, Rolled Diet (cm^2)	91.2 ± 0.34	96.2 ± 0.80	<0.001

[z]LS Means ± SEM = Least squares mean ± standard error of the mean

The cattle were primarily shipped when they reached a target backfat by ultrasound and therefore no significant differences were expected in slaughter backfat. However, due to diet interactions and fewer red cattle meeting the minimum fat measurements, black steers fed the RB diet displayed significantly more carcass average fat than red steers (Table 4.5). In this population, 78% of the E^D/E^D cattle and 70% of the E^D/e cattle reached the 12 mm US backfat measurement target before slaughter, whereas only 41% of the e/e cattle reached the desired 12 mm of backfat.

4.4 Discussion

The E^+/E^+ genotype was absent in these cattle (Table 4.1). Although this genotype appears to be fixed in breeds such as Brown Swiss and Jersey (Berryere et al. 2003), these breeds are not common contributors to beef cattle in Western Canada. Since the allele frequency of E^+ was 5.0%, it may not be cost effective for feedlot owners to

have cattle genotyped for this allele. Using the *MC1R* genotyping method reported here, where the *e* allele is detected first, cattle with the E^+ allele would be classified as E^D. However, if red coat colour was also considered, then the e/E^+ red could be distinguished, but the E^D/E^D cattle would not be distinguished from the E^D/E^+ or the E^+/E^+ cattle.

Significant differences in weight at the beginning of the feeding period, found between cattle of different *MC1R* genotypes (Table 4.2) and coat colour (Table 4.3), suggest that cow-calf producers who market calves at this time might also see a profit differential between the groups. Cattle of E^D/E^D genotype, fed the PB diet, were heavier than cattle of *e/e* genotype from start to day 141 of the finish period (Table 4.2).

Although the weight differences were evident throughout this study, fat is accrued during the finish period (Owens et al. 1995). More cattle of E^D/E^D genotype reached the finish backfat target sooner and therefore had fewer days on feed than the *e/e* red cattle (Table 4.2). Conversely the red cattle of *e/e* genotype were slaughtered with significantly higher shipping weights, resulting in increased hot carcass weights when compared to E^D/E^D cattle (Table 4.4).

Obese *MC4R* knockout mice were found to have increased appetites because there was no functional *MC4R* that would bind α-MSH to decrease appetite (Huszar et al.1997). It is tempting to speculate that the differences observed in weight of the cattle were related to different α-MSH binding to MC4R, among cattle of different *MC1R* genotypes. Although α-MSH binds to MC1R, MC3R, MC4R and MC5R (Cone 2005), the mutation in *MC1R* of red cattle prevents binding (Klungland et al. 1995). Mutations in other species that cause a decrease in the production of the peptide in either *POMC*, the gene that encodes α-MSH, or in *MC4R*, affect fat deposition in a gene dosage effect manner (Cone 2005).

As in any study documenting associations, it is also possible that the *MC1R* mutation causing red coat colour is in linkage disequilibrium with another mutation in another gene that causes weight and fat deposition differences in cattle. Since red versus black coat colour is caused by this *MC1R* mutation, coat colour could be an indirect selection marker for the *MC1R* genotype or a genotype at a closely linked gene.

Slight differences in magnitude of effect were noticed between cattle on the two diets (Table 4.2 - 4.5). This could be considered a nutrigenetic effect (Kussman et al.

2006), whereby the effect of genotype is not equal across all diets. The RB diet may be more slowly digested allowing for adequate rumen buffering, compared to the pelleted diet (Beauchemin et al. 2001). Cattle fed the rolled diet had significantly higher gains than those fed the pelleted diet (Williams et al. 2008). The black cattle may be genetically capable to utilize the PB diet more readily than the red cattle.

While these data present a significant difference in the growth parameters of feedlot cattle of different *MC1R* genotype or resulting colour in Western Canada, the diet interactions present perhaps offer some indication that potential tailored feeding by genotype or colour would help cattle reach finish more efficiently. By changing diet composition fed to the *e/e* or red cattle, increased muscle mass and fat deposition may occur more quickly.

A crucial indicator of efficiency for a feedlot is the overall length of time an animal requires to reach finish. This trial was established with a target end point of 12mm of backfat, and for those cattle that did not reach this endpoint in a timely fashion, 680 kg (Williams et al. 2008). Cattle that gained well were shipped prior to those animals needing longer to reach optimal backfat. The mean trial length or days on feed, for each group of cattle, is a strong indicator of economic efficiency per *MC1R* genotype or coat colour. On both diets (Table 4.2), the E^D/E^D cattle needed significantly less time to reach slaughter criteria than *e/e* cattle. Likewise the mean days on feed was lower for the black cattle than the red cattle (Table 4.3). These data suggest that by sorting cattle of different coat colour into two separate pens, the feedlot operator would see a difference of approximately 22 days in the length of time necessary for the animals in each pen to reach a desirable finish. It is also likely that the uniformity of the cattle in the pen, for several slaughter traits, would be improved. However, sorting cattle into three pens based on the three common genotypes might only be worthwhile on some diets, i.e. such as the pelleted diet in this study (Table 4.2).

Since differences in growth patterns based on *MC1R* genotypes or resulting colour were observed, it was anticipated that the overall carcass composition would be different also (Table 4.4 & 4.5). An additive effect based on the number *e* alleles was observed for carcass l. dorsi area (Table 44). Since carcass and shipping weights were higher in the *e/e* cattle, it is not surprising that they also had larger l. dorsi area.

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Similar differences in finish and carcass traits were observed between cattle of different coat colour (Table 4.5), as on genotype. Therefore sorting by coat colour may be sufficient to see a difference in the carcass traits within a pen of cattle, avoiding the cost of *MC1R* genotyping.

4.5 Acknowledgements

5.0 MELANOCORTIN 4 RECEPTOR POLYMORPHISM IS ASSOCIATED WITH CARCASS FAT IN BEEF CATTLE.[*]

5.1 Introduction

Melanocortin 4 receptor (*MC4R*) has been implicated in the control of appetite in mammals (Huszar et al. 1997). MC4R is part of the central melanocortin pathway located in the hypothalamus, an area of the brain in which appetite is regulated (Gantz et al. 1993). This 332 amino acid protein consists of one exon, and like the other four melanocortin receptors, is a 7 transmembrane G protein coupled receptor (Gantz et al. 1993; Mountjoy et al. 1994; Scioth et al. 2003). When the agonist, α-melanocyte stimulating hormone (α-MSH) is bound to MC4R, appetite is decreased (Huszar et al. 1997).

MC4R is an excellent candidate gene for association studies in livestock with its role in appetite control. An association between *MC4R* genotype and increased carcass growth and fatness was reported in swine (Kim et al. 2000a,b). An Asp298Asn mutation resulted in increased growth rates, daily gains and backfat deposition. This has been replicated in other studies (Houston et al. 2004; Kim et al. 2006; Meidtner et al. 2006).

Studies focused on the role of *MC4R* in beef cattle production have been relatively rare in comparison to those in humans or swine. Haegeman et al. (2001) obtained the coding sequence using bovine cDNA cloning with primer walking to map *MC4R* to BTA24 (Genbank AF265221). Thue et al. (2001) also mapped *MC4R* to BTA24 and later Buchanan et al. (2005) showed that a Val286Leu SNP showed a trend with increased hot carcass weights. 5'UTR polymorphisms g.-293 C>G and g.-129 A>G were shown to be associated with increased weight and average daily gain at six months of age in Nanyang cattle, but not at 24 months (Zhang et al. 2008). Zhang et al. (2008) suggested

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[*] This chapter was prepared as a Short Communication for Animal Genetics. It has been modified since to incorporate the suggestions of committee members.

this lack of continuity in the older cattle was due to the change in diet, from milk to a forage based diet.

Beef cattle production in recent years has become much more focused on producing cattle that will develop an optimal carcass, a balance of lean meat yield and carcass fat. As marker assisted selection and DNA testing becomes more widely available and understood, producers are looking for more information for their decision making. The aim of this study was to determine if *MC4R* could be used as a genetic test to assist in future selection of animals with the potential for increased carcass qualities.

5.2 Materials and methods

5.2.1 Cattle

Three hundred and eighty two random crossbred Canadian steers were purchased at weaning from auction marts across Saskatchewan and assembled at the University of Saskatchewan Beef Research Station. Care and housing for the cattle followed guidelines set by the Canadian Council for Animal Care. These steers were backgrounded for 152 days prior to being shipped to Poundmaker Agventures feedlot in Lanigan, SK for finishing for 131 days. These Canadian steers were fed a diet consisting primarily of barley silage and grain with a gradual increase in percentage of barley grain (Appendix G). Growth measurements were obtained at regular intervals throughout the feeding period. The cattle were shipped over a period of two weeks to XL Beef Inc., Moose Jaw, SK. Carcass measurements were obtained from the Canadian Beef Grading Agency graders at the slaughter plant.

