ANALYSIS OF *PMCH* AND *LEP* GENOTYPES AND STUDY OF THE *ITM2A* GENE AS A BASIS FOR SELECTION OF BEEF REPLACEMENT HEIFERS

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ABSTRACT

The need for a more reliable method to select beef heifers to retain as replacement dams has become a concern in the beef industry. Two polymorphisms described in *leptin* (*LEP*), p.Arg25Cys, and pro-melanin concentrating hormone (*PMCH*), g.-134A>T, have already been shown to improve carcass quality in beef cattle. This study was designed to evaluate any additional advantages of these polymorphisms in terms of heifer conception and calving success and lactational milk yield measured indirectly by their calves' early ADG while they were primarily on lactation.

A dominant effect of the dam's *PMCH T* allele was observed on improved calf early ADGs in Simmental heifer dams, although not in Angus heifer dams. This effect could be useful in cow-calf operations where calves were suckling their dams for a longer period before sending the cow-calf pairs out to summer pasture. The dam *LEP* genotype did not show an effect on their calves' ADG. This was assumed to be due to low body fat reserves of the heifer dams at the age of two years, allowing for only low levels of leptin. Even though heifer conception was not affected by their *LEP* and *PMCH* genotypes, it would be worth evaluating their rebreeding success in the presence of these SNPs in the future.

The *Integral Membrane Protein 2A* (*ITM2A*) was hypothesized as a candidate gene for frame size in cattle. DNA fragments from 20 cattle, matching the predicted exons of the cattle *ITM2A* gene, were sequenced to determine whether genetic variation existed. However, the sequence obtained based on the predicted cattle *ITM2A* sequences appeared to be a

pseudogene, rather than the actual cattle *ITM2A* gene, because exons 1, 2, 3 and 5 contained stop codons. Since frame size has been reported to be associated with the reproductive performance of beef dams and their calves' growth characteristics, it would be useful to characterize this gene once an improved cattle genome assembly is available.

Key words: *LEP, PMCH*, *ITM2A*, Angus, Simmental, ADG, lactation, frame size, pseudogene

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

ADG	Average daily gain
ANOVA	Analysis of Variance
Arg	Arginine
BCS	Body condition score
BIF	Beef Improvement Federation
Cys	Cysteine
dH_2O	Deionized water
DNMT1	*DNA methyltransferase*
E4BP4	Adenovirus E4 Promoter Binding Protein
bp	Base Pair
cDNA	Complimentary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ITM2A	*Integral Membrane Protein 2A*
ITM2B	*Integral Membrane Protein 2B*
ITM2C	*Integral Membrane Protein 2C*
kb	Kilobase
LEP	*Leptin*
Mbp	Mega basepair
MCH	*Melanin-Concentrating Hormone*
$MgCl_2$	Magnesium chloride
mRNA	Messenger ribonucleic acid
NEI	*Neuropeptide-Glutamic-Acid-Isoleucine*
NGI	*Neuropeptide-Glycine-Glutamic Acid*
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase Chain Reaction Restriction Fragment Length Polymorphism

PMCH	*Pro-Melanin ConcentratingHormone*
PMEL	*Premelanosome Protein*
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
UMD	University of Maryland
UTR	Untranslated Region
WBDC	Western Beef Development Centre
XCI	X-chromosome inactivation
Xsit	*X Inactive Specific Transcript*

1.0 GENERAL INTRODUCTION

Commercial beef cattle cow-calf operations typically breed cows in the summer followed by the birth of their calves in the following spring. Some purebred breeders still breed in spring for January calving. At the time of weaning calves in the fall, a portion of female calves (heifers) are selected as future breeding animals and overwintered, whereas the others are sold for beef production.

Profits to commercial cow-calf producers are a function of several factors including calf weaning weights and prices, cow rebreeding success, cow productive life, etc. (Mitchell *et al*. 2009). Hence, to maintain a quality and profitable cow herd, a constant input of new, high performing replacement females is essential. The costs associated with rearing and managing replacement heifers including high quality well formulated feed, shelter, and bedding are very large. If these replacement heifers are properly selected and managed, it can significantly affect their lifetime productivity. Thus, selecting and raising the best heifers with favorable characteristics as replacement heifers is a major investment for the farmer, despite the high cost incurred for their management.

Selecting a good set of replacement heifers is a challenging task. At present most ranchers select their replacement heifers based on a few common criteria such as: weight at birth and weaning, performance records (such as average daily gain from birth to weaning) and visual appraisal (Pala & McCraw 2005; Engelken 2008). Although this manual selection

seems straightforward, there is some controversy in the literature regarding the effectiveness of using weaning weights as an indicator of future heifer performance (Pala & McCraw 2005; Engelken 2008). These criteria could also differ significantly depending on the management system under which the heifers were raised and selection based on visual appraisal is very subjective. This raises the need for a reliable guide that could help ranchers to verify their decision in choosing the best animals as their replacement stock.

A possible aid to address this issue would be to search for molecular markers in genes involved directly or indirectly in reproduction or calf quality and possibly develop a DNA test, which will allow the genotype of heifers to be available prior to selection and choose the most suitable replacement heifers with greater efficiency. Although there have been studies carried out on selecting beef replacement heifers (Pala & McCraw 2005), application of genetic approaches for this purpose has been minimal.

Three genes; *leptin* (*LEP*), *pro-melanin concentrating hormone* (*PMCH*) and *integral membrane protein 2A* (*ITM2A*) were chosen for this study, as polymorphisms in these genes were reported to affect traits that could be important for heifer selection such as fat deposition (Buchanan *et al*. 2002; Helgeson & Schmutz 2008), milk production (Buchanan *et al*. 2003), calf weaning weights (DeVuyst *et al*. 2008) and height (Tukiainen *et al*. 2014). If associations were found between these gene markers and the above mentioned traits in the Angus and Simmental heifers used in the study, the cow-calf producers could then use these findings in the selection of best replacement heifers.

2.0 LITERATURE REVIEW

2.1 Performance targets for heifers as herd replacement stock

A calf, with desirable growth traits is the main objective in a commercial beef cow-calf operation. Hence, the performance efficiency of beef heifers, and subsequently cows, is key to the biological and economical sustainability of the enterprise. The success of this objective is mainly attributable to the heifers' ability to conceive early in the breeding season, maintain their pregnancy and calve approximately at the age of two years, as well as their ability to provide a good lactational milk yield for their calves (Lamb 2013; Diskin & Kenny 2014).

2.2 Factors affecting heifer performance

2.2.1 Heifer weight at weaning and breeding

Heifer weaning weight is considered a major criterion for prediction of the performance of replacement beef heifers (Pala & McCraw 2005; Engelken 2008; BIF 2010). Weaning weight of the dam was positively associated with calf weaning weights in Angus, Simmental, Hereford and Charolais (r=0.18) (Pala & McCraw 2005). However, above a heifer weaning weight of approximately 272 kg, their calf's weaning weights did not increase with increasing dam weaning weight (Pala & McCraw 2005). Higher weaning weights of heifer dams have been shown to be detrimental to their milk production due to

underdeveloped mammary glands, leading to lighter calves (Buskirk *et al*. 1996). Therefore, the reliability of heifer weaning weight as an indicator of the heifer's future production is debatable. In contrast to this, weaning weights have shown positive effects on heifer fertility and rebreeding. Heifers with weaning weights of 267 kg had 80% conception rate, whereas heifers with weaning weights less than 167 kg had only a 39% conception rate (Pala & McCraw 2005). These findings suggested that increased heifer weaning weights could help ranchers to have higher conception and rebreeding rates and heavier calves. However, overweight heifers provide no added advantage in terms of calf weaning weights (Pala & McCraw 2005).

2.2.2 Frame size

Frame size is a way of describing the skeletal size and is defined by the hip height of a heifer/cow at a particular age (Vargas *et al*. 1999; Senturklu *et al*. 2015). Even though increased size of an animal is often preferred in the beef industry, this may not be true in terms of heifer reproductive traits.

In Brahman cattle, it was reported that smaller heifers had higher reproductive performance such as early onset of puberty, higher calving rate and higher calf survival rate (Figure 2.1). However, the dam frame score did not affect the survival rates of the calves of mature cows (Vargas *et al*. 1999). Similar effects of frame size on calving rates were observed for second and third parity cows (Buttram & Willham 1989; Vargas *et al*. 1999). Nonetheless, calf birth weight, weaning weight and pre-weaning average daily gain were

significantly higher in larger framed first calf heifers (Stewart & Martin 1981; Vargas *et al*. 1999).

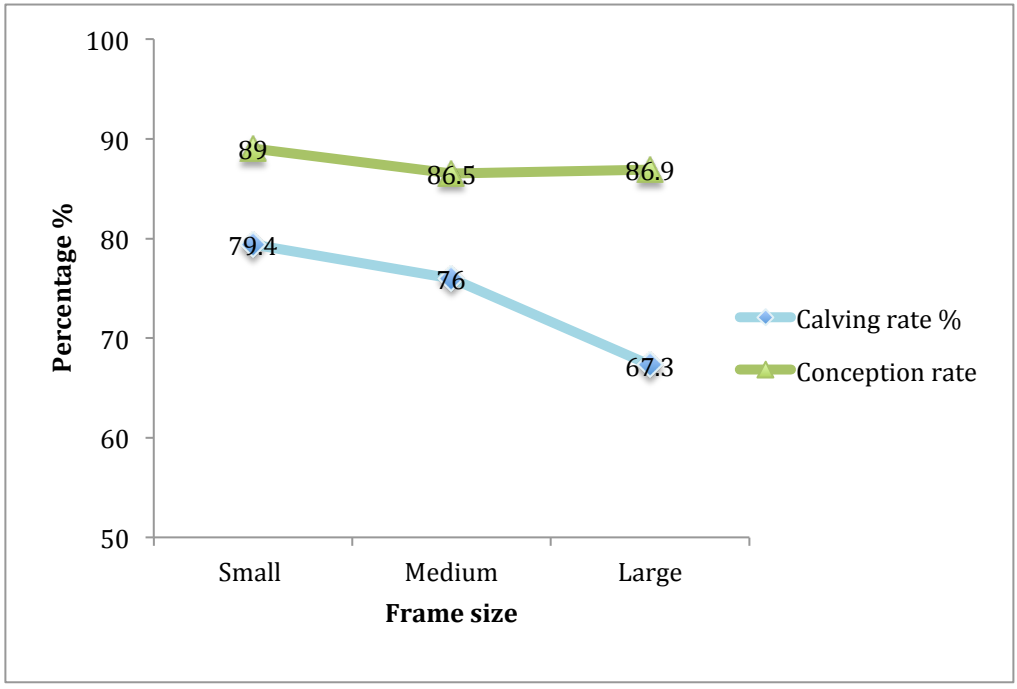


Figure 2.1: The effect of frame size on conception rate and calving rate in first calf heifers. The frame size was a significant (P<0.05) source of variation and calving rate, but not for conception rate. Small heifers were 110 cm at the hip, medium heifers were 115 cm and large heifers were 120 cm at 18 months of age (Adapted from Buttram & Willham 1989).

2.2.3 Body condition score (BCS)

Total body energy reserves/body condition at specific stages of a production cycle is considered an important factor affecting the reproductive traits of heifers and cows. A subjective scoring system ranging from 1-5 or 1-9 (1=severely emaciated to 5 or 9 =very

obese) is used to reflect the fatness or condition of beef cows (BIF 2010). The scoring is generally done by either visual appraisal or palpation of the areas including last half of the ribs, edge of the loin, hooks and pins, tail head, brisket and shoulder area (BIF 2010).

Increased BCS (4, 5 and 6 scores) at parturition has resulted in higher percentage of cows exhibiting estrus early in the breeding season and higher pregnancy rates *(DeRouen et al*. 1994; Spitzer *et al*. 1995; Bohnert *et al*. 2013). Nevertheless, according to Schmutz *et al*. (2000), the heifer BCS did not affect the ovulation rate or conceptions rate in beef heifers. However, ultrasound backfat had a negative correlation on number of embryos produced suggesting that excess fat can be detrimental to heifer ovulation rate (Schmutz *et al*. 2000).