A population of 985 American steers was used for validation. These cattle were managed at Cactus Feeders research feedlot in Northern Texas. These steers were slaughtered over the course of two days at Tyson Fresh Meats in Amarillo, Texas. These steers were crossbred with some proportion of *Bos indicus*. These cattle were fed a typical American feedlot finishing diet (Appendix H) and all were treated with Zilpaterol. Carcass measurements were obtained from the USDA graders at Tyson Fresh Meats.

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5.2.2 PCR, sequencing and PCR RFLP

The standard PCR protocol used throughout this analysis utilized a 15 µl PCR reaction. This reaction mixture included 1.5 µl 10xPCR reaction buffer (Fermentas), 0.90 µl of 25mM $MgCl_2$, 0.3µl of 10mM dNTPs, 1.0 µl of 10 pm/µl forward primer, 1.0 µl of 10 pm/µl reverse primer, 0.5 U *Taq* polymerase (Fermentas), and 1.0µl of extracted DNA of ~ 50-100 ng in concentration. The cycling protocol was 4 min denaturing at 94º C, with 38 cycles of 50 s at 94º C, 50 s at 60º for annealing, 72º C for 50 s ending with four minutes of extension at 72º C.

Sequencing of the *MC4R* gene was performed using primers *MC4R* Gen Forward [5'-GCAAAGACCTGCATGCCTCCGACT-3'] and *MC4R* End Reverse [5'-CTGTCTCTGAGAAACACACATAGT-3'] designed from the existing bovine *MC4R* sequences NW_001494269 (Shotgun) and AF265221 (mRNA). The Standard PCR protocol was used to amplify an 1845 bp product. This product was then excised from an agarose gel and extracted using a gel extraction kit (Qiagen). This product was sent to the Plant Biotechnology Institue (PBI) Lab where an Applied Biosystems sequencer was used for sequencing of the entire *MC4R* gene including the 5'UTR and 3'UTR regions.

A PCR RFLP was designed for one variant, g.989G>A. Primers MC4R 3'UTR F [5' GACCCTCTGATTTATGCCCTG-3'] and MC4R 3'UTR R [5'-GCTGTGGCTGATACAGACTGT-3'] were used to amplify a product of 195 bp. Using the enzyme *Fspb1* (Fermentas), a natural cut site was present and g.989A animals showed DNA fragments at 73 and 122 bps on a 4 % agarose gel. Steers with the g.989G allele showed fragments at 22, 73 and 100 bps (Appendix B, Figure B.1).

5.2.3 Statistical analysis

The statistical software SAS (SAS Institute Inc., Cary, NC) was used to perform statistical analysis on the various carcass traits within each population using the SAS procedure NPAR1WAY. A Mann-Whitney U test determined if there was a significant association ($P <0.05$) between the means of the two different genotypes for each carcass trait.

5.3 Results and discussion

Sequence was obtained from 20 random crossbred *Bos taurus* cattle. One novel Ser330Asn polymorphism was discovered (Genbank FJ430565) which changed a G to an A at nucleotide 989, in addition to the previously published polymorphisms (Thue et al. 2001; Zhang et al. 2006; Zhang et al. 2008). Only g.989GA or g.989GG genotypes were detected in the feedlot steers. No homozygous g.989AA cattle were found in either population. A minor allele frequency of 0.01 was found for the Canadian population and 0.02 in the American validation population.

Significant carcass effects were observed in the Canadian steer population and were validated in the American steer population. Fat deposition appears to be significantly different between cattle of the two g.989 genotypes (Table 5.1 and 5.2). The Canadian g.989GA cattle showed significantly higher grade fat (*P*=0.036) and lower lean meat yield (*P*=0.032) (Table 5.1). Similarly, in the validation population of the American steers, cattle with a g.989GA genotype had increased backfat (*P*=0.031) resulting in a less desirable stamped yield grade (*P*=0.022). The g.989GG steers had significantly larger ribeye area when compared to the g.989GA steers in the validation population (Table 5.2).

Table 5.1 Carcass data for the 382 Canadian steers in relation to *MC4R* Ser330Asn genotype (Mean ± SEM).

Trait	g.989GA	g.989GG	P-Value
	n=8	*n=374*	
Grade Fat, mm	11.5 ± 1.65	8.3 ± 0.19	0.036
Average Fat, mm	12.1 ± 1.33	9.7 ± 0.19	0.084
Ribeye Area, cm^2	96.9 ± 3.71	101.7 ± 0.61	0.368
Lean Meat Yield, %	58.0 ± 1.56	61.1 ± 0.16	0.032

Table 5.2 Carcass data for the American steer population of 985 steers in relation to *MC4R* Ser330Asn genotype (Mean ± SEM)

Trait	g.989GA	g.989GG	P-Value
	n=20	*n=965*	
Fat Thickness, mm	12.5 ± 0.93	10.6 ± 0.13	0.031
Marbling score[z]	40.7 ± 1.30	38.3 ± 0.23	0.078
Ribeye Area, cm^2	87.7 ± 2.65	94.2 ± 0.39	0.031
Stamped Yield Grade[y]	2.2 ± 0.17	1.8 ± 0.03	0.022

[z] 10= practically devoid, 100= Abundant
[y] 1=Highest yield grade, 5=Lowest yield grade

Lean meat yield was significantly higher for the Canadian g.989GG steers due to decreased average and backfat measurements (Table 5.1). The lean meat yield percentage necessary to make yield grade one in Canada is 59 % (Canadian Beef Grading Agency, 2009). The lean meat yield percentage observed in the g.989GG cattle would classify the carcasses as Canadian yield grade one, unlike the g.989GA cattle which would be classified into the Canadian yield grade two. Similarly the stamped yield grade for the American steer population is more desirable in the g.989GG cattle as they have a lower fat to lean tissue ratio than the g.989GA cattle (Table 5.2). In both grading systems a yield grade score of one is desirable. The Canadian yield grade is a scale of three, while the American yield grade is a five point scale. Considering that muscle accretion occurs prior to fat deposition (Owens et al. 1995), when shipped at an earlier date these g.989GA cattle could possibly have increased lean meat yield at an earlier time period which cannot be shown in this analysis because of the way that these cattle were slaughtered.

These data suggest that *MC4R* does play a contributing role in carcass development of cattle. Humans and rodents with *MC4R* polymorphisms have been shown to have an increased risk of obesity and increased overall weight gains (Huszar et al. 1997; Farooqi et al. 2000; Vaisse et al. 2000; Farooqi et al. 2003). With these polymorphisms in humans and one variant in swine (Kim et al. 2000b) resulting in increased adipose tissue deposition, it is not surprising that the g.989 *MC4R* genotype was associated with increased fat in two populations of beef cattle.

The Asp298Asn polymorphism in pigs has been reported to have an effect on overall carcass composition (Kim et al. 2000a,b; Houston et al. 2004). Pigs with the Asn298 allele were reported to have increased backfat and average daily gain. Using in-vitro gene expression, Kim et al. (2004), found that cells with the Asn298 variant could not stimulate cAMP production in response to bound α-MSH and therefore postulated that pigs homozygous Asn were able to gain weight and become fatter more efficiently than pigs homozygous for the Asp298 allele. The wild-type variant Asp298 was capable of regular *MC4R* signaling and stimulation of cAMP, in turn reducing appetite (Kim et al. 2004).

MC4R sequence alignment across species (Supplemental Figure 1) shows that the Ser330 amino acid is highly conserved across mammals, although not in the chicken. The Ser330Asn cattle polymorphism discovered in this study and Asp298Asn pig polymorphism (Kim et al. 2000a,b) both lie within the seventh transmembrane of the melanocortin 4 receptor. Scioth et al. (1998) suggested that the seventh transmembrane of melanocortin receptors is a region of high homology and important for ligand binding. It is possible polymorphisms in this region could prevent proper signaling of the receptor and reduce efficiency of the receptor. In the present study this may be the cause of increased carcass fat in cattle carrying the g.989A allele.

This study suggests that the Ser330Asn *MC4R* polymorphism could be utilized as a method of selecting for increased lean meat yield or carcass grade fat, depending on the goals of the beef cattle operation. While the minor allele frequency of the g.989A allele does occur at a low frequency, genotyping for this polymorphism may be more cost effective when used in a panel of DNA tests.

Supplemental Figure 1. *MC4R* amino acid alignment across species.

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Cattle   MNSTQPLGMH TSLHSWNRSA HGMPTNVSES LAKGYSDGGC YEQLFVSPEV
Pig      ----HHH--- ----F----T Y-LHS-A--P -G----E--- ----------
Dog      ----LQH--- ----F----T Y-QHG-AT-- -G---P---- ----------
Human    -VNSTHR--- ----L----S YRLHS-A--- -G-------- ----------
Mouse    ----HHH--Y ----L----S Y-LHG-A--- -G--HP---- ----------
Chicken  --F-HR-TL QP--F--Q-N GLHRG*A--P S---H-S--- ----------

Cattle   FVTLGVISLL ENILVIVAIA KNKNLHSPMY FFICSLAVAD MLVSVSNGSE
Pig      ---------- ---------- ---------- ---------- ----------
Dog      ---------- ---------- ---------- ---------- ----------
Human    ---------- ---------- ---------- ---------- ----------
Mouse    ---------- ---------- ---------- ---------- ----------
Chicken  -----I---- --V------- ---------- ---------- ----------

Cattle   TIVITLLNST DTDAQSFTVD IDNVIDSVIC SSLLASICSL LSIAVDRYFT
Pig      ---------- --------N ---------- ---------- ----------
Dog      ---------- --------N ---------- ---------- ----------
Human    ---------- --------N ---------- ---------- ----------
Mouse    ---------- --------N ---------- ---------- ----------
Chicken  --------NI --------IN ---------- ---------- ----------

Cattle   IFYALQYHNI MTVKRVAITI SAIWAACTVS GVLFIIYSDS SAVIICLITV
Pig      ---------- ------G-I- -C---V---- ---------- ----------
Dog      ---------- ---RRVG-I- -C-------- -I-------- T--------M
Human    ---------- ------G-I- -C-------- -I-------- ---------M
Mouse    ---------- ---R--G-I- -C-------- ---------- --------SM
Chicken  ---------- ------GVI- TC-------- -I-------- -V------SM

Cattle   FFTMLALMAS LYVHMFLMAR LHIKRIAVLP GSGTIRQGAN MKGAITLTIL
Pig      ---------- ---------- ---------- -T------- ----------
Dog      ---------- ---------- ---------- -T------- ----------
Human    ---------- ---------- ---------- -T-A----- ----------
Mouse    -----V---- ---------- ---------- -T------T- ----------
Chicken  -----I---- ------M--- M---K----- -T-P------ ----------

Cattle   IGVFVVCWAP FFLHLIFYIS CPQNPYCVCF MSHFNLYLIL IMCNSIIDPL
Pig      ---------- ---------- ---------- ---------- ----------
Dog      ---------- ---------- ---------- ---------- ----------
Human    ---------- ---------- ---------- ---------- ----------
Mouse    ---------- -----L---- ---------- ---------- ----AV----
Chicken  ---------- ---------- --Y------- -----F---- ----------

Cattle   IYALRSQELR KTFKEIICCS PLGGLCDLSS RY
Pig      ---------- --------Y ---------- --
Dog      ---------- --------Y ---------- --
Human    ---------- --------Y ---------- --
Mouse    ---------- --------FY ----I-E-- --
Chicken  ---F------ --------C N-R-----PG -Y
```

*Sequences used for this *MC4R* amino acid alignment include *Bos taurus* (AAI48893), *Sus scrofa* (NP_999338.1), *Homo sapiens* (NP_005903), *Mus musculus* (NP_058673), *Canis lupus familiaris* (NP_001074193), *Gallus gallus* (NP_001026685).
**The region highlighted in gray corresponds to the Ser330Asn polymorphism detected in this study.

6.0 AGOUTI RELATED PROTEIN (AGRP) CHARCTERIZATION AND ASSOCIATION ANALYSIS IN BEEF CATTLE

6.1 Introduction

Ollmann et al. (1997) and Graham et. al. (1997) both concluded that AGRP acts as a potent antagonist of the MC3R and MC4R regulating weight gain by preventing the signalling of α-MSH at these melanocortin receptors in the hypothalamus of mice. Neurons which co-express both, AGRP and Neuropeptide Y (NPY), are found in the ARC of the hypothalamus and are located within the same region as leptin receptors. When in a fed situation rats have increased circulating leptin which will bind to leptin receptors in the arcuate nucleus of the hypothalamus and block the expression of AGRP and NPY (Stutz et al. 2005). When the animal is in a fasted state, less leptin is available in the arcuate nucleus and therefore AGRP and NPY are expressed sending orexigenic signals stimulating the appetite of the animal, suggesting that the animal search out food (Korner et al. 2000; Stutz et al. 2005). Graham et al. (1997) was the first to report that mice over-expressing AGRP displayed obese phenotypes which were longer bodied and had late-onset hypergylcemia and hyperinsulinemia. The expression of AGRP was also noted at increased levels in diabetic obese mice (Ollmann et al. 1997; Shutter et al. 1997; Graham et al. 1997).

Polymorphisms in the human *AGRP* sequence include a promoter mutation at g.-38C>T, demonstrating differences in promoter activity, as well as a g.199G>A polymorphism resulting in an Ala67Thr amino acid change (Schwartz et al. 1996; Argyropoulos et al. 2002). Schwartz et al. (1996) showed an association between the CC genotype and obesity, and also type 2 diabetes.

With these findings in rats and humans, we anticipated that *AGRP* would play a role in feeding and overall fatness of beef cattle. There has been no *AGRP* cattle association studies reported. One *Bos taurus* mRNA sequence has been published thus far (Genbank AJ002025). The aim of this study was to characterize *AGRP* in *Bos taurus*

and perform subsequent association analysis on polymorphisms found in beef cattle, with growth and carcass qualities.

6.2 Materials and methods

6.2.1 Cattle

Sixteen cattle from the Canadian Beef Reference Herd (CBRH) were sequenced to obtain the entire *AGRP* gene. An additional 22 random crossbred beef steers were sequenced for cDNA. Three purebred Holsteins and four Brahma bulls were sequenced to determine if certain polymorphisms were present in different breeds of cattle.

A population of 382 random crossbred Canadian beef steers was genotyped for polymorphisms discovered through *AGRP* sequencing. The beef behaviour steers were purchased at weaning from several auction marts across Saskatchewan and housed at the University of Saskatchewan Beef Research Center. The cattle were fed for a backgrounding period of 152 days prior to being shipped to Poundmaker Agventures at Lanigan, SK for 131 days finishing. The diet fed to these cattle was a barley based silage and grain ration with increasing percentages of barley grain fed through the finishing period (Appendix G). Growth measurements were obtained at regular intervals (Pugh 2007). Cattle were slaughtered over a course of two weeks at a slaughter facility in Moose Jaw, SK. The slaughter criteria, all within two weeks, for these cattle will allow for detection of potential fat deposition differences between genotypes.

6.2.2 PCR, sequencing, PCR-RFLP and genetic analyzer