Spitzer *et al*. (1995) and Bohnert *et al*. (2013) reported that, primiparous and multiparous cows with a higher BCS at parturition had heavier calves at birth without increased incidences of dystocia. Further, higher BCS at late gestation had positive effects on number of live calves at birth and weaning (Bohnert *et al*. 2013).

In beef cows, the weight gain of the calf during the first 30 days of life was positivley affected by the body condition of the dam, thus suggesting that cows with a higher BCS provided more nutrition to their calves, via a better lactational milk yield or quality (Stěhulová *et al*. 2013).

2.2.4 Milk production

In beef cow-calf production, milk produced by the dam was the primary source of nutrients to its calf before they were sent to pasture, and thus is considered an important component affecting the pre-weaning calf's growth (Clutter & Nielsen 1987; Beal *et al*. 1990; Freking & Marshall 1992b; Marston *et al*. 1992; Meyer *et al*. 1994; Brown & Brown 2002; Liu *et al*. 2015). However, the total amount of milk produced by the dam and the amount of each milk component varied as lactation progressed. Clutter and Nielsen (1987) have depicted average 205-day milk production in three groups of crossbred beef cows based on their genetic potential for milk production (Figure 2.2). The milk yield increased up to 50 to 60 days following parturition for all groups, and declined throughout the remainder of the lactation. According to Marston *et al*. (1992), Angus cows have reached a peak milk yield approximately 67 days postpartum, whereas Simmental cows reached their peak milk production at 80 days postpartum.

Several studies have reported the association of dam lactational milk yield with their calf's pre-weaning growth in beef cattle (Rutledge *et al*. 1971; Clutter & Nielsen 1987; Marston *et al*. 1992; Meyer *et al*. 1994). Approximately 60% of the variance in 205-day calf weaning weights was attributed to the direct influence of their dam's milk yield (Rutledge *et al*. 1971). Higher milk producing cows with a maximum daily production of 9.66 kg weaned calves with 16.9 kg greater 205-day weaning weights compared to cows with a maximum daily production of 7.04 kg (Clutter & Nielsen 1987). A 1 kg increase in overnight milk

production has been associated with a 17.1 kg increase in 205-day calf weaning weight (Freking & Marshall 1992a).

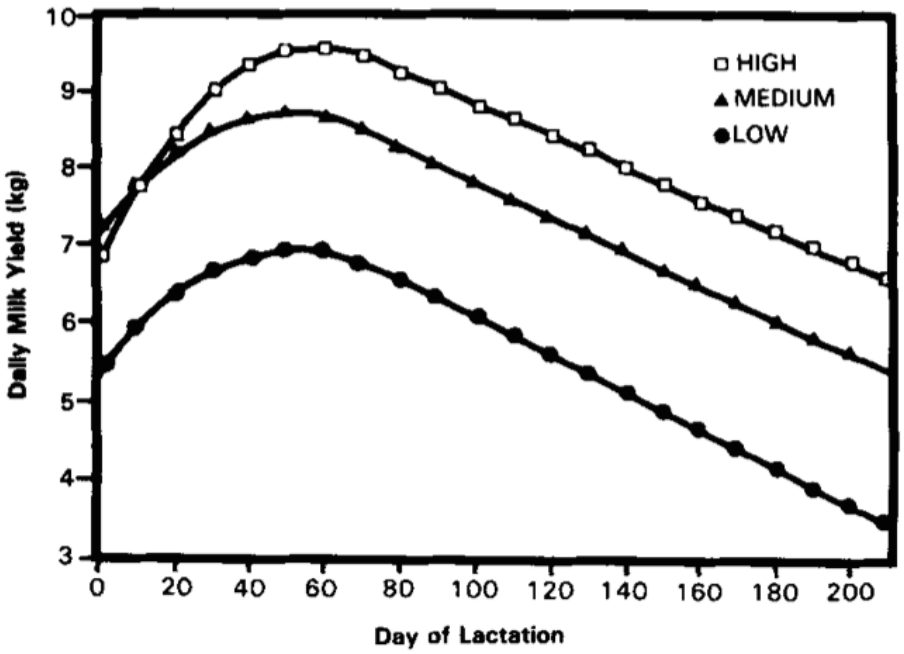


Figure 2.2: Average 205-day dam milk production curves for three milk groups based on their genetic potential for milk production. The higher milking group included Angus × Milking Shorthorn cows, the medium milking group included Angus × Red Poll cows and the low milking group included Angus × Hereford cows. The maximum milk production was estimated to be at day 58 of lactation for the high producing group with a 24-hour yield of 9.66 kg. The maximum milk production was at day 50 of lactation for both medium producing cows with 8.75 kg maximum daily production and low producing cows with 7.04 kg milk production (Taken from Clutter & Nielsen 1987 with permission from *Journal of Animal Science*).

In addition to weaning weights, a positive relationship was also reported between dam milk yield and their calf's pre-weaning ADG (Clutter & Nielsen 1987; Beal *et al*. 1990;

Brown & Brown 2002; Liu *et al*. 2015). A recent study conducted by Liu *et al*. (2015) has reported a linear relationship between 24 hour dam milk yield and their calf's pre-weaning ADG for Brangus cows sired by British breeds such as Hereford and tropical breeds such as Bonsmara, Brangus and Romosinuano. A quadratic relationship was observed for Brangus cows sired by European breeds such as Charolais and Gelbvieh (Figure 2.3).

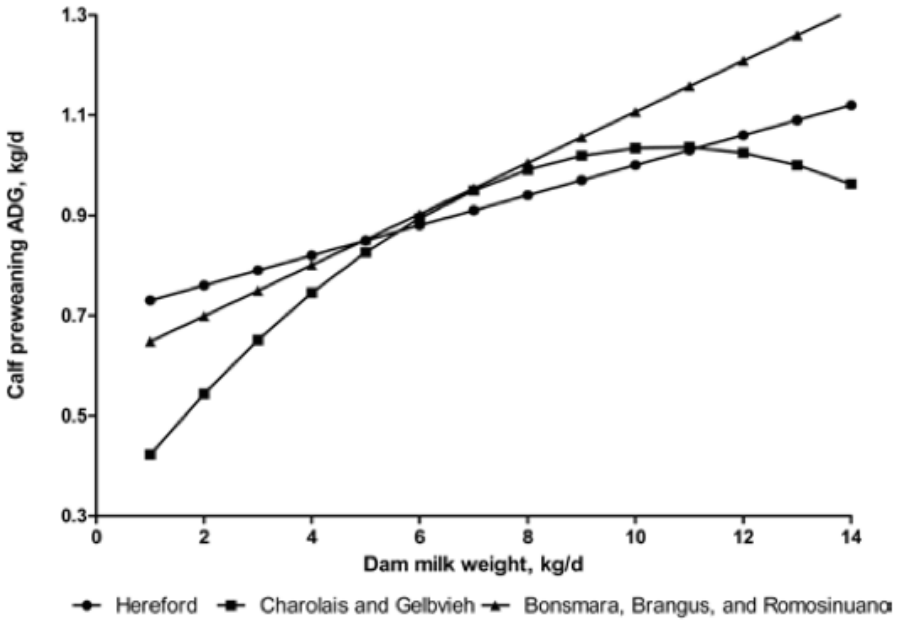


Figure 2.3: Regression on calf pre-weaning weight gain on dam milk yield. Calf pre-weaning ADG had a linear relationship with their dam's milk yield in the British breed of Hereford ($P<0.01$, $r^{2=}0.4156$) and tropical breeds of Bonsmara, Brangus and Romosinuano ($P<0.01$, $r^2=0.3549$) and quadratic relationship in the European breeds of Charolais and Gelbvieh ($P<0.01$, $r^2=0.5283$). The maximum of the quadratic equation for European breeds was 10.63 kg/d. The value indicated that milk yield beyond this point was not beneficial to the calf. The initial linear portion of the quadratic curve was steeper for European breeds compared with the linear slopes of British and tropical breeds suggesting the effect of dam breed composition on the association between calf pre-weaning gain and dam milk yield (Taken from Liu *et al*. 2015 with permission from *Journal of Animal Science*).

The milk production of a cow and its calf's growth performance are largely affected by the parity of the cow in both the dairy and beef industries. Marston *et al*. (1992) reported that primiparous Angus cows produced 354 kg less milk during a 205-day lactational period than multiparous cows that ranged from age 3-10 years. Primiparous Simmental cows produced 429 kg less milk than multiparous cows aged 3-10 years. Gaskins and Anderson (1980) reported that 2 year old Angus × Hereford and Simmental × Angus crossbred beef cows produced approximately 2 kg less milk per day compared to 4 year old cows. Clutter and Nielsen (1987) reported that the 205-day milk yield of dams increased significantly from 1172 kg at the age of 2 years, to 1552 kg at the age of 5 years, but no significant increase occurred after 5 years of age. Boggs *et al*. (1980) reported that dam milk production increased from 3-8 years of age and decreased thereafter. Cows 5-8 years of age produced 0.51 kg/d more milk compared to younger cows at 3 years of age and 0.77 kg/d more compared to older cows aged 9 years and above. According to Triplett *et al*. (1995), mature beef cows weaned calves with 11 kg greater weights and 0.07 kg/d greater ADGs compared to calves weaned from first calf heifers.

2.3 Potential genes that impact heifer and calf productivity

2.3.1 The *Leptin* (*LEP*) gene

Leptin is the hormone product of the *Leptin* (*LEP*) gene, consist of three exons and two introns (Taniguchi *et al*. 2002; Figure 2.4). The gene is mapped to chromosome 6 in

mice (Zhang *et al*. 1994), chromosome 7q31.3 in humans (Green *et al*. 1995), and chromosome 4 in cattle (Stone *et al*. 1996). The hormone is synthesized predominantly by white adipose tissues and very small amounts by brown adipose tissues (Zhang *et al*. 1994; Auwerx & Staels 1998). As a result, plasma leptin concentrations are positively correlated with the adipose tissue mass in the body, being high in animals with high fat levels and being low in animals with lower fat deposits (Houseknecht *et al*. 1998).

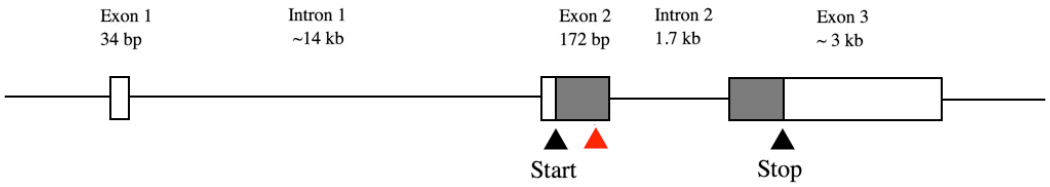


Figure 2.4: Schematic of genomic structure of the *Bos taurus LEP* gene. Boxes indicate the three exons and the coding regions are highlighted in grey, whereas open boxes represent the untranslated regions. The ATG start and the stop codon are represented in black arrows. The c.Arg25Cys SNP is represented in a red arrow (Adapted from Buchanan *et al*. 2002 and Taniguchi *et al*. 2002).

2.3.1.1 Leptin hormone functions in reproduction in cattle

Leptin is a signaling factor that controls body homeostasis by interacting with specific receptors located in the central nerve system, mainly the hypothalamus and the peripheral tissues (Auwerx & Staels 1998; Delavaud *et al*. 2002). Serum leptin has been shown to have a role in reproduction in cattle, controlled via the central nervous system (Houseknecht *et al*. 1998; Meikle *et al*. 2004; Clempson *et al*. 2011). It has been reported

that adequate concentrations of circulating leptin were required to initiate the onset of puberty in dairy cows (Liefers *et al*. 2003; Zieba *et al*. 2005) and tended to rise before puberty and throughout the pregnancy (Liefers *et al*. 2003; Zieba *et al*. 2005; Clempson *et al*. 2011). However, a decrease in serum leptin concentration was observed shortly before calving in dairy cattle and reached the lowest levels in early lactation, attributed to negative energy balance (Liefers *et al*. 2003; Meikle *et al*. 2004). An *in vitro* study conducted by Boelhauve *et al*. (2005) reported that leptin affected fertilty in cattle at the ovarian level by increasing early embryo development. Serum leptin concentrations were positively correlated with BCS in dairy cattle (Ehrhardt *et al*. 2000; Meikle *et al*. 2004), which according to Spitzer *et al*. (1995) and Vargas *et al*. (1999) has a close relationship with conception rates. Kulig, 2005 speculated that leptin may inform the hypothalamus about the energy reserves of the cow to support pregnancy and lactation, thus permitting the cow to reproduce.