Similar to the previously described standard PCR protocol (McLean and Schmutz 2009), a 15 µl PCR reaction was used for sequencing and genotyping. 1.5 µl 10xPCR reaction buffer (Fermentas) was mixed with 0.3 µl of 10mM dNTPs, 0.90 µl of 25mM MgCl$_2$, 1.0 µl of 10 pm/µl forward primer, 1.0 µl of 10 pm/µl reverse primer, 0.5 U *Taq* polymerase (Fermentas), 9.2 µl of dH$_2$0 and 1.0 µl of extracted DNA of ~ 50-100 ng in concentration. The cycling protocol was standard with a 4 min denaturing step at 94º C

followed by 38 cycles of 50 s at 94º C, 50 s at varying annealing temperatures for each primer set, 72º C for 50 s completed with a dwell period of four minutes at 72º C.

Sequencing the *AGRP* gene was done by amplifying a 1385 bp product using primers AGRP 5'UTR and AGRP 3'UTR (Table 6.1) with an annealing temperature of 63º C. These primers were designed using a published *Bos taurus* mRNA sequence (Genbank AJ002025 (mRNA); NW_001493595.2 (shotgun)). This product was then extracted from a one percent agarose gel using a gel extraction kit (Qiagen). A second forward primer, AGRP Intron 3F was used to obtain clear sequence for the entire gene from this 1385 bp product. This product was sent to the Plant Biotechnology Institute (PBI) Lab where an Applied Biosystems sequencer was used to sequence the entire *AGRP* gene including the 5' and 3' untranslated regions.

Table 6.1. Primer sequences used for amplifying *AGRP* products

Primer Name	Sequence (5' – 3')
AGRP 5'UTR F	AGCTCCTAGGTCCCTGTCCTG
AGRP 3'UTR R	AGCGGTTCCTGGTGCTCTAAGA
AGRP Intron 3F	TTAGCAGAGGTAACTGCTCAGGGC
AGRP Exon 4R	CTAGGTGCGGCTGCAGGGGTTC
AGRP Indel 3F	GTGTGGCCACAGTCTTTAAAT
AGRP Indel 3R	GAACATGGGCCTCCAAAGGCACG

Genotyping for the g.715G>A polymorphism was performed using a PCR-RFLP designed with primers, AGRP Intron 3F and AGRP Exon 4R and an annealing temperature of 59º C. A 381 bp product was amplified with these primers. The g.715G allele cut at 345 bp and 36 bp while the g.715A allele remained uncut at 381 bps. This RFLP was performed on a 4% agarose gel to detect the 36 bp difference between the two alleles.

Genotyping for the g.439_440delTC polymorphism was performed using a 3130 xl genetic analyzer from Applied Biosystems. This genetic analyzer system utilized a

PCR protocol of 0.3 μl of labelled primers, AGRP Indel 3F and AGRP Indel 3R, 7.5 μl of Applied Biosystems 2X Amplitaq Gold master mix (2.5 mM $MgCl_2$), 5.9 μl HPLC grade dH_2O and 1.0 μl of extracted DNA (50 ng in concentration). A mycycler® thermal cycler (Bio-Rad) was used to amplify this PCR product. The cycling protocol consisted of 7 min denaturing at 95º C followed by 40 cycles of 20 s at 95º C, 30 s of at 52º C annealing, 60 s at 72º C, followed by a 7 min dwell time of 72º C. This PCR product was then diluted to a 1 in 50 concentration. 1 μl of this dilution was then heated in 0.3 μl of Gene Scan 600 Liz size standard (Applied Biosystems) and 8.7 μl of formamide at 95º C for 5 min and cooled on ice for two min prior to being run on the 3130 xl genetic analyzer. Genotypes were then determined using the program GeneMapper 3.7 (Applied Biosystems 2004)

6.2.3 Statistical analysis

The mixed model of SAS (SAS Institute Inc., Cary, NC) was used to perform an ANOVA analysis on steer growth and carcass measurements from the crossbred steers genotyped. This model included genotype for each SNP as a fixed effect as shown in the following model:

$$Y_{ij} = μ + Genotype_i + e_{ij}$$

Where Y_{ij} represents the dependent variable (Ribeye area, Backfat, etc.) ; μ is the mean for this trait; genotype represents the effect the *AGRP* genotype has on this overall mean; e_{ij} accounts for the random error for each observation.

6.3 Results

Sequencing of the *AGRP* gene led to the discovery of six polymorphisms throughout the entire region of the gene (Figure 6.1). Only one of these SNPs resulted in an amino acid change, Pro17Ser. However, this polymorphism was only detected in a Brahma bull. Sequencing of the 16 CBRH cattle as well as 22 random crossbred steers showed that all of these polymorphisms occurred with a very rare frequency in beef breeds (Table 6.2).

5'UTR 561 bp | 751 bp | 3'UTR

| 201 bp | 243 bp | 117 bp | 130 bp | 157 bp | 94 bp | 187 bp | 183 bp | 224 bp |

Start

EXON 1   EXON 2   EXON 3   EXON 4

Stop

439 bp | 478 bp

49 bp   470 bp   533 bp   715 bp
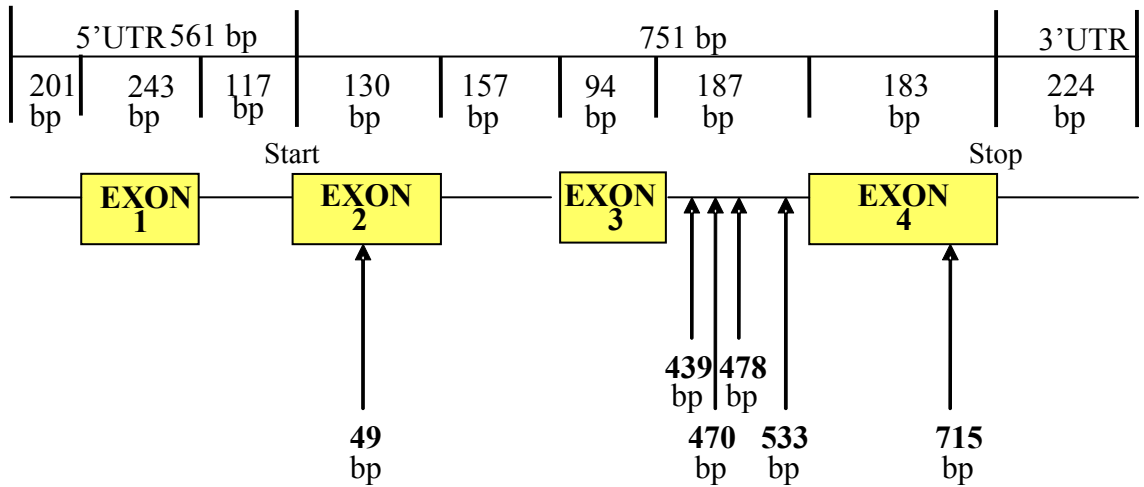
Figure 6.1. *AGRP* gene structure of the cow. Polymorphism location within the mRNA is depicted by arrows

Table 6.2 *AGRP* sequence and genotype analysis using several different types of cattle

Polymorphism	Population									
	Beef Purebreds		Beef Crossbreds		Beef Behaviour		Holstein		Brahma	
	N	MAF^z	N	MAF	n	MAF	n	MAF	n	MAF
Pro17Ser	11	0.00	22	0.00	-	-	3	0.00	4	0.13
g.439_440delTC	16	0.16	22	0.05	342	0.08	3	0.00	4	0.00
g.470C>T	14	0.00	22	0.00	-	-	3	0.33	4	0.13
g.478C>T	14	0.00	22	0.00	-	-	3	0.00	4	0.13
g.533G>C	14	0.00	22	0.00	-	-	3	0.00	4	0.13
g.715G>A	12	0.13	22	0.07	382	0.20	3	0.00	4	0.13

^{z} Minor allele frequency

The cattle amino acid sequence of *AGRP* is 79% homologous to pig and 75 % homologous to the human (Figure 6.2). In this amino acid alignment, the underlined regions are where polymorphisms that were detected in this sequence analysis would exist in the *AGRP* mRNA. The g.49C>T polymorphism which results in a Pro17Ser amino acid change occurs in an area with low conservation across species. The g.715G>A polymorphism exists in a highly conserved region of *AGRP*, but the amino acid lysine (K) is not altered (Figure 6.2).

The first polymorphism of priority for association analysis was g.439_440delTC. This polymorphism was chosen because mRNA production might be affected through potential splicing problems. Twenty-two random crossbred steers were sequenced around the region of g.439_440delTC to determine their genotype. Testes tissues from these same animals had been obtained to perform cDNA analysis to determine if protein production was affected. However, since none of these animals were homozygous for the deletion, quantitative PCR was not performed.

Two polymorphisms were chosen to pursue further to determine if there was an association between these polymorphisms and growth and carcass traits of beef cattle. The g.439_440delTC and g.715G>A polymorphisms were selected as the most likely to occur in beef cattle at relatively high allele frequencies. Due to the high nucleotide conservation across species and the incidence of the g.715G>A SNP in beef cattle, this polymorphism was genotyped in cattle also (Figure 6.2). The ANOVA analysis did not find any significant differences between either polymorphism, g.439_440delTC or g.715G>A, and growth or carcass traits (Table 6.3).

```
                                                                    ↓
Cattle    MLTAVLLSCA LLLAMPPLQG AQMGPAPLEG IGRPEEALFL ELQGLSLQPS
Pig       ---TM----- ----M-TML- -QI-L----- -G-LDQ---P ---D-G---P
Sheep                           ------ V---D----- ----------
Human     ----AV---- ----L-ATR- ----L-PM-- -R--DQ--LP --P--G-RAP
Mouse     ----M----V ----L--TL- V---V---K- -R--DQ---P -FP----NGL
Rat       MLTAMLLSCV LLLALPPTLG VHMGVAPLKG IRRSDQALFP -FS----***
                                          ↓

Cattle    LKRITEEQAE ESLLQEAEAK ALAEVLDPEG RKPRSPRRCV RLHESCLGHQ
Pig       ---T-A-R-- -A---Q---- -L-------- -KA--P---- ----------
Sheep     ----M----- -A-------- ---------- ---------- ----------
Human     --KT-A---- -D-----Q-L -E**---LQD -E---S---- --------Q-
Mouse     K-TTADR*-- -V---K---L -E**----QN -ES------- --------Q-
Rat       K-TAADR*-- DV---K---L -E**----QN -ES------- --------Q-


Cattle    VPCCDPCATC YCRFFNAFCY CRKLGTTTNP CSRT
Pig       ---------- ---------- ------AT-- ----
Sheep     ---------- ---------- --------
Human     ---------- ---------- ------A--- ----
Mouse     ---------- ---------- ------A--L ----
Rat       ---------- ------T--- ------G-TN LCSRP
```

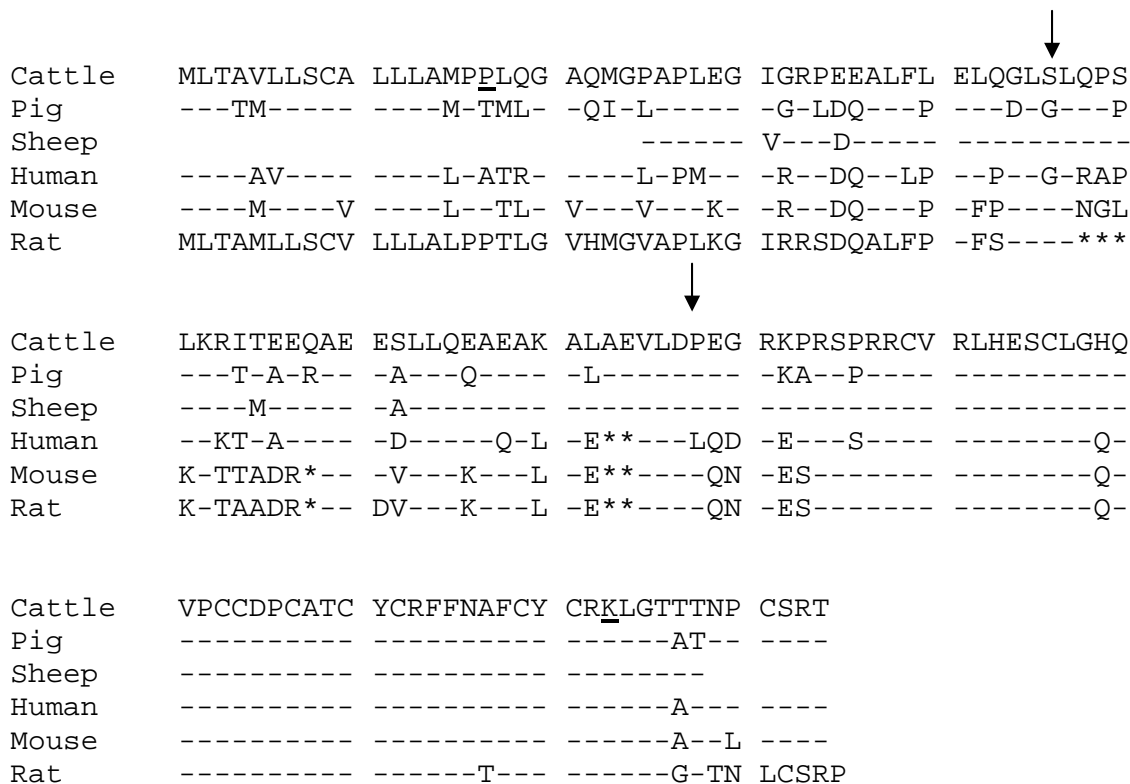**Figure 6.2** Amino acid alignment of the AGRP gene across various species of animals. Sequences used for the AGRP align include Bos taurus (NM_173983), Sus scrofa (NP_001011693), Homo sapiens (AAK96526), Mus susculus (NP_031453), Rattus norvegicus (EDL92383) Ovis aries(AAT41659). Amino acids underlined are where the polymorphisms found in this analysis are located in the mRNA of AGRP across species

Table 6.3 P-values from the association analysis of two *AGRP* SNPs in the crossbred Canadian beef steer population using a one way ANOVA.

Trait	Polymorphism	
	g.439_440delTC	**g.715G>A**
Start of Backgrounding Weight	0.192	0.588
End of Backgrounding Weight	0.597	0.486
Poundmaker Shipping Weight	0.813	0.493
Backgrounding ADG	0.881	0.913
US End of Backgrounding Backfat[1]	0.830	0.288
US End of Backgrounding Ribeye Area	0.567	0.374
End of Finishing Weight	0.447	0.237
Finishing ADG	0.558	0.349
Total Weight Gains	0.647	0.249
Hot Carcass Weight	0.615	0.836
Average Fat	0.128	0.716
Grade Fat	0.301	0.683
Carcass Ribeye Area	0.600	0.969
Marbling	0.480	0.502
Lean Meat Yield	0.156	0.882

[1] US, Ultrasound

6.4 Discussion

Six polymorphisms were detected in the *AGRP* cattle sequence (Table 6.2). Only two of these occurred in *Bos taurus* cattle chosen for sequencing in this study. One of four Brahma bulls sequenced was heterozygous for five of these six polymorphisms. In the present study, four of the polymorphisms occurred with such a rare allele frequency they were not studied further. Further characterization of these SNPs could be possible in a population with more *Bos indicus* influence.

The nature of these polymorphisms would appear to have little effect on protein production as the majority of these SNPs occur in the intron or result in a silent mutation. Intronic mutations have been shown to affect splicing ability of a protein. Burrows et al. (1998) has shown that although intron branch site mutations are quite rare they appear to affect splicing ability causing exon skipping or poor efficiency of splicing the intron.

Chamary and Hurst (2009) reported that to date there are over fifty genetic disorders that have been linked to silent mutations. Silent mutations have been often overlooked and appear to impact protein function in certain cases. If these mutations do not affect the proper intron-exon splicing mechanisms it is possible that these SNPs may affect the folding or result in premature degradation of the mRNA (Chamary and Hurst 2009). Kudla et al. (2006) reports that differences in GC content results in varying levels of expression, GC rich genes often have increased mRNA expression compared to GC poor genes. A single GC-AT difference such as that of the g.715G>A SNP may cause minor changes in expression (Kudla et al. 2006).

Two of these SNPs were genotyped in this Canadian steer population. These two polymorphisms were genotyped in this population as it was thought the intronic deletion may have an effect on splicing and impact protein production as the lariat or intron branch site may have been altered with this deletion. The g.715G>A polymorphism was also genotyped because of its location in an area of high conservation of the protein (Figure 6.2) and its incidence in beef cattle (Table 6.1) compared to other polymorphisms found through sequencing.

The human mutations shown to have an association with obesity occur at g.-38C>T and Ala67Thr (Schwartz et al. 1996; Argyropoulos et al. 2002; Tracy et al. 2007). Neither of these polymorphisms occur in a region close to the polymorphisms studied in this analysis. However, knockout rat studies have shown that the critical portion of AGRP for stimulating expression of the protein and in turn appetite, is amino acids 83-134 (Pritchard et al. 2004). The silent mutation, g.715G>A is within this critical region for expression of AGRP, at amino acid 123.

Based on the research in humans and rats (Schwartz et al. 1996; Argyropoulos et al. 2002; Tracy et al. 2007), it was anticipated that AGRP would have had an effect on appetite stimulation, metabolism and in turn adipose deposition in beef cattle. In the

group of steers analyzed the mean grade fat was an 8 mm, with a range of 1-20 mm. The degree of fatness in this population may not have been high enough to show significant differences, particularly since it was late onset obesity that was caused by *AGRP* genotypes. This association analysis suggests that the g.439_440delTC and g.715G>A polymorphisms do not result in an association with growth or carcass differences in Canadian crossbred beef cattle (Table 6.3). There was no evidence that this silent mutation or intronic deletion affect the overall production, expression or function of AGRP in Canadian beef cattle.

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7.0 GENERAL DISCUSSION

Three genes implicated in the appetite pathway were analyzed in this association analysis with carcass qualities in beef cattle. The results of this analysis suggest *MC1R* and *MC4R* both modify growth and/or fat deposition of beef cattle. In this analysis the polymorphisms found and subsequently genotyped in *AGRP* did not appear to influence growth of these Canadian crossbred beef cattle.

α-MSH plays a critical role in the appetite pathway in beef cattle. Variants in both *MC1R* and *MC4R* resulted in carcass differences (Table 4.4, Table 4.5, Table 5.1 and Table 5.2). α-MSH has high affinities to bind to both MC1R and MC4R (Cone et al. 1996; Mountjoy et al. 1997). It is possible that variants occurring in these genes may result in different levels of α-MSH binding, and in turn significant differences in fat deposition for cattle of different genotypes. Although α-MSH levels were not analyzed in this study, the data suggest that a major portion of circulating α-MSH binds to MC1R. Jordan and Jackson (1998) questioned whether mice with an a^y allele were obese because MC1R was antagonized by agouti signalling protein or whether there were other receptors downstream of the MC1R causing this obese phenotype. MC4R may be downstream of the MC1R and receive less α-MSH in cattle with eumelanin pigment, as black cattle were able to gain weight more rapidly than red cattle.

Significant differences in fat deposition were observed in cattle of Ser330Asn *MC4R* genotype (Table 5.1 and Table 5.2), suggesting differences in the amount of α-MSH bound to the receptor, based on genotype. Kim et al. (2004) observed differences in agonist activity of 293 cells transfected with the different Asp298Asn porcine alleles. cAMP production was significantly decreased in cells carrying the Asn allele, as less α-MSH was bound to MC4R and could explain why the pigs homozygous for the Asn allele had increased weight gains and backfat deposition (Kim et al. 2004). It is highly possible that cAMP production is reduced in cattle with g.989GA genotype due to reduced affinity for α-MSH compared to g.989GG cattle that produce a serine instead of an asparagine.

Future studies, such as cAMP production of cells transfected with the different Ser330Asn alleles, could be done to evaluate the role and functionality that this Ser330Asn polymorphism plays in *MC4R* and ligand binding.

Several polymorphisms in *MC4R* were identified and studied. Seven unpublished polymorphisms and four previously reported variants (Thue et al. 2001; Zhang et al. 2006; Zhang et al. 2008) were detected through sequencing of sixteen cattle of the Canadian Beef Reference Herd, twenty crossbred feedlot steers, four dairy cows and four Brahma bulls (Appendix B, Table B.1). Genotyping the previously published Val286Leu in a population of crossbred beef steers and subsequent association analysis did not validate the trend for an increased hot carcass weight reported by Buchanan et al. (2005) (Appendix C).