2.3.1.2 Beef cattle c.Arg25Cys SNP and its functions

A non synonymous amino acid substitution: c.Arg25Cys, has been discovered in exon 2 of the *LEP* gene, with a cytosine (*C*) to thymine (*T*) base change in cattle (Buchanan *et al*. 2002; Buchanan *et al*. 2003). The amino acid substitution was located in the fourth codon from the N-terminus of the mature leptin protein. This substitution imparted a functional difference to the leptin molecule by adding a cysteine bridge site in the A-helix of the molecule (Buchanan *et al*. 2002). The presence of an additional unpaired cysteine in the leptin molecule could destabilizes the existing disulfide bridge between the two cysteines and

leads to a disruption of the binding of leptin to its receptor, which then prevents the leptin signaling to the hypothalamus (Buchanan *et al*. 2002). The *T* allele or cysteine at this position was associated with increased gene expression (Buchanan *et al*. 2002) and several other economically important traits in cattle.

The c.Arg25Cys SNP was found to be associated with fat measurements in beef carcasses (Buchanan *et al*. 2002; Schenkel *et al*. 2005). Both average fat and grade fat were significantly affected by genotype. The animals homozygous for the *T* allele were associated with fatter carcasses whereas the animals homozygous for the *C* allele were associated with leaner carcasses. According to Kononoff *et al*. (2005), beef cattle homozygous for the *T* allele have been graded AAA, approximately 7% more often than *C*/*T* or *C*/*C* animals. Buchanan *et al*. (2002) reported a significant difference in allele frequencies across breeds. The British breeds such as Angus and Hereford, which are characterized by their early maturity and ability to deposit more fat at a younger age (Gregory *et al*. 1994), had a higher frequency of the *T* allele, while, more heavily muscled and leaner continental breeds such as Simmental and Charolais had a higher frequency of the *C* allele.

The *LEP* c.Arg25Cys SNP has also been found to affect milk production in mature dairy cows in terms of total milk yield and protein yield without changing the milk fat yield (Buchanan *et al*. 2003). Over the entire lactation, the *T*/*T* homozygous cows produced 1.5 kg/d more milk compared to the *C*/*C* homozygous cows. This association was more prominent in early lactation (approximately the first 100 days) with 2.44 kg/d milk yield and declined to 1.74 kg/d between 101 and 200 days (Buchanan *et al*. 2003). Similarly, the *T*/*T*

homozygous cows had a 0.072 kg/d higher milk protein yield in their early lactation than the *C/C* homozygous cows (Buchanan *et al*. 2003). Buchanan *et al*. (2003) speculated that the *T/T* homozygous animals may have had increased *LEP* gene expression and body fat reserves similar to that observed in beef cattle.

Based on the findings of Buchanan *et al*. (2003), DeVuyst *et al*. (2008) hypothesized that, if the effect of the *LEP* c.Arg25Cys SNP on dairy cow milk production was carried over to beef cattle, it was possible that the *T/T* and *C/T* beef cows would wean calves heavier than the *C/C* cows. As they had hypothesized, the crossbred *T/T* beef cows in their study weaned calves that were 12.44 kg heavier, and the *C/T* cows weaned calves that were 8.81 kg heavier than the crossbred *C/C* beef cows.

From an economic perspective, the c.Arg25Cys SNP has shown positive impacts on cow productive life and cow-calf profitability, with the use of a bio-economic stochastic simulation (Mitchell *et al.* 2009). *T/T* cows remained 1.94 years more and *C/T* cows remained 1.65 years more in the cow herd than *C/C* cows. Further, based on the simulated productive lives and calf weaning weights, Angus, Simmental and commercial beef cows homozygous for *T* allele gave statistically higher economic returns than cows homozygous for *C* allele, proving that managing *T/T* animals was more profitable for the rancher than *C/C* animals (Mitchell *et al.* 2009).

2.3.2 The *Pro Melanin Concentrating Hormone* (*PMCH*) Gene

Mammalian *PMCH* gene is known to encode three neuropeptides: Melanin-Concentrating Hormone (MCH), Neuropeptide-Glutamic acid-Isoleucine (NEI), and putatively Neuropeptide-Glycine-Glutamic acid (NGE; Nahon *et al*. 1989; Breton *et al*. 1993). The *PMCH* gene consists of three exons and two introns (Breton *et al*. 1993; Figure 2.5). Abundant research has been carried out on MCH, a little on NEI, while the knowledge on NGE remains negligible.

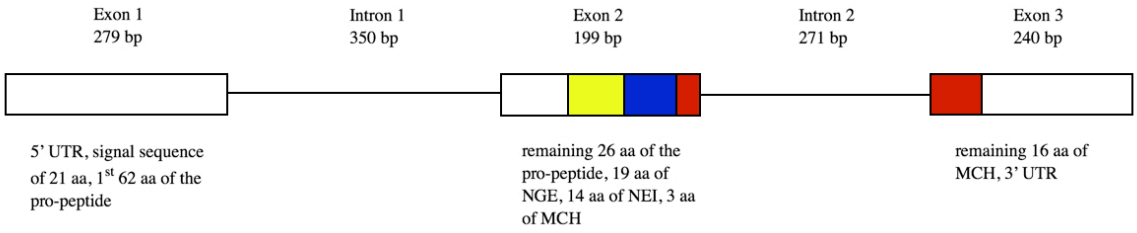


Figure 2.5: Schematic of genomic structure of *Homo sapiens PMCH* gene. Yellow indicates the coding sequence of NGE, blue indicates NEI and red indicates MCH (Adapted from Breton *et al*. 1993 and Helgeson 2007).

2.3.2.1 Peptide functions

MCH is an orexigenic neuropeptide that plays a role in the regulation of appetite/food intake and metabolism in mammals (Shimada *et al*. 1998; Gavrila *et al*. 2005; Mul *et al*. 2010). The *MCH* expression has been shown to increase during fasting in mice (Qu *et al*.

1996; Shimada *et al*. 1998) and in humans (Gavrila *et al*. 2005). Mice deficient in MCH were lean and had reduced body weights due to reduced feed intake and increased metabolic rate (Shimada *et al*. 1998). However, once MCH was administered centrally, it promoted their feed intake and led to obesity (Shimada *et al*. 1998). MCH deficient mice also had reduced leptin levels, which should result in increased feed intake. However, in this context, low leptin levels resulted in a reduced feed intake suggesting that MCH may be necessary for the hyperphagic response of leptin hormone (Shimada *et al*. 1998).

NEI and *MCH* have shown variations in expression during pregnancy and lactation in rats (Knollema *et al*. 1992; Sun *et al*. 2004), whereas Garcia *et al*. (2003) reported decreased hypothalmic *NEI* and *MCH* expression in pregnant and lactating rats. Furthermore, MCH and NEI have been involved in stimulating the oxytocin release from the posterior pituitary glands in rats (Parkes & Vale 1993). MCH is also known to have effects on regulation of luteinizing hormone release in rats, either directly at the level of the pituitary gland or indirectly via the stimulation of hypothalamic gonadotropin-releasing hormone (Murray *et al*. 2000; Naufahu *et al*. 2013). Even though MCH knockout mice are fertile (Shimada *et al*. 1998; Adams *et al*. 2011), they had low success rates in mating, estrous synchronization and smaller and lighter litters with lower survival rates compared to wild type mice (Adams *et al*. 2011).

2.3.2.2 Beef cattle PMCH gene g.-134A>T SNP and carcass fat measurements

Genes that are involved in the appetite pathway are of particular interest in the beef cattle industry, as animals with higher weights and optimal fat layers are more desirable than their counterparts. Thus, the effect of *PMCH* as a candidate gene for the regulation of carcass traits in cattle was investigated and a 5' nucleotide alteration from adenosine-to-thymine at position -134 relative to the translation start codon was identified (Helgeson & Schmutz 2008; Figure 2.6). The SNP was reported to be associated with carcass traits in beef cattle including average fat and grade fat of meat (Helgeson & Schmutz 2008). The *A/A* homozygotes had the highest fat levels and the *T/T* homozygotes had the least fat levels, while the *A/T* animals had intermediate fat levels (Helgeson 2007).

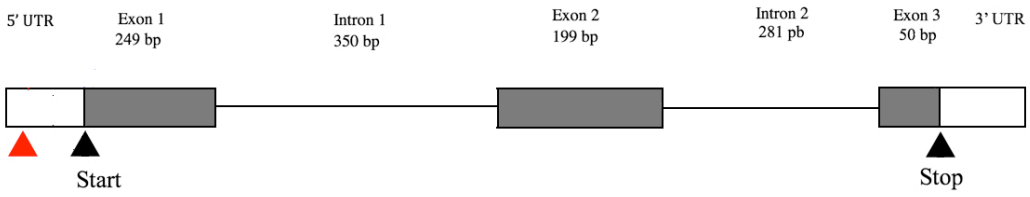


Figure 2.6: Schematic of genomic structure of *Bos taurus PMCH* gene. Boxes indicate the three exons and the coding regions are highlighted in grey, whereas open boxes represent the untranslated regions. Start and stop codons are indicated in black arrows and the g.-134A>T SNP is indicated in a red arrow (Adapted from Helgeson 2007).

2.3.2.3 Function of *PMCH* SNP

As this nucleotide alteration was located in the promoter region of the *PMCH* gene, Helgeson and Schmutz (2008) postulated that it may affect the transcription rate of the gene. It has been found that in the presence of a *T* allele at the -134 position, a binding site was created for a transcriptional repressor known as Adenovirus E4 promoter binding protein (E4BP4). The SNP is located at the seventh residue of the E4BP4 consensus sequence. In the presence of an *A* allele the consensus binding site was disrupted, thus E4BP4 does not bind to the *PMCH* promoter (Figure 2.7). E4BP4 was assumed to be responsible for the decreased transcription of the *PMCH*. Therefore, the observation that animals homozygous for the *T* allele had lesser fat than the animals homozygous for the *A* allele supported the hypothesis that the decreased transcription of the gene in the presence of a *T* allele is the means by which g.-134A>T SNP exerts a functional effect on carcass fat deposition in beef cattle (Helgeson & Schmutz 2008).

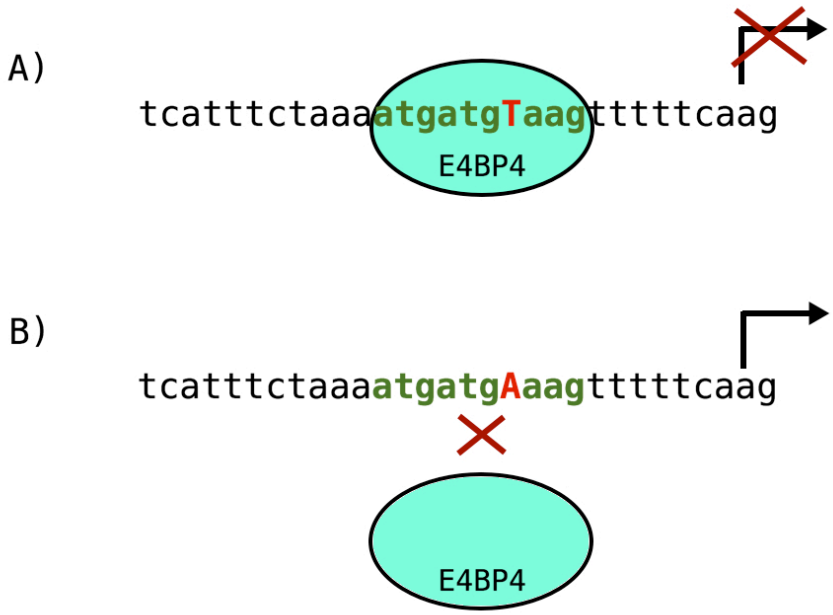


Figure 2.7: Schematic of suggested mechanism of the *PMCH* SNP action. A) In the presence of a *T* allele shown in upper case, it creates a binding site for E4BP4 repressor, thus inhibits the transcription of the gene. B) In the presence of an *A* allele shown in upper case, the E4BP4 repressor cannot bind to the promoter, thus the transcription of the gene was not hindered (Adapted from Helgeson 2007).