Prior to finding the Ser330Asn polymorphism several SNPs were evaluated in a group of approximately 380 crossbred beef steers fed for similar days on feed. The polymorphisms selected for further association analysis were chosen based on their likelihood to affect the function of *MC4R*. A computer program, P-Match (Biobase, Biological Database 2009), was used to determine that the polymorphisms g.-192T>G and g-129A>G had potential to alter the site for c-rel transcription factor binding. Alleles g.-192T and g.-129G were favourable for introduction of a c-rel binding site. The g.-129G allele also promotes the biding of the Elk-1 transcription factor. 5'UTR polymorphisms often are associated with instability of the protein and poor translational efficiencies as the 5'UTR region often regulates gene expression through a series of promoters (Van der Velden and Thomas 1999; Bashirullah et al. 2001).

In this population the minor allele was very rare for both 5'UTR polymorphisms. This low allele frequency does not necessarily rule out a causative effect on the appetite of beef cattle. However in this population of Canadian crossbred cattle no significant associations were detected.

3'UTR polymorphisms have been known to lower the stability of the mRNA, localization and translation (Grzybowska et al. 2001) and could potentially have an impact on the phenotype of cattle possessing these polymorphisms. Future research in *MC4R* could be to study whether the four 3'UTR polymorphisms, g.*159C>G, g.*181T>C, g.*361C>A, and g*577C>T have an effect on a trait. Since the minor allele

frequency of the g.*159C>G polymorphism is 0.17 (Appendix B, Table B.1), it appears to be more promising for further study. These 3'UTR SNPs, *181T>C, g.*361C>A, and g*577C>T occurred more commonly in the Brahma bulls sequenced (Appendix B, Table B.1) and therefore there is potential for further association analysis to be conducted in a Brahma population. Although Brahma cattle are rarely raised in Canada, they are common in southern climates.

$AGRP$ was included in this analysis as it has been shown to block the binding of α-MSH to the MC4R, in turn resulting in a larger appetite and late onset obesity in humans (Schwartz et al. 1996; Argyropoulos et al. 2002). Of the polymorphisms selected for further association analysis, no significant differences were detected for any growth or carcass traits in the population of 380 crossbred beef cattle. Future research in $AGRP$ should be directed to determining the effect of the Pro49Ser polymorphism in a beef cattle population with a significant $Bos\ indicus$ influence.

As reviewed earlier, growth patterns in beef cattle can be manipulated by several factors including nutrition, hormones, genetics and mature body size (Owens et al. 1993; Owens et al. 1995). By manipulating the growth curve of cattle, two distinct carcass types result. A heavier, leaner, more muscled carcass with increased lean meat yield or a carcass considered to be higher quality with more intramuscular fat and adequate muscling, but a lower lean meat yield. Mutations in two of the three genes analyzed in this study have altered the carcass composition of the beef steers studied. This implies their growth curves were modified depending on $MC1R$ or $MC4R$ genotype.

$MC1R$ takes into consideration three of these main factors for modifying growth curves of cattle, as black cattle were heavier at take in time at the feedlot, had similar ADG as red cattle, however reached point four of their growth curve (Figure 2.2), depositing backfat and intramuscular fat sooner than red cattle (Table 4.3 and Table 4.5). The red cattle would have extended their growth curve slowing down adipose tissue deposition, and reached a higher mature body weight than black cattle (Table 4.5).

A nutrigenetic component was also present in this association analysis as differences were more dramatic for cattle fed the pelleted diet (Table 4.2). This is thought to be due to more rapid digestion of a pelleted than a rolled diet (Williams et al. 2008). The black, E^D/E^D or $E^D/e,$ genotype cattle were more capable of converting this feed into

protein and adipose tissue than the *e/e* red cattle. This suggests that diets tailored to each genotype group of cattle may allow one to shift the genetic potential for these cattle. Red cattle may be able to deposit fat more readily if receiving a higher energy diet, and a higher protein diet fed to the black cattle may increase hot carcass weight while still promoting adipose tissue deposition.

The Ser330Asn *MC4R* polymorphism also supports the benefits of using genetics to improve carcass quality and modify beef cattle growth. This polymorphism showed that carcasses of cattle of the g.989GA genotype had approximately 39% more backfat and average fat than g.989GG cattle (Table 5.1 and Table 5.2). Importantly this additional fat did not lower hot carcass weight (P=0.255 Canadian Steers and P=0.389 American Steers) and producers would receive similar dollars for each carcass when sold on a carcass weight basis. It appears the *MC4R* 330Asn allele partitions growth towards fat, while still maintaining protein tissue accretion similar to cattle with the 330Ser allele.

The steers with a *MC4R* genotype g.989GA did have a significantly lower lean meat yield and could fall into the yield grade two categories for both Canadian and U.S. grading schemes (Canadian Beef Grading Agency 2009; United States Department of Agriculture 1997). Understanding Figure 2.1 and Figure 2.2, one can assume that as hot carcass weight was not significantly different and because the g.989GA cattle had excess carcass and grade fat, that these cattle could have been sent for slaughter earlier than the g.989GG cattle, improving lean meat yield while not sacrificing hot carcass weights or yield grades.

Longissimus dorsi area was affected by this *MC4R* mutation in the larger group of American steers (Table 5.2), however was not significantly different in the smaller group of Canadian crossbred steers (Table 5.1). One potential reason could be due to a hormonal x genetic interaction. Zilpaterol hydrochloride was fed to the American steers. This hormone has been shown previously to increase ADG, HCW, dressing percentage and longissimus muscle size (Elam et al. 2009). The g.989GG steers were able to increase longissimus size much more so than the g.989GA, resulting in increased lean meat yield. There may be a further need to evaluate the effect of genetic variants with zilpaterol hydrochloride. Elam et al. (2009) also observed limited fat deposition in cattle fed zilpaterol hydrochloride, however in this instance the g.989GA steers were able to

overcome this effect and had increase grade fat while showing a trend for increased marbling compared to the g.989GG cattle (Table 5.2). Engler (2009) stated that there was potential to use this β-agonist for cattle of CC and CT leptin genotype. However when feeding the agonist to TT cattle, there was little impact on growth. Cost savings resulted by not needing this hormone for a group of cattle in a feedlot.

One area of research that could be expanded is the potential interactions between *MC1R* and *MC4R* mutations. In the groups of cattle available for this study, very few groups of cattle were an appropriate mix of red and black. A large scale study would be needed to incorporate the three genotypes in both genes and the very low minor allele frequency of the *MC4R* Ser330Asn polymorphism to accurately study an interaction. The allele frequency of the *MC4R* Ser330Asn polymorphism did not differ across six different breeds of beef cattle (Appendix B, Table B.2). There is no need to account for *AGRP* in this analysis as of yet, as there has not been an association with growth or carcass traits within this gene. This may be necessary in *Bos indicus* cattle after further characterization of *AGRP* is complete. Mutations in *POMC* (Deobald 2009) could also be incorporated in this interaction model, as this gene is responsible for the production of α-MSH, possibly affecting the amount of α-MSH available for binding to MC4R.

7.1 Application

Beef cattle producers will be able to utilize the results of the *MC1R* and *MC4R* association analysis in a way that fits their operation. The opportunities presented by *MC1R* reach all sectors of beef production. Cow-calf producers can utilize *MC1R* genotype or simply coat colour as a way to increase weaning weights and profits when sold at the auction marts. When evaluating their cow inventory, producers could utilize the theory behind this study as they may choose to have black cows limiting the amount of feed needed to maintain their cow herd. The producers could use a red, *e/e*, sire to increase slaughter weights as the calves would be heterozygous and still finish significantly earlier than homozygous red calves. Conversely, a red cow herd could be utilized and when planning to market the calf crop at weaning a homozygous black sire could be used to produce black calves with increased weaning weights and potentially higher shipping weights for their customers.