2.3.3 The *Integral Membrane Protein 2A* (*ITM2A*) gene

The *ITM2A* is a gene that codes for a transmembrane type II protein and belongs to a family composed of at least two other members: *ITM2B* and *ITM2C* in human and mouse (Deleersnijder *et al*. 1996). There is high homology (over 95%) between human and mouse for the same protein in this family. Homology between the three proteins is around 40% within the same species, mostly in the carboxy-terminal region (Deleersnijder *et al*. 1996).

The porcine ITM2A protein has 89% homology with human and 88% homology with mouse (Liu *et al*. 2008).

In humans and rodents, the *ITM2A* was predominantly expressed in endochondral bone derived structures including, ribs, vertebrae, long bones and in skeletal muscle (Deleersnijder *et al*. 1996), hair follicles, skin and tongue (Tuckermann *et al*. 2000). It was also expressed in T-cells and low levels were seen in brain, heart, lung, stomach and uterus in mice (Kirchner & Bevan 1999). Further, Rengaraj *et al*. (2007) has shown that a low level of *ITM2A* was detected in testicular interstitium during sexual maturation in mice. In swine, the gene was overexpressed in fat and spleen and moderate to weak expression was observed in lung and muscle. Minimal levels were expressed in liver, small intestine, large intestine and kidney (Liu *et al*. 2008). However, there are no findings reported on ruminant *ITM2A* to date.

The *ITM2A* gene consists of six exons and 5 introns (Figure 2.8). The first intron is long (~3400 bp) compared to rest of the introns in both human and mouse (Pittois *et al*. 1999). The 5' UTR contains many binding sites for transcription factors involved in chondrogenesis and osteogenesis (Pittois *et al*. 1999). The transmembrane domain of the ITM2A protein is contained entirely in exon 2, with the carboxy terminal region being extracellular (Deleersnijder *et al*. 1996; Pittois *et al*. 1999). The gene is mapped to the X chromosome, position XA2-XA3 in mouse and to position Xq13.3-Xq21.2 in human (Pittois *et al*. 1999). In humans, it has been reported that the region including the *ITM2A* gene escapes X-chromosome inactivation (Tukiainen *et al*. 2014).

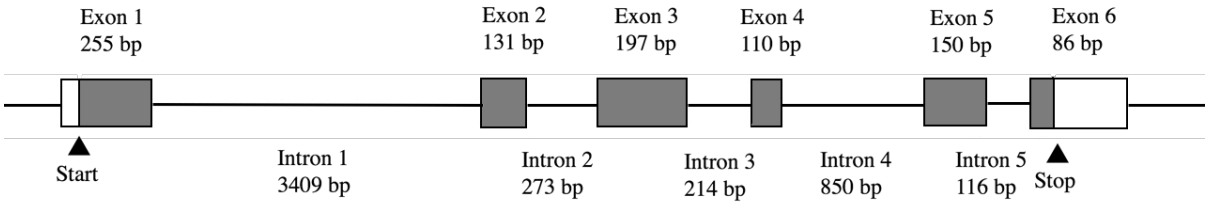


Figure 2.8: Schematic of genomic structure of *Homo sapiens ITM2A* gene. The exons are represented in boxes and the arrows indicate the ATG start codon and TGA stop codon.

2.3.3.1 X-chromosome inactivation

Sex chromosome dimorphism leads to a genetic imbalance between XX females and XY males in mammals. Therefore, the dosage compensation of X linked genes in mammals is achieved by silencing one of the X chromosomes in female somatic cells (Lyon 1961). This process is known as the X-chromosome inactivation (XCI) or lyonization.

In placental mammals, two forms of XCI have been reported: imprinted/paternal XCI and random XCI. The imprinted inactivation of the paternal X chromosome occurs soon after fertilization at the four cell stage of embryonic development and is only limited to the extra embryonic tissues, which later become the placenta. Shortly after this, random XCI of either the maternal or paternal X chromosome takes place in the inner cell mass of the embryo, which gives rise to the embryonic somatic cells (Heard & Disteche 2006; Payer & Lee 2008; Augui *et al*. 2011). An untranslated RNA encoded by the *X inactive specific transcript* (*Xist*) gene determines the choice of which X chromosome undergoes random XCI and initiates the XCI in the chosen chromosome (Penny *et al*. 1996). The *Xist* is transcribed from the center of

the inactivation of the chosen chromosome and coats its entire length. It has been reported that upon initiation, the *Xist* has a lesser role in silencing the chromosome. A diverse set of epigenetic regulators are associated with *Xist*, in heterochromatin formation and silencing the inactivated X chromosome, as well as in maintaining the inactivation throughout the subsequent cell divisions, except in the germ line where the X-reactivation occurs (Plath *et al*. 2003; Silva *et al*. 2003; Payer & Lee 2008; Chan *et al*. 2011).

In addition, DNA methylation is a key factor in maintenance of the inactive state of the chromosome. Deletion of the *DNA methyltransferase* gene (*DNMT1*) resulted in X-reactivation in the embryos (Sado *et al*. 2000). DNA methylation is also important in the maintenance of the repressed state of *Xsit* on the active chromosome (Sado *et al*. 2000). *Xist* is unmethylated in the inactive X chromosome, therefore it facilitates initiation and maintenance of the inactive state of the chromosome. In the active X chromosome, the *Xist* is methylated and silent, and thus prevents it undergoing inactivation (Beard *et al*. 1995).

However, it has been reported that some genes in the inactive X chromosome escape inactivation and are expressed from both the active and the inactive X chromosomes. In humans between 15-25% of X-linked genes were reported to escape inactivation completely or partially (Carrel & Willard 2005). The proportion of the genes escaping inactivation differs between different regions of the X chromosome, where evolutionary younger, unconserved parts are the most susceptible regions for escaping inactivation (Carrel & Willard 2005). These genes could lead to sexual dimorphic traits in humans (Carrel & Willard 2005; Tukiainen *et al*. 2014).

2.3.3.2 The *ITM2A* gene functions

The *ITM2A* gene has been identified as a contributor in the immune system in mice (Kirchner & Bevan 1999). In thymus, it has been shown that there is upregulated expression of *ITM2A* in positively selected CD4 and CD8 thymocytes that interact with major histocompatibility complexes. However, it was seen that the expression was higher in mature $CD4^+$ thymocytes compared to $CD8^+$, suggesting a unique requirement of *ITM2A* in the development of $CD4^+$ thymocytes (Kirchner & Bevan 1999). The expression of ITM2A was low in peripheral organs such as spleen and lymph nodes compared to thymus (Kirchner & Bevan 1999). Thus, it is proposed that the *ITM2A* acts as a novel marker in thymocyte development and T cell activation (Kirchner & Bevan 1999).

In mice, the *ITM2A* gene has been identified as a novel marker for chondrogenic differentiation (Deleersnijder *et al*. 1996) and found to have upregulated expression across the growth plate of long bones that contain resting and proliferative chondrocytes (Tuckermann *et al*. 2000). This upregulation of the *ITM2A* expression was predominantly associated with the early stages of chondrogenic differentiation (Deleersnijder *et al*. 1996; Van den Plas & Merregaert 2004). However, the overexpression of *ITM2A* has been shown to have inhibitory effects on chondrogenic differentiation (Van den Plas & Merregaert 2004; Boeuf *et al*. 2009). Cells of the mature bones did not express *ITM2A* at significant levels suggesting a lesser involement of the gene in the osteogeneic pathway (Van den Plas & Merregaert 2004). Even though *ITM2A* has been identified as a marker for chondrogenesis, the exact function of the gene in this process is not known.

2.3.3.3 Human *ITM2A* rs1751138 G>A SNP

A recent chromosome X wide association study conducted by Tukiainen *et al*. (2014) in a Finnish and a Swedish human population discovered a SNP (rs1751138 G>A), located approximately 35 kb upstream of the transcription start site of the *ITM2A* gene. The evidence suggested that this region, including the *ITM2A* gene, escapes from X chromosome inactivation, thus showing a lack of dosage compensation at this region in the majority of women (Tukiainen *et al*. 2014). In this context, the allelic effect of the SNP was estimated to be twice as high in women compared to men, as females have two X chromosomes that are transcriptionally active. This has lead to sex differential expression of *ITM2A* in whole blood (Tukiainen *et al*. 2014).

The minor *A* allele of the SNP rs1751138 G>A was associated with shorter stature as well as increased expression of *ITM2A* in females. The overexpression of *ITM2A* has been proposed to inhibit the initiation of chondrogenesis in adipose tissue derived mesenchymal cells (Boeuf *et al*. 2009). Hence, the greater dosage of the *ITM2A* in women could explain some considerable height differences observed in men and women in the same family and suggests *ITM2A* as a functional candidate gene for the sexual dimorphism in stature. Further, these findings highlight *ITM2A* as a potential contributor to the abnormal height phenotypes observed in individuals with an unusual number of X chromosomes (Tukiainen *et al*. 2014), such as X0 females who are always short (Ranke *et al*. 1983).

2.4 Pseudogenes

Pseudogenes are characterized by their close resemblance to one or more genes that derive from the same ancestral gene, and by their non-functionality (Vanin 1985; Mighell *et al*. 2000). The homology of the pseudogene to its functional ancestral gene is achieved by sequence alignment and computing the percentage of identical base pairs (van Baren & Brent 2006). The non-functionality of pseudogenes are a result of their failure to either transcribe, translate or encode a protein (Vanin 1985).

Pseudogenes fall under two general categories: processed and non-processed (Vanin 1985). Processed pseudogenes are inserted into the genome as complementary DNA, generated from reverse transcription of a single stranded mature RNA (Vanin 1985). Therefore, most of the processed pseudogenes have characteristics similar to a mature RNA such as: lack of introns and the 5' untranslated region and presence of a poly A sequence at 3' end (Vanin 1985; Mighell *et al*. 2000; Dewannieux & Heidmann 2005; Figure 2.9). Processed pseudogenes are usually clustered at a common locus in the genome, but can also occur in isolation (Mighell *et al*. 2000). Since these genes are already processed, most of the processed pseudogenes cannot code for a protein, as they are unable to undergo transcriptional and translational modifications.

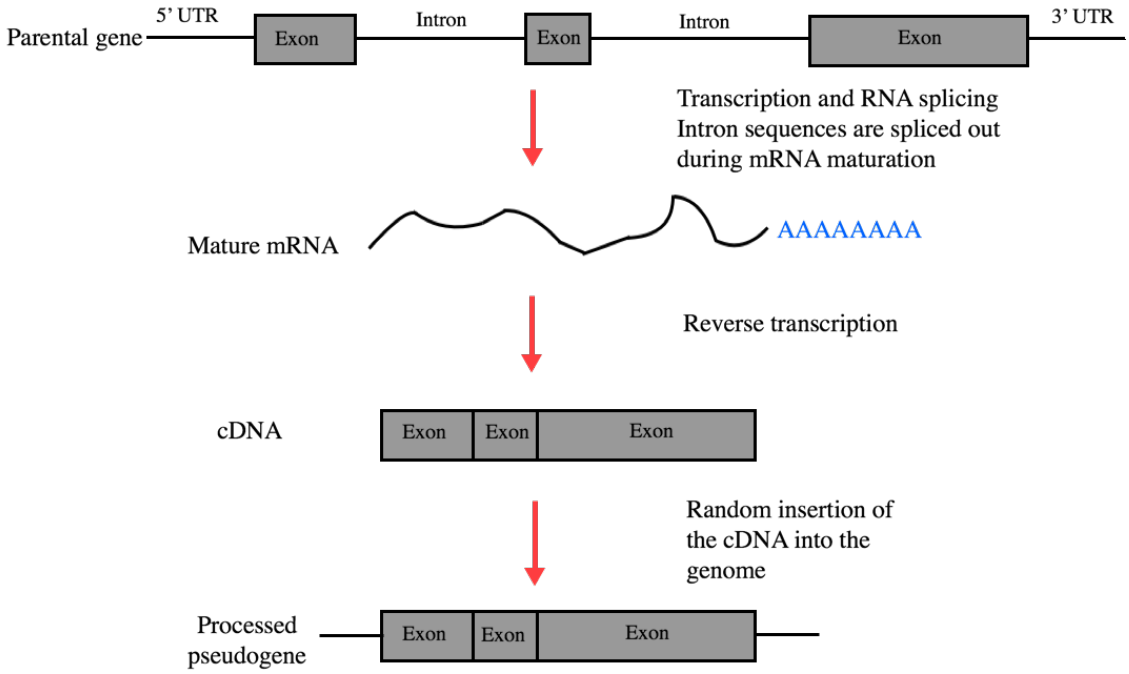


Figure 2.9: Schematic representation of processed pseudogene formation. Solid boxes represent the exons and the linear bold lines represent intervening introns and flanking sequences (Adapted from Vanin 1985 and Zhang 2003).

Non-processed pseudogenes arise by duplication of genomic DNA segments during the development of complex genomes (Mighell *et al*. 2000; Zhang 2003). Once a gene is duplicated, it usually has similar characteristics as the original gene such as intervening introns and a 5' untranslated region (Zhang 2003). However, gene duplication often generates functional redundancy, as having two functional genes could lead to gene dosage (Zhang 2003). Pseudogenization of the duplicated gene is achieved by introducing mutations such as premature stop codons, frameshifts, etc. that disrupt the structure and function of the

gene. These mutations are not deleterious to the organism and are not removed by selection (Zhang 2003). Pseudogenes generated by duplication of genomic DNA are more likely to be adjacent to their functional genes, but they could also be inserted into different chromosomes (Mighell *et al*. 2000). Pseudogenes have also been found within introns (Dierick *et al*. 1997) and the promoter regions (Samuelson *et al*. 1990) of other functional genes in humans. When these genes are present within exons of another functional gene, they alter the normal function of the host gene, thus are rapidly selected against and removed from the genome (Mighell *et al*. 2000).

3.0 ANALYSIS OF *PMCH* AND *LEPTIN* GENOTYPES AS A BASIS FOR SELECTION OF BEEF REPLACEMENT HEIFERS

3.1 Abstract

Selecting a good set of replacement heifers is critical to maintaining a quality beef herd. The objective of this study was to develop a DNA test for the reliable selection of beef replacement heifers. Two cohorts of Angus (n=94) and three cohorts of Simmental (N=111) heifer dams were examined. Two single nucleotide polymorphisms (SNPs): p.Arg25Cys in *leptin* (*LEP*) and g.-134A>T in pro-melanin concentrating hormone (*PMCH*), previously discovered in the Department of Animal Science, University of Saskatchewan were assessed. We hypothesized that there could be a correlation of these alleles with improved heifer performance. The association of calf average daily gain (ADG) to pasture as an indirect measurement of heifer dam milk production with the heifer SNP genotypes was evaluated. A significant association was observed with dam *PMCH* genotype and calf ADG to pasture in Simmental heifer dams (F=7.76, P=0.007), although not in Angus heifer dams. Increased ADG showed a dominant relationship with the *PMCH T* allele, suggesting that it was associated with the dam's lactational milk yield. A combined effect with the dam *LEP* genotype was not observed. Associations with heifer pregnancy and calving success with these genotypes were also assessed, but were not observed.

Key words: *LEP, PMCH*, Angus, Simmental, ADG, pregnancy, lactation

3.2 Introduction

The ability of a heifer to conceive and produce a calf with desirable growth traits in a timely manner is a major aspect of a sustainable and economically viable beef cow-calf operation (Mitchell *et al*. 2009; Stěhulová *et al*. 2013). Given this, it is apparent that selecting replacement heifers that would produce the best calves is vital. A novel approach to accomplish this task would be to search for genetic markers that are directly or indirectly associated with optimal calf performance from heifers.

Two previously discovered single nucleotide polymorphisms (SNP) in the *leptin* (*LEP*) gene: p.Arg25Cys (Buchanan *et al*. 2002) and the promoter region of the pro-melanin concentrating hormone (*PMCH*) gene: g.-134A>T (Helgeson & Schmutz 2008) were selected to genotype heifers. *LEP T* and *PMCH A* alleles were associated with higher carcass fat deposition in beef cattle (Buchanan *et al*. 2002; Helgeson & Schmutz 2008). Both alleles have shown an additive effect with the trait. Studies have demonstrated that body fat reserves contribute to pregnancy and calving success in beef cows (Rae *et al*. 1993; DeRouen *et al*. 1994; Spitzer *et al*. 1995; Bohnert *et al*. 2013). Therefore, these SNPs may indirectly affect heifer reproductive performance.

It was also hypothesized that both *LEP* and *PMCH* SNPs may affect lactational milk yield in beef heifer dams. The *LEP T* allele was associated with increased milk production in dairy cows (Buchanan *et al*. 2003) and beef calf weaning weights (DeVuyst *et al*. 2008). The *T* allele has shown an additive effect with these traits.

Two neuropeptides coded by the *PMCH* gene, neuropeptide-glutamic acid-isoleucine (NEI) and melanin-concentrating hormone (MCH; Nahon *et al*. 1989; Breton *et al*. 1993) have been shown to stimulate oxytocin release (Breton *et al*. 1993; Parkes & Vale 1993), as well as variation in expression during lactation in rodents (Knollema *et al*. 1992; Parkes & Vale 1993; Garcia *et al*. 2003; Sun *et al*. 2004). This suggests a possible role of these neuropeptides in lactation in other mammals. Because the g.-134A>T SNP occurs in the *PMCH* promoter region in beef cattle, this SNP may affect the transcription of *NEI* and *MCH* and regulate lactation in heifer dams.

Effect of the dam SNP genotypes on the ADG of their calves, while they were on a predominantly milk diet, was considered a suitable measure to indirectly assess the dam lactational milk yield. We hypothesized that *LEP* and *PMCH* SNPs could be potential genetic markers for identification of the best replacement heifers based on their calves' early ADG, presumably based on dam milk production and their body fat reserves which would ultimately lead to pregnancy and calving success.

3.3 Materials and Methods

3.3.1 Animal Populations

Two purebred beef breeds, Angus and Simmental, were used in the study. Numerically both breeds are among the top five common beef breeds in Canada

(http://www.canadianbeefbreeds.com/resources/statistics/), thus highly relevant to Canadian ranchers.

Five cohorts of heifer dams were studied from a large Angus and a Simmental herd (Table 3.1). Simmental heifers formed part of a privately owned herd in west-central Saskatchewan. The Angus heifers formed part of the beef herd at the Western Beef Development Centre (WBDC) ranch near Lanigan, SK. Simmental W and Y heifers were bled in summer 2011 and Simmental A heifers and Angus Z and A heifers were bled in summer 2013 to obtain DNA.

Table 3.1: Numbers of Angus and Simmental heifers from each year.

Ranch	**Angus (WBDC)**		**Simmental (Braithwaite Ranch)**		
Cohort	Z heifers	A heifers	W heifers	Y heifers	A heifers
Number (N)	42	52	23	57	32
Heifer year of birth	2012	2013	2009	2011	2013
First calf year of birth*	2013	2015	2011	2013	2015

* For which weights were collected

Phenotypic data were collected from heifer dams and their calves. Heifer data included pregnancy status determined by a licensed veterinarian in October, at approximately 20 months of age (5-6 months pregnant) for Simmental and at 18 months of age (2-4 months pregnant) for Angus heifers. Calving results were obtained in the following spring: January/February for Simmental and March/April for Angus heifers. Calf phenotypic data included birth weight, weight before sending them to pasture in May/June and weaning weight in October. Calf ADG from birth to until they were sent out to pasture (ADG to pasture) and ADG from birth to weaning (ADG to weaning) was calculated.

3.3.2 DNA Extraction and Genotyping

Blood samples had been obtained previously by venipuncture of the jugular or tail veins. Genomic DNA was extracted from fresh whole blood using a salt extraction method (Montgomery & Sise 1990; Appendix A). Extracted DNA was stored at -20^0 C until analyzed.

LEP was genotyped using a Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) assay previously described by Buchanan *et al*. (2002). The SNP creates a restriction site for the *Kpn2I*/*BSPE1* enzyme (T^CCGGA) with a purposeful mismatch primer (Table 3.2). Three genotypes are discernible: *C*/*C*, *T*/*T* and *C*/*T*. The *C* allele was cleaved into two fragments of 75 and 19 bp, while the *T* allele remained uncut at 94 bp.

PMCH genotyping was carried out at Quantum Genetics, Saskatoon, SK using real-time PCR (RT-PCR). The RT-PCR was carried out in a 4 µl reaction containing 30-50 ng of

genomic DNA, 1X PCR buffer, 0.2 mM dNTP, 5 mM $MgCl_2$, 0.5 μM of primer mixture (containing forward and reverse primers and Texas Red and Cy5 dyes; Table 3.2), 0.4 U of Roche FastStart Taq DNA polymerase and 1.44 μl deionized water (dH_20). The RT-PCR reaction was carried out in a BioRad CFX384 real time system. Intial denatuaration was at 95 ºC for 12 min, followed by 44 cycles of: 95 ºC for 10 s and 58 ºC for 30 s. The output was analysed using the BioRad CFX manager 3.1 software.

Table 3.2: Sequences of primers used for PCR-RFLP assay and RT- PCR.

Gene	Primer	Sequence
LEP	*Obese* ex2MM – For	ATGCGCTGTGGACCCCTGTATC
	Obese ex2MM – Rev	TGGTGTCATCCTGGACCTTCC
PMCH	*PMCH* – For	GTTGGTTTCTATCTGATGAGTC
	PMCH – Rev	AATGAAGTGAAATTTCTCAGTC

3.3.3 Trait Adjustments

Prior to statistical analysis, the calf traits were adjusted to be equivalent for comparison using standard calculations. Calf weights were sex corrected to heifer equivalents. The correction factors were 1.05 for steers and 1.1 for bulls (Fredeen 1968). ADG to pasture was calculated using the sex corrected May/June weight minus the sex corrected birth weight, divided by days of age. ADG to weaning was calculated using the sex corrected weaning weight minus the sex corrected birth weight, divided by days of age.

3.3.4 Statistical Analysis

Allele frequencies for each SNP in the Angus and Simmental cattle were calculated. The data regarding Angus Z and A cohorts and data regarding Simmental W, Y and A cohorts were pooled within breed for further analysis, after testing for differences among cohorts (Appendix B). Means and standard error of means for calf ADG to pasture and ADG to weaning were calculated. The dominant effect of dam *PMCH T* allele on calf ADG was analyzed by one-way analysis of variance (ANOVA) in SAS statistical software version 9.4 (SAS Institute Inc., Cary, NC, USA) using the mixed procedure. The model used was:

$$Y_{ij} = \mu + PMCH_i + e_{ij}$$

where Y_{ij} was the trait measured (calf ADG) for the i^{th} individual, μ was the overall population mean of the dependent variable, $PMCH_i$ was the effect of the dam genotype and e_{ij} was the random error for each experimental unit (i.e. individual).

The *LEP T* allele was hypothesized to have an additive effect on calf ADG, and was analyzed by linear regression in StatView statistical software version 5 (SAS Institute Inc., Cary, NC, USA). An interaction effect of the dam's *LEP* genotype and the *PMCH* genotype on its calf's ADG was analyzed by linear regression in StatView statistical software.

The effect of the genotypes on pregnancy and calving success were assessed by chi-square analysis in SAS statistical software version 9.4 (SAS Institute Inc., Cary, NC, USA). A trait was considered to be significantly affected by genotype when $P<0.05$ and trends declared when $0.05<P<0.1$.