The feedlot sector and those retaining ownership or finishing their own calves must assess the market at time of feeding. *MC1R* genotype and/or coat colour alone represent an excellent feedlot sorting tool. Determining where your markets will be is key to determining what colour of cattle to feed. Black cattle, E^D/E^D and E^D/E, will finish much more quickly than red cattle saving significant dollars in feed and yardage costs. However if the cost of meat per pound is higher than the cost of feed and yardage, it will be more lucrative to feed red cattle as they will have a higher shipping and hot carcass weight. Black cattle will be more suited to a value-added grid as they have a propensity to deposit adipose tissue more quickly than red cattle. In the current study a significant number of red cattle (41%) never met the fat requirements of the trial endpoint.

The polymorphism found in *MC4R*, Ser330Asn, will not have as much of an effect on the cow-calf industry as *MC1R*. Seed-stock producers may wish to select for cattle with the ability to deposit fat more readily or wish to select for a leaner, more muscled carcass animals. In the later case, it may be beneficial to implement genotyping for the Ser330Asn polymorphism in their herd. Whether genotyping is only done for the herd bulls or also dams will depend on how aggressive the producer is to improve meat quality. The commercial cow-calf producer will likely not find it economically beneficial to genotype their cows or herd sires as a large percentage of these producers market at weaning time when adipose deposition has not begun.

Feedlot operators will find the most benefit to genotype steers for the Ser330Asn in *MC4R*. Although there is a low minor allele frequency (0.01) in Canadian steers and (0.02) in American steers, when dealing with large feedlots this Ser330Asn polymorphism may represent significant savings to these feedlots. Whether this is through increasing uniformity of their cattle, not overfeeding calves that have reached their maximal protein and adipose growth earlier than the rest of the pen or by targeting the g.989GA steers towards a grid rather than a live weight market. This test will be successful when marketed with another genetic test for feedlot sorting since cost of testing would be lower.

Meat quality across North America has the potential to improve taking these two association analyses into consideration. With improved uniformity, the ability to sort and

ship cattle at an appropriate time for slaughter for that genotype, increased carcasses should be available for consumers. These two genetic tests have the ability to improve overall meat quality and consumer acceptance, areas that Lorenzen et al. (1993) suggest we focus on. With these improvements consumers should find meat at their local meat stores which will meet their expectations and improve the overall beef eating experience.

8.0 CONCLUSIONS

This association analysis involving three genes in the appetite pathway of beef cattle has found two practical applications for the beef production industry. With the need to improve production efficiency and overall profitability to maintain the sustainability of the beef industry knowing the genetic potential of the producer's cattle is highly beneficial.

Depending on market situation certain genotypes or certain carcasses will be more desirable and this may change from year to year. Knowing ahead of time what the genetic potential is for each group of calves can assist producers in purchasing plans for seed stock or feeder calves. Feedlots may choose to simply sort cattle based on coat colour, without genotyping the cattle for *MC1R* and will still see significant differences in time on feed and carcass lean meat yield. Those with the capacity to further sort their cattle may choose to be more progressive and sort based on *MC1R* genotype or in addition to other genetic tests MC4R Ser330Asn polymorphism could be included to increase overall carcass uniformity for their feedlot pens sent for slaughter.

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10.0 APPENDICES

Appendix A. *MC1R* genotype frequencies of additional populations

     Population A consists of Canadian crossbred *Bos taurus* beef cattle fed at the University of Saskatchewan Beef Research Center over the winter of 2004. These steers were fed five different diets of varying inclusion levels of sunflower seeds.

     Population B consists of Canadian crossbred *Bos taurus* beef cattle fed at the University of Saskatchewan Beef Research Center in the winter of 2005. These cattle received three different diets, oats, corn or barley.

Table A.1 *MC1R* Genotype frequencies of two additional populations

Genotype	Population A	Population B
	n	*n*
E^D/E^D	3	25
E^D/E^+	0	4
E^D/e	15	42
E^+/e	30	18
e/e	209	146

Appendix B. *MC4R* allele frequencies

Table B.1. *MC4R* sequence and genotype analysis using several different types of cattle.

Polymorphism	Population									
	Beef Purebreds		Beef Crossbreds		Beef Behaviour		Holstein		Brahma	
	n	MAF[1]	*n*	MAF	*n*	MAF	*n*	MAF	*n*	MAF
g.-192T>G	16	0.03	19	0.00	384	0.004	3	0.00	4	0.13
g.-129A>G	16	0.03	19	0.00	383	0.004	3	0.00	4	0.13
g.39C>T	16	0.00	19	0.00	-	-	4	0.00	4	0.13
g.534G>A	16	0.03	19	0.00	-	-	4	0.00	4	0.00
g.710C>T	16	0.03	19	0.00	-	-	4	0.00	4	0.00
g.856C>G	16	0.40	19	0.29	378	0.28	4	0.38	4	0.63
g.989G>A	16	0.00	19	0.03	382	0.01	4	0.00	4	0.00
g.*159C>G	12	0.17	19	0.05	-	-	4	0.00	3	0.00
g.*181T>C	13	0.00	19	0.03	-	-	3	0.00	3	0.17
g.*361C>A	10	0.00	19	0.00	-	-	2	0.00	3	0.17
g.*577C>T	10	0.05	19	0.03	-	-	2	0.00	3	0.33

[1] Minor allele frequency

Table B.2. Allele frequencies of the g.989G>A polymorphism in unrelated animals of different beef cattle breeds.

Breed	*n*	Allele	
		G	*A*
Angus	57	0.99	0.01
Charolais	50	1.00	0.00
Simmental	48	1.00	0.00
Hereford	50	1.00	0.00
Limousin	15	1.00	0.00
Wagyu	38	1.00	0.00
Holstein	51	0.94	0.06

Appendix C. Association analysis of selected *MC4R* SNPs with production traits in the behaviour steer population.

Trait	Polymorphism		
	g.-192T>G[1]	g.-129A>G[1]	g.856C>G[2]
Start of Backgrounding Weight	0.425	0.437	0.017
End of Backgrounding Weight	0.328	0.336	0.705
Shipping to Poundmaker Weight	0.374	0.388	0.652
Backgrounding ADG	0.518	0.517	0.288
US End of Backgrounding Backfat[3]	0.901	0.909	0.726
US End of Backgrounding Ribeye Area	0.123	0.124	0.118
End of Finishing Weight	0.425	0.434	0.657
Finishing ADG	0.722	0.728	0.859
Total Weight Gains	0.577	0.585	0.638
Hot Carcass Weight	0.754	0.748	0.743
Average Fat	0.702	0.700	0.790
Grade Fat	0.600	0.594	0.981
Carcass Ribeye Area	0.132	0.133	0.914
Marbling	0.926	0.928	0.500
Lean Meat Yield	0.965	0.965	0.699

[1] Probability based on Mann-Whitney U, non-parametric statistical analysis
[2] Probability based on Anova, parametric statistical analysis
[3] US, Ultrasound

Appendix D. Genbank Submissions

EU366349 1855 bp *Bos taurus* breed Limousin melanocortin 4 receptor (*MC4R*) gene,
       complete cds.

EU366350 1871 bp *Bos indicus* breed Brahma melanocortin 4 receptor (*MC4R*) gene,
       complete cds.

EU366351 1871 bp *Bos taurus* breed Angus melanocortin 4 receptor (*MC4R*) gene,
       complete cds.

EU374211 1515 bp *Bos taurus* breed Limousin agouti-related protein (*AGRP*) gene,
       complete cds.

EU374212 1308 bp *Bos indicus* breed Brahma agouti-related protein (*AGRP*) gene,
       complete cds.

EU374213 1322 bp *Bos taurus* breed Holstein agouti-related protein (*AGRP*) gene,
       complete cds.

FJ430565 1845 bp *Bos taurus* breed Crossbred melanocortin 4 receptor (*MC4R*) gene,
       complete cds.

Appendix E. *MC4R* **Protocol for genotyping additional** *MC4R* **polymorphisms**

The PCR cocktail consisted of 1.5 µl of 10 x PCR reaction buffer, 0.3 µl dNTPs, 0.9 µl MgCl$_2$, 1.0 µl of forward and reverse primers 10 pm/µl in concentration, 0.1 µl of *taq* polymerase (Fermentas) and 9.2 µl of dH$_2$0. A common cycling pattern of 4 min of dwell at 72° C followed by 38 cycles of 50 s denaturing at 95° C, 50 s annealing at the specified temperature in Table E.1 and 50 s of extension at 72° C followed by another four min dwell period at 72° C.

Table E.1 *MC4R* PCR-RFLP primers and genotyping protocols

PCR - RFLP	g.-192T>G	g.-129A>G	g.856C>G
Forward Primer	MC4R Gen F 5'-GCAAAGACCTGC ATGCCTCCGACT-3'	MC4R Gen F 5'- CAAAGACCTGC ATGCCTCCGACT-3'	MC4R F 5'- TACCCTGACC ATACTGATCG -3'
Reverse Primer	MC4R 5'UTR MM R 5'-CTTTCAAGTGTG GCTCTGGTCAGG -3'	MC4R RFLP R 5'- CCTTTCTCCAG T CTTGACTTGC-3'	MC4R R 5'- AGAGCAACAAA TGATCTCTTTG -3'
Annealing Temperature	60° C	59° C	50° C
Enzyme	*Mbo 1*	*Tai 1*	*Tai 1*

Appendix F. Diet formulations for the feedlot steers in the *MC1R* analysis

Table F.1 Diet formulations for the feedlot steers in the *MC1R* analysis. Taken from Williams et al. (2008) with permission

Ingredient (all values DM Basis)	Backgrounding		Finishing	
	Pelleted Barley	Rolled Barley	Pelleted Barley	Rolled Barley
Total mixed diet, % of diet				
Barley silage	23.0	23.0	7.0	7.0
Brome grass hay	28.0	28.0	-	-
Barley straw	3.0	3.0	-	-