3.4 Results

3.4.1 *LEP* and *PMCH* allele frequencies

LEP and *PMCH* genotypes for each heifer dam were generated (Appendix C and D) and the allele frequencies were calculated (Table 3.3). For *LEP*, the minor allele was *C* in the Angus herd and *T* in the Simmental herd. For *PMCH*, the minor allele was *T* for both Angus and Simmental, except for the Simmental A cohort. The two cohorts of Angus heifers have a similar frequency for *PMCH* alleles, whereas, there was more of a difference in allele frequencies among the three cohorts of Simmental heifers. Furthermore, the frequency of both *LEP T* and *PMCH A* alleles, that are associated with higher fat deposition (Buchanan *et al*. 2002; Helgeson & Schmutz 2008), is higher in Angus than in Simmental.

Table 3.3: Allele frequency comparison between Angus and Simmental heifer dams.

Allele	**Allele Frequency**				
	Angus		Simmental		
	Z heifer dams (n=42)	A heifer dams (n=52)	W heifer dams (n=23)	Y heifer dams (n=56)	A heifer dams (n=32)
LEP T	0.56	0.62	0.39	0.28	0.42
LEP C	0.44	0.38	0.61	0.72	0.58
PMCH A	0.90	0.94	0.61	0.57	0.42
PMCH T	0.10	0.06	0.39	0.43	0.58

3.4.2 Association of *PMCH* genotype with performance data in Simmental and Angus heifer dams

ADG to pasture data were available for Simmental W, Y and A heifer dams and ADG to weaning data were available for only W and Y heifer dams. Assessing the calf ADGs to pasture in Simmental, it was observed that calves from dams having a *PMCH A/A* genotype had lower ADGs to pasture compared to calves from dams having *A/T* and *T/T* genotypes. There was no significant difference between the calves' ADGs of heifer dams with *A/T* and *T/T* genotypes based on ANOVA (F=0.12, P=0.73; Table 3.4). These results suggested that the *PMCH T* allele has a dominant effect on calf ADG to pasture. As a result, data regarding the *T/T* and *A/T* genotypes were combined and analysed, using ANOVA, against the data regarding *A/A* genotype. Heifer dams with *A/A* genotypes had calves with significantly lower ADGs to pasture (F=7.76, P=0.007; Table 3.4). This association was not observed for calf ADG to weaning (F=1.12; P=0.48; Table 3.4).

Table 3.4: Association of calf ADG and dam *PMCH* genotype in Simmental heifers.

Dam *PMCH*	**Calf ADG to pasture[1] (kg/d)**		**Calf ADG to weaning[1] (kg/d)**	
	Number (n=111)	W, Y and A heifer dams	Number (n=79)	W and Y heifer dams
T/T	18	0.97 ± 0.069[a]	9	1.09 ± 0.051
A/T	66	0.95 ± 0.033[a]	47	1.08 ± 0.028
A/A	27	0.79 ± 0.055[b]	23	1.11 ± 0.034

[1]Means and standard errors
[a,b] Means with different letters are significantly different (P<0.05)

The *T* allele was not homozygous in any Angus heifers as the frequency of this allele was rare (Table 3.5). ADG to pasture data were available for Angus Z and A heifer dams and ADG to weaning data were available for only Z heifer dams. Neither calf ADG to pasture (F= 0.82; P=0.29) nor ADG to weaning (F=0.98; P=0.36) was affected by *PMCH* genotype in Angus heifers based on ANOVA.

Table 3.5: Association of calf ADG with dam *PMCH* genotype in Angus heifers.

Dam *PMCH*	Calf ADG to pasture[1] (kg/d)		Calf ADG to weaning[1] (kg/d)	
	Number (n=94)	Z and A heifer dams	Number (n=42)	Z heifer dams
A/T	14	0.96 ± 0.052	8	1.01 ± 0.047
A/A	80	1.02 ± 0.026	34	1.05 ± 0.024

[1]Means and standard errors

For Simmental heifers, pregnancy and calving data were not available for W heifers. Neither Simmental (Table 3.6) nor Angus (Table 3.7) heifers showed a significant association between their *PMCH* genotype and their pregnancy success (Simmental χ^2= 0.263, P=0.88; Angus χ^2= 1.719, P=0.19) or calving success (Simmental χ^2= 1.938, P=0.18; Angus χ^2= 0.814, P=0.62) based on Chi-Square analysis.

Table 3.6: Association of reproductive performance data and *PMCH* genotype of Simmental heifers.

Dam *PMCH*	Number (n=88)	Pregnancy[1]	Calving[2]
T/T	15	68%	91%
A/T	54	69%	100%
A/A	19	75%	100%

[1]Percentage of animals that were pregnant of the total.
[2]Percentage of the pregnant animals that calved.

Table 3.7: Association of reproductive performance data in *PMCH* genotype of Angus heifers.

Dam *PMCH*	Number (n=94)	Pregnancy[1]	Calving[2]
A/T	14	95%	100%
A/A	80	86%	96%

[1]Percentage of animals that were pregnant of the total.
[2]Percentage of the pregnant animals that calved.

3.4.3 Association of *LEP* genotypes with the calf performance data of Simmental and Angus heifer dams

The additive effect of dam *LEP* genotype on calf ADG was evaluated by regression analysis. Neither Simmental (Table 3.8) nor Angus (Table 3.9) heifer dams showed a significant association with their *LEP* genotype and calf's ADG to pasture (Simmental r=0.081, P=0.49; Angus r= 0.129, P=0.27) nor with calf's ADG to weaning (Simmental r=0.126, P=0.38; Angus r=0.128, P=0.29).

Table 3.8: Association of calf ADG and dam *LEP* genotype in Simmental heifers.

Dam *LEP*	Calf ADG to pasture[1] (kg/d)		Calf ADG to weaning[1] (kg/d)	
	Number (n=110)	W, Y and A heifer dams	Number (n=78)	W and Y heifer dams
C/C	46	0.93 ± 0.054	33	1.08 ± 0.034
C/T	52	0.86 ± 0.045	41	1.09 ± 0.021
T/T	12	1.05 ± 0.072	4	1.14 ± 0.072

[1]Means and standard errors

Table 3.9: Association of calf ADG with dam *LEP* genotype in Angus heifers.

Dam *LEP*	Calf ADG to pasture[1] (kg/d)		Calf ADG to weaning[1] (kg/d)	
	Number (n=94)	Z and A heifer dams	Number (n=42)	Z heifer dams
C/C	17	0.96 ± 0.035	9	0.96 ± 0.041
C/T	43	1.01 ± 0.063	19	1.08 ± 0.027
T/T	34	1.03 ± 0.032	14	1.03 ± 0.034

[1]Means and standard errors

Significant associations were not observed between dam *LEP* genotype and their pregnancy (χ^2= 1.194; P=0.55) nor calving success (χ^2= 1.249; P=0.45) for Simmental (Table 3.10). Significantly more Angus heifers with a *LEP C/T* genotype became pregnant than their homozygous counterparts (χ^2= 5.404, P=0.04; Table 3.11). This effect did not show an

additive association with the *LEP T* allele. Calving success was not affected by the *LEP* genotype in Angus (χ^2= 1.173; P=0.57; Table 3.11).

Table 3.10: Association of reproductive performance data in *LEP* genotype of Simmental heifers.

Dam *LEP*	Number (n=88)	Pregnancy[1]	Calving[2]
C/C	40	71%	96%
C/T	37	65%	100%
T/T	11	82%	100%

[1]Percentage of animals that were pregnant of the total.
[2]Percentage of the pregnant animals that calved.

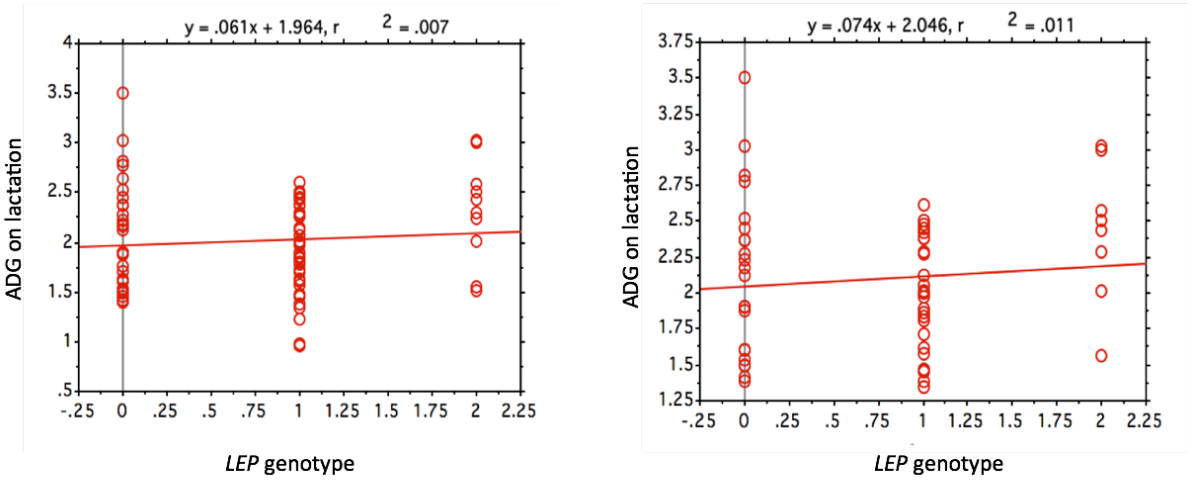
Table 3.11: Association of reproductive performance data in *LEP* genotype of Angus heifers.

Dam *LEP*	Number (n=94)	Pregnancy[1]	Calving[2]
C/C	17	88%	93%
C/T	43	100%	98%
T/T	34	88%	97%

[1]Percentage of animals that were pregnant of the total.
[2]Percentage of the pregnant animals that calved.

3.4.4 Combined effect of dam *LEP* and *PMCH* genotypes on calf ADG in Simmental heifer dams

Since Simmental dam *LEP* genotype alone did not affect their calf's ADG, the data were re-analysed to determine if there was an interactive effect of dam *LEP* and *PMCH* genotypes on their calf's ADG. Data from heifers with either *PMCH A/T* or *T/T* genotype were combined, due to the dominance effect of *T* allele observed on calf ADG to pasture. Based on the proposed additive effect of the *T* allele on dam milk production (Buchanan *et al*. 2003), we hypothesized that heifer dams with a *T/T* genotype for *LEP* and either *A/T* or *T/T* genotype for *PMCH* would have calves with highest ADGs, heifer dams with a *C/T* genotype for *LEP* and either *A/T* or *T/T* genotype for *PMCH* would have calves with intermediate ADGs and heifer dams with a *C/C* genotype for *LEP* and either *A/T* or *T/T* genotype for *PMCH* would have calves with lowest ADGs. In order to determine if there was an interaction, the regression of this interaction was compared to the regression of heifer *LEP* genotype alone on calf ADG (Figure 3.1). Although the slopes of the regression line were different, the fit of the lines was not significant.



(a) *LEP* (b) *LEP* and *PMCH A/T* and *T/T*

Figure 3.1 (a) Comparison of regression of *LEP* genotype alone. (b) *LEP* genotype and *PMCH A/T* and *T/T* genotype interaction. 0 codes for *LEP C/C* genotype, 1 codes for *LEP C/T* genotype and 2 codes for *LEP T/T* genotype. The slope of the regression line was 0.061, r=0.081 and P= 0.49 for *LEP* genotype alone and the slope of the regression line was 0.074, r=0.103 and P=0.45 for *PMCH A/T* and *T/T* genotype interaction.

3.5 Discussion

3.5.1 Allele frequencies

The two cohorts of Angus heifers had similar allele frequencies for *PMCH* g.-134A>T SNP, but there was more of a difference in allele frequencies between the W, Y and A Simmental heifers (Table 3.2). For *PMCH*, the minor allele was *T* for both Simmental and Angus heifers with an overall frequency of 0.47 for Simmental and 0.08 for Angus. Helgeson (2007) reported somewhat higher *PMCH T* allele frequencies among 18 Simmental (0.58) and 55 Angus (0.17) purebred bulls.

The *LEP* p.Cys25Arg SNP allele frequencies were also less variable among the two Angus cohorts compared to the three Simmental cohorts (Table 3.2). Among Angus, the minor allele was *C* with an overall fequency of 0.41. Conversely, the minor allele was *T* among Simmental heifers with a frequency of 0.36. Similar results have been reported by Buchanan *et al*. (2002) for Angus and Simmental bulls with a 0.42 *C* allele frequency among 60 Angus bulls and 0.32 *T* allele frequency among 17 Simmental bulls.

In accordance with previously reported data by Buchanan *et al*. (2002) and Helgeson and Schmutz (2008), the allele frequencies observed in this study were different between the two breeds with the Angus heifers having greater frequency of the *LEP T* allele and the *PMCH A* allele (Table 3.2). The *LEP T* (Buchanan *et al*. 2002) and the *PMCH A* (Helgeson & Schmutz 2008) are the alleles that are associated with higher fat deposition in beef cattle.

3.5.2 Effect of *PMCH* and *LEP* genotype on calf ADG

3.5.2.1 *PMCH*

Calves of Simmental dams having *A/T* and *T/T PMCH* genotypes had 0.18 kg/d greater ADG from birth to the time they went to pasture (ADG to pasture) compared to calves from dams having an *A/A* genotype (Table 3.4). Based on the literature, this difference is likely caused by a difference in lactational milk yield and/or quality of the heifer dams. Liu *et al*. (2015) reported a positive linear relationship between 24 hour dam milk yield and their

calf's pre-weaning ADG for cows sired by British breeds (r^2=0.41) and a quadratic relationship for cows sired by European breeds (r^2=0.53; Figure 2.4). Similarly, a strong positive correlation (r =0.75) was reported by Beal *et al*. (1990) between dam 180-day milk production and their calf's pre-weaning gain in Angus crossbred cows.

Once the Simmental cow-calf pairs were sent to pasture, the calf ADG from birth to weaning in fall (ADG to weaning) was not affected by the dam *PMCH* genotype (Table 3.4). This further supports our speculation that the difference in calf ADGs observed before they were sent to pasture, while they relied primarily on lactation, was caused by a difference in milk yield of their dams. According to Clutter and Nielsen (1987) the correlation between calf intake of milk and their 205-day pre-weaning ADG decreased from 0.58 (at day 52) to 0.16 (at day 205) in crossbred beef cows as lactation progressed (Figure 3.2). This confirms the importance of the dam's milk on their calf's growth during early lactation.

The association of the *PMCH* genotype on calf ADG to pasture was not observed in Angus heifers (Table 3.5). One possible reason could be the absence of heifers with the *PMCH T/T* genotype in both Angus cohorts. Perhaps most importantly, the management system under which these two breeds were reared was very different. The Simmental herd was managed as a typical western Canadian purebred herd. The heifers were bred in early April, calved January/February and the cow-calf pairs were sent to pasture in May. On average, they were primarily on their dams' milk for 115 days. On the other hand, the Angus herd was managed as a typical cow/calf herd selling cattle for beef. The heifers were bred in early July, calved March/April and the cow-calf pairs were sent to pasture in early June. This

meant they were primarily on their dams' milk for only about 50 days. In the current study, it is possible that by the time the Angus calves were sent to pasture at approximately 50 days of age, they started eating and digesting grass in considerable amounts in addition to suckling. Therefore, it is likely that the Angus calves did not rely primarily on their dams' milk long enough to see the association of dam *PMCH* genotype on calf early ADG observed in Simmental.

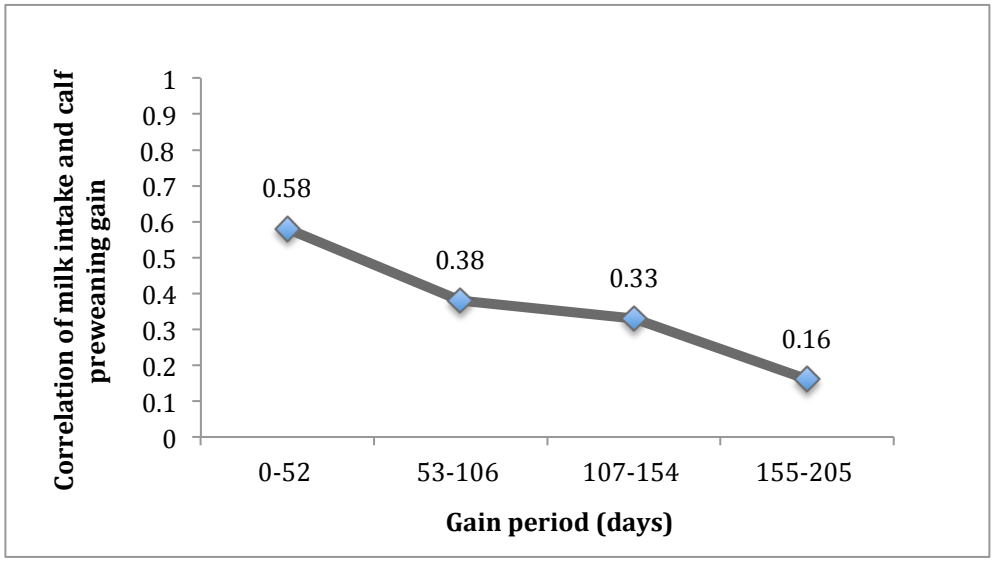


Figure 3.2: The correlation between intake of milk and calf pre-weaning gain. The correlation has decreased as the lactation progressed (Adapted from Clutter & Nielsen 1987. The graph was redrawn using data from 1981 and 1983 study years).

Both Angus and Simmental calves had access to hay, provided to their dam from the time they were born. In addition, Simmental calves were given creep feed starting from the age of approximately 40 days until they were sent to pasture (Appendix E). Even though Simmental calves, but not Angus calves, were creep fed before being turned out on pasture, it is unlikely this was a major factor that affected their ADGs to pasture. Although the creep feed probably affected calf ADGs to some extent, it would be expected to affect all the Simmental calves equally and not cause an increase only in calves of dams with *PMCH A/T* and *T/T* genotypes.

3.5.2.2 Function of the *PMCH* SNP on heifer lactation

The -134A>T *PMCH* SNP is located in the promoter region of the gene (Helgeson & Schmutz 2008; Figure 2.4). A transcriptional repressor known as Adenovirus E4 promoter binding protein (E4BP4) binds to the *PMCH* promoter only in the presence of the *T* allele at this locus and is postulated to decrease the transcription of the gene, whereas not in the presence of an *A* allele (Helgeson & Schmutz 2008; Figure 2.11). MCH is the neuropeptide produced by the *PMCH* gene that is associated with appetite and food intake and metabolism in humans and rodents (Shimada *et al*. 1998; Gavrila *et al*. 2005; Mul *et al*. 2010). The observation that the *T/T* homozygous animals had less fat deposition than the *A/A* homozygous animals suggested that decreased transcription of the gene and the production of decreased MCH in the presence of a *T* allele is the means by which this SNP exerted an effect on carcass fat traits in beef cattle (Helgeson & Schmutz 2008).

Improved heifer lactational milk yield in the presence of a *T* allele at this locus could suggest that the transcription of a second protein of the *PMCH* gene, NEI, could also be affected by the same promoter mutation. Based on the proposed mechanism of the SNP (Helgeson & Schmutz 2008), even though inhibition of *MCH* transcription in the presence of a *T* allele is unfavorable for fat deposition, a decrease in transcription of *NEI* and *MCH* could be favorable for milk production in heifers. Garcia *et al*. (2003) reported a decreased hypothalmic *NEI* and *MCH* expression in pregnant and lactating rats supporting our hypothesis that *NEI* and *MCH* could act as lactation repressors, which would suggest that decreased transcription of these genes are favourable in lactation. However, Knollema *et al*. (1992) and Sun *et al*. (2004) have shown contradictory results and reported increased expression of these neuropeptides in lactating rats. Therefore this suggestion is by no means conclusive, but provides a possible explanation for the action of the *PMCH* SNP on heifer lactational milk yield.

3.5.2.3 *LEP*

An additive effect of the *LEP T* allele was observed with increased milk yield in Holstein dairy cows, predominantly during the first 100 days of lactation (Buchanan *et al*. 2003). In beef cattle, the same *LEP T* allele has shown an additive effect on carcass fat deposition (Buchanan *et al*. 2002). Buchanan *et al*. (2003) speculated that dairy cows may also have had increased body fat reserves in the presence of *LEP T* allele, which may have contributed to their increased milk production. Heifers were not included in this study.

DeVuyst *et al*. (2008) found an effect of dam *LEP* genotype and their calf's weaning weight in crossbred beef cattle and speculated that the effect was due to increased milk yield of the dam. Both heifers and cows were included in this study. However, this additive effect of *LEP T* allele on dam's milk production was not observed for Simmental (Table 3.8) nor Angus (Table 3.9) heifer dams as observed with ADG in the current study, as we had hypothesized. Based on the literature, we suggest that heifers have not deposited as much body fat at calving at the age of two years as their mature counterparts. Hence, the effect of the dam *LEP* genotype observed on increased milk production in dairy cows, probably due to increased cow body fat reserves (Buchanan *et al*. 2003) and increased beef calf weaning weights probably due to increased dam lactational milk yield (DeVuyst *et al*. 2008), may not be observed in heifers.

3.5.4 Effect of *PMCH* and *LEP* genotype on heifer reproductive success

It was reported that cows with higher body fat reserves at late gestation had approximately 10% more live calves at birth and at weaning compared to cows with a lower body fat reserves (Bohnert *et al*. 2013). The subsequent pregnancy rate was also 92% for cows with high fat reserves whereas it was only 79% for cows with low fat reserves (Bohnert *et al*. 2013). In previous studies, the *PMCH A* allele (Helgeson & Schmutz 2008) and *LEP T* allele (Buchanan *et al*. 2002) were shown to have an additive effect based on an increase in fat characteristics in beef cattle. We hypothesized that the *PMCH* and *LEP* favorable alleles would show an additive effect on heifer conception and calving success, as indirectly

affected by their increased body fat reserves. However, we did not observe such an association. It is likely that the effects of higher body fat reserves observed on calving success in mature cows at late gestation may not be prominent in heifers, as they have not deposited much fat at calving at the age of two years. It is also possible that heifers have sufficient body fat to facilitate their first conception, but only rebreeding first calf heifers would be impaired by low body fat reserves due to mobilization of fat tissues and nutrients towards the continued growth of first calf heifers.

Twelve percent more *LEP C/T* heifers became pregnant than their *T/T* and *C/C* counterparts (χ^2= 8.126, P=0.04) (Table 3.11). This association did not show an additive effect with the *LEP T* allele and therefore this could be a spurious association.

4.0 ATTEMPTS AT THE CHARACTERIZATION OF *ITM2A* GENE IN *Bos taurus* CATTLE

4.1 Abstract

The *integral membrane protein 2A* (*ITM2A*) is a gene involved in early cartilage development and has been shown to have higher expression in endochondral bone derived structures. A single nucleotide polymorphism (SNP); rs1751138 G>A has been discovered upstream of the translational start site of the *ITM2A* gene in two human populations. The *A* allele was associated with shorter stature in women. The gene was not characterized yet in cattle. Only a predicted sequence was available. Since stature/frame size was considered an important determinant of reproductive performance in beef heifers and cows, attempts were made to characterize the *ITM2A* gene in Angus and Simmental heifers to search for polymorphisms that could be associated with heifer performance. The sequence obtained from twenty heifers did not reveal the presence of SNPs. However, these sequences and the UMD sequence included stop codons within the predicted exons. Amplification of this putative cattle *ITM2A* cDNA was not successful. Therefore, it is suggested that a pseudogene, rather than the actual cattle *ITM2A* gene was captured.

Key words: *ITM2A*, Angus, Simmental, frame size, pseudogene

4.2 Introduction

The *integral membrane protein 2A* (*ITM2A*) is a gene that codes for a transmembrane type II protein in mouse and human (Deleersnijder *et al*. 1996) and pig (Liu *et al*. 2008). The gene consists of six exons and five introns (Figure 2.5) and was mapped to chromosome X, position XA2-XA3 in mouse and position Xq13.3-Xq21.2 in human (Pittois *et al*. 1999). The *ITM2A* was predominantly expressed in endochondral bone derived structures, skin and lung in humans and rodents (Deleersnijder *et al*. 1996; Tuckermann *et al*. 2000) and in spleen and muscle in pigs (Liu *et al*. 2008). The *ITM2A* gene is implicated in early cartilage development (Deleersnijder *et al*. 1996; Tuckermann *et al*. 2000; Van den Plas & Merregaert 2004). The overexpression of the gene has shown inhibitory effects on chondrogenic differentiation (Van den Plas & Merregaert 2004; Boeuf *et al*. 2009).