Barley grain, rolled	-	35.0	-	83.0
Canola meal	-	6.0	-	5.0
Ground barley/canola meal pellet	41.0	-	88.0	-
Supplement	5.0	5.0	5.0	5.0
Supplement, % of supplement				
Barley grain	51.0	51.0	42.0	42.0
Tallow	3.4	3.4	3.3	3.3
Molasses	3.6	3.6	3.5	3.5
Limestone	8.8	8.8	19.6	19.6
Rumensin premix [z]	9.2	9.2	7.7	7.7
Trace mineral salt	9.4	9.4	9.3	9.3
LS 106	14.6	14.6	14.6	14.6
Chemical Analysis, % of total mixed diet				
Crude protein	12.4	12.1	14.5	14.9
Calcium	0.59	0.58	0.58	0.52
Phosphorus	0.31	0.29	0.38	0.37
Formulated Energy Content				
NEm (Mcal kg^{-1})	1.55	1.55	1.94	1.94
NEG (Mcal kg^{-1})	0.95	0.95	1.29	1.29

[z] Rumensin/Tylan premix: 3.21% monensin sodium; 2.7% Tylan 40

Appendix G. Diet formulations for the Canadian Steers

Table G.1 Backgrounding diet fed to the Canadian Steers. Taken from Pugh (2007) with permission

Ingredient	Dates Fed		
(%, as fed)	11/29/05 - 12/13/05	12/14/05 - 03/20/06	03/21/06 - 05/02/26
Barley Silage	40	50	50
Barley Grain	20	25	35
Pellet	5[z]	5[y]	5[y]
Hay	35	10	20
Straw	0	10	0

[z] JM starter pellet
[y] RB1 pellet

Table G.2 Pellet formulation for the backgrounding diet fed to the Canadian Steers. Taken from Pugh (2007) with permission

Ingredient (%, as fed)	Pellet Name	
	JM Starter	RB1
Barley Grain	7	-
Tallow	3	-
Molasses	3.5	3.2
Canola Meal	6.1	68.3
Ground Limestone	6.5	10
TM Salt	4.5	4.5
JM Rumensin Premix[z]	5.5	5
Lab Supplement[y]	9	9

[z] Contains 97% barley grain and 3% Rumensin
[y] Contains barley mixed with Vitamins A & D

Table G.3 Finishing diet fed to the Canadian Steers. Taken from Pugh (2007) with permission.

Ingredient	% as fed
Barley Grain	66.8
Barley Silage	17
Wet Distillers Grain[z]	10
Supplement	4.2
Grass Hay	2

[z] The steers also had access to thin stillage as a fluid source

Appendix H. Diet formulations for the American Steers

Table H.1 Diet formulations for the American steers in the *MC4R* analysis.

Ingredient (%, dry matter)	Adaptation Diet (3 weeks)	Finishing Diet
Corn	53.0	77.0
Alfalfa Hay	32.5	-
Fat	-	3.5
Corn Silage	7.7	10.0
Supplement	6.8^z	9.5^y

^{z}Adaptation supplement
^{y}Finishing supplement

Table H.2 Supplement formulations for the American steers in the *MC4R* analysis.

Adaptation Supplement	Finishing Supplement
Rumensin	Rumensin
Trace Minerals	Trace Minerals
Macro Minerals	Macro Minerals
Vitamins A, D, E	Vitamins A & D
	Tylan
	Zilpaterol Hydrochloride^z

^{z} Zilpaterol Hydrochloride was implemented in the supplement in the last phase of the finishing period

Appendix I. Cattlemen's Magazine Article

The following is a copy of an article written by Kim McLean and Sheila Schmutz, published in the Cattlemen's Magazine, May 2008. Reproduced with permission.

Should you
sort feedlot pens by coat colour?

This study says you should

With the implementation of DNA-assisted selection, producers have been concentrating on new ways to select breeding stock. While still in its infancy, the DNA selection way of thinking appears to be very complex, which may explain why adoption of this new technology has been so slow. Whether producers know it or not, they have been doing their own DNA-assisted selection for quite some time simply by deciding the colour of cattle they want to breed. Often these decisions were based on what colour was hot in the market at the time. We wanted to find out if there was any science behind why particular coloured cattle were popular, and why producers still have a colour preference for cattle?

The science of pigmentation and appetite

Cattle are red or black, or shades thereof, due to the Melanocortin 1 Receptor (*MC1R*) gene. *MC1R* is considered to be the main gene controlling the production of coat colour pigment in cattle. There are three common *MC1R* alleles found in beef cattle, E^D, E^+ and e. The E^D allele binds alpha-Melanocyte Stimulating Hormone (α-MSH) and allows production of black pigment, this allele is considered dominant and therefore only one copy of this allele is needed to give a black (or grey which is a dilute black) calf. The recessive red allele e creates a non-functional receptor that will not bind the α-MSH hormone allowing for production of a red or yellow coat colour. In order to make a red, yellow, or cream coat colour, the calf will need two copies of this allele. The E^+ allele relies on either the α-MSH hormone or another hormone, agouti, binding at the pigment cell to decide whether red or black hair is made, or some of each as in brindle. Four of the five major Canadian cattle breeds are black or red because of their genotype at *MC1R*. If an animal is said to be "Double black" or "True red" it will have been this *MC1R* gene that the cattle were DNA tested for.

In this same gene family, Melanocortin 4 Receptor (*MC4R*), binds the same α-MSH hormone to cause a reduction in appetite. Appetite has a complicated regulatory mechanism involving the hormone α-MSH. If the hormone is bound to the receptor, a signal is sent to the brain to stop eating as the animal thinks it is full. If no α-MSH is bound to this receptor, the animal will think it is still hungry and keep eating. It is thought that this *MC4R* gene is one of the largest genetic contributors to human obesity.

We hypothesized that due to α-MSH binding to the *MC1R* making cattle black that there would be less α-MSH circulating in the animal to bind to the *MC4R*. As a result these black or smoke-coloured cattle would have an increased appetite leading to increased weights and differences in carcass quality as more fat would be deposited on the carcass. We conducted a study at the University of Saskatchewan to determine if there was an association between the *MC1R* genotype and appetite causing production and carcass differences.

In order to test this hypothesis and our pilot study, 328 weaned feedlot steers were randomly purchased and fed to finish at the University of Saskatchewan feedlot. These were "rainbow cattle" representing several breeds with several different hide colours drawn from various herds across Saskatchewan. In this study we compared cattle that were red, white and tan to cattle that were black, smoke and grey, therefore there was no particular breed analyzed. Only colours were reported. This population was used as we felt that it was representative of the western Canadian beef feedlot industry.

The steers were also on a feeding trial comparing pelleted and rolled diets. This did make interpretation of our results slightly more difficult; however it also allowed us to examine how different diets affect overall production when cattle of different genotypes or colours are fed. This is a new field of research called nutrigenomics where feed is tailored to suit the genetics of the animals.

From the start of the trial it was evident that there were differences in weights of cattle of different colours. The black cattle were significantly heavier than red cattle (35 pounds) at the start of the trial and that increased to 40 pounds by the end of backgrounding phase.

However, when we analyzed just by genotype or colour the overall weight change (ADG) from the start of feeding to 141 days was not significantly different.

Perhaps the most informative data analyzed in this study was the length of time individual steers took to finish. The cattle were ultrasounded bimonthly and shipped when they reached an estimated 12 mm of backfat. By looking at the length of time this took, we were able to determine which genotypes finished faster. Not all cattle reached this target.

On the pelleted diet the number of black alleles significantly decreased the length of time to finish. Steers with two copies

of the black allele took about 185 days, and steers with one copy of the black allele took 197 days while the recessive red cattle took 218 days. Similar results were seen on the rolled diet, however both groups of black cattle finished at an average 185 days on feed and the red cattle needed 212 days.

By simply comparing colour, using all 328 steers, a difference of 21 days to finish was observed with the black cattle finishing significantly sooner than the red cattle.

Shipping weight and hot carcass weights show a slightly different picture. The recessive red steers were 46 pounds heavier at shipping than the homozygous black cattle and 36 pounds heavier than black cattle of either type. Not too surprising as they had been fed longer to finish. Similar trends in hot carcass weights were observed. The red cattle had an average hot carcass weight of 836 pounds and the black cattle 807 pounds.

What's it mean?

These results suggest feedlots could profit by simply sorting cattle for color. The black cattle will meet backfat quality criteria and finish sooner than red cattle with no loss in dressing percentage or yield. Sorting for this trait should improve the uniformity of when a pen of cattle finish and reduce the losses on cattle being shipped too early or too late.

— *Kim McLean and Sheila Schmutz*

Kim McLean is a graduate student in animal science and Sheila Schmutz is a professor of animal genetics at the University of Saskatchewan