A recent study conducted by Tukiainen *et al*. (2014) in humans, suggested that the *ITM2A* gene and the region surrounding it escape from X chromosome inactivation, resulting in a lack of dosage compensation at this locus. This has lead to sex differential expression of the *ITM2A* in whole blood in humans (Tukiainen *et al*. 2014). A single nucleotide polymorphism (SNP): rs1751138 G>A, located approximately 35 kb upstream of the transcription start site of the *ITM2A* gene has been discovered in a Finnish and a Swedish human population (Tukiainen *et al*. 2014). As a result of lack of dosage compensation, the allelic effect of this SNP was estimated to be twice as high in women compared to men. Homozygosity for the minor *A* allele was associated with shorter stature in women, as well as

increased expression of *ITM2A*. As over expression of the gene inhibits the initiation of chondrogenesis (Boeuf *et al*. 2009), the greater dosage of *ITM2A* in women could cause the considerable height differences observed between men and women.

We hypothesized that *ITM2A* could affect stature/frame size in cattle. A smaller frame size (approximately 115 cm at hip) has been associated with increased reproductive performance in beef heifers and cows including a 39-day earlier onset of puberty (Vargas *et al*. 1999), 12% higher calving rate (Buttram & Willham 1989) and 33% more calf survival (Vargas *et al*. 1999) compared to larger frame (approximately 120-135 cm at hip) heifers and cows (Figure 2.1). However, larger framed cows have produced calves with better growth characteristics including 8 kg greater birth weights, 34 kg greater weaning weights and 0.15 kg/d greater ADG from birth to weaning (Vargas *et al*. 1999).

The *ITM2A* gene has not been studied yet in cattle. Therefore, the objective of this study was to characterize the *ITM2A* gene to search for polymorphisms that could affect heifer reproductive performance directly or indirectly in replacement heifer selection.

4.3 Material and methods

4.3.1 Samples

DNA previously extracted from the blood of ten Angus and ten Simmental heifers from a related study (Chapter 3) was examined for single nucleotide polymorphisms (SNP). Samples from tissues including lung, spleen, skin, muscle, ear cartilage, cartilage of trachea

were obtained from two, 51 day old Holstein steers at the Livestock Research Barn, University of Saskatchewan for cDNA studies.

4.3.2 DNA/RNA extraction and cDNA synthesis

Genomic DNA was extracted from whole blood using a salt extraction method (Montgomery & Sise 1990; Appendix A). The tissue samples were immediately placed in RNA*later*® (Ambion, Austin, TX) and frozen until subsequent RNA extraction. Total RNA was extracted following the protocol as outlined in the Qiagen RNeasy mini kit (Qiagen, Mississauga, ON). Synthesis of cDNA from RNA was performed according to the protocol described in the Quanta qscript cDNA supermix (Quanta Biosciences, Gaithersburg, MD).

4.3.3. Genomic DNA and cDNA PCR

Polymerase chain reaction (PCR) primers were designed to amplify exons 1, 2, 3-4 and 4-6 including the intervening introns (Table 4.1; Figure 4.1) from genomic DNA based on the predicted bovine *ITM2A* mRNA sequence (GenBank XM_003588148.2) and the region corresponding to the *ITM2A* gene in bovine UMD 3.1.1 (GenBank NW_003104721.1.) X chromosome whole genome sequences.

RNA sequence was not available for this gene in cattle. The cDNA primers were designed to amplify exons 1-5, 2-3, 2-4, 3-5, and 5-6 based on the predicted cattle *ITM2A* mRNA sequence (GenBank XM_003588148.2) and the human *ITM2A* mRNA sequence (GenBank CR456875.1) (Table 4.1; Figure 4.2). Premelanosome protein (*PMEL*) gene was run on skin and ear samples as a control to check the quality of cDNA.

Table 4.1: Sequences of primers used for genomic and cDNA PCR using predicted cattle *ITM2A* mRNA sequence and UMD 3.1.1 sequence.

Amplified Region	Primer sequence	Expected product size (bp)	Annealing Temperature (°C)
Exon1	***ITM2A 5'-For*** ACTTCCATTCCAGCATTTGG ***ITM2A In1-Rev*** CAAAGCCAACGTAGAGCACA	Genomic 519	59
Exon 2	***ITM2A In1-For*** TGGGTTTCAAAGGCAGTAAAATG ***ITM2A In2-Rev*** GTCTCAGTGAACTCCGGGAA	Genomic 391	61
Exon 3-4	***ITM2A In2-For*** TCCTCAGCTTGATAACTGGTACA ***ITM2A Ex4-Rev*** TTTAGGGGCATCAGACAGCA	Genomic 1071	61
Exon 4-6	***ITM2A In3-For*** GTTGGTGATGGACAGGAAGG ***ITM2A 3' stop-Rev*** TGGCAAAAGCTTGAAGTG GT	Genomic 1009	65
Exon 2-4	***ITM2A Human Ex2-For**** AGGCCTTTCATTCATCTTGGC ***ITM2A Ex4-Rev*** TTTAGGGGCATCAGACAGCA	cDNA 321	60
Exon 5-6	***ITM2A Ex5-For*** GGTTCATGAAGATCTGGTTGC ***ITM2A Ex6-Rev*** TCCAGCACTTATCAATGGCA	cDNA 157	59

*Primer sequence was similar for both human and cattle

Table 4.2: Sequences of primers used for cDNA PCR using human *ITM2A* mRNA sequence.

Amplified Region	Primer sequence	Expected product size (bp)	Annealing Temperature (°C)
Exon 1-5	***ITM2A Human Ex1-For*** GCCGTGCAAAAGGAGGAG ***ITM2A Human Ex5-Rev*** TGCCAAGGTTACTAACATCACG	cDNA 604	61
Exon 2-3	***ITM2A Human Ex2-For*** AGGCCTTTCATTCATCTTGGC ***ITM2A Human Ex3-Rev*** TTGCTGCAGGGTCACTATCA	cDNA 247	60
Exon 3-5	***ITM2A Human Ex3-For*** TGATAGTGACCCTGCAGCAA ***ITM2A Human Ex5-Rev*** TGCCAAGGTTACTAACATCACG	cDNA 232	60

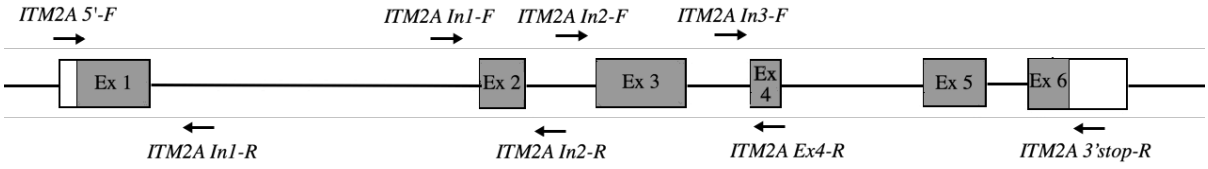


Figure 4.1: Schematic of the binding locations of the DNA primers on the predicted *ITM2A* gene of cattle. Forward primers are shown above and reverse primers are shown below. The exons are indicated as shaded boxes.

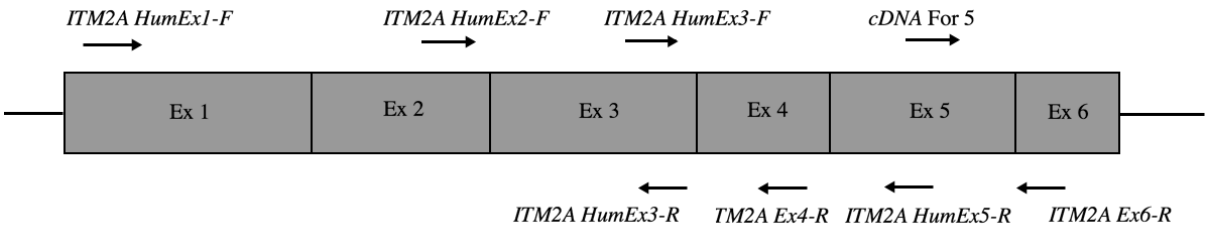


Figure 4.2: Schematic of the binding locations of *ITM2A* complimentary DNA (cDNA) primers. Forward primers are shown above and reverse primers are shown below the schematic of *ITM2A* cDNA.

PCR was carried out according to a previously developed assay in our lab (Appendix F). To amplify the *ITM2A* gene, 30-50 ng of genomic DNA was added to each 14 µl reaction containing 1.5 ml 10X PCR buffer with $(NH_4)_2SO_4$, 10 mM dNTP, 1.5 mM $MgCl_2$, 0.5 U of *Taq* Polymerase (Fermentas Co., Carlsbad, CA, USA), 10 pmol of each primer (Table 4.1) and 9.5 µl of deionized water (dH_2O). The reaction was carried out in a BioRad T100™ thermo cycler. Initial denaturation was 4 min at 95 °C, followed by 34 cycles of: denaturation for 50 s at 95 °C, annealing for 50 s at specific temperatures (Table 4.1) and 1 min extension at 72 °C with a final extension of 10 min at 72 °C.

The PCR reaction cocktail for cDNA amplification was the same as for the genomic DNA amplification. The amplification cycle was: initial denaturation for 4 min at 95 °C, followed by 34 cycles of: denaturation for 40 s at 95 °C, annealing for 40 s at specific temperatures (Table 4.1) and 50 s extension at 72 °C. The reaction concluded with a final extension of 5 min at 72 °C. Genomic PCR products were analyzed on a 1% agarose gel

whereas cDNA products were analyzed on a 2% agarose gel with a 1 kb plus DNA ladder (Invitrogen).

4.3.4 Sequencing

Amplified genomic DNA PCR products were excised from the agarose gel and extracted following the Omega Biotech Gel Extraction Kit protocol and quantified using a NanoDrop™ Lite spectrophotometer. The extracted products were then sent to the National Research Council of Canada, Plant Biotechnology Institute in Saskatoon for Sanger dideoxy sequencing on an Applied Biosystems Sequencer. The sequences were analyzed using Sequencher 4.8 software (Gene Codes, Ann Arbor, MI, USA).

4.4 Results

4.4.1 Genomic DNA sequence analysis

Based on the predicted bovine *ITM2A* sequence, a genomic sequence was obtained from exon 1, exon 2 and exons 3-6. These sequences obtained from 20 cattle did not reveal the presence of any SNPs.

These sequences were aligned to each other and to both the predicted *ITM2A* gene in Btau scaffold sequence (GenBank NW_001508786.2) of the Texas assembly and the *ITM2A* gene scaffold sequence of the University of Maryland (UMD) assembly (GenBank NW_003104721.1). The *ITM2A* sequences obtained were all identical but did not match the

region corresponding to the Btau sequence perfectly. Both the sequences obtained and the UMD scaffold sequences included stop codons within exons 1, 2, 3 and 5.

Our cattle sequences were also compared to the *ITM2A* amino acid sequence reported from human (GenBank CA_G33156.1), mouse (GenBank NP_032435.2) and pig (GenBank NP_001026968.1; Figure 4.3). Although the cattle sequence was similar to other species, the presence of stop codons and extended cattle exon 6 suggest that these cattle genomic fragments do not represent the exons of the *ITM2A* gene.

4.4.2 cDNA sequence analysis

Due to the presence of stop codons within the predicted cattle exons, attempts were made to amplify cattle *ITM2A* cDNA from sequencing parts of predicted exons 2, 4, 5, and 6 before or after the stop codons, using liver, spleen, ear cartilage, lung and skin cDNA samples. It was not possible to obtain cDNA sequence from any of these primer combinations or tissues, leading us to conclude this is a pseudogene. Likewise sequencing exons 1-5, 2-3 and 3-5 using human *ITM2A* primers was also attempted but no cDNA product was obtained. These tissues were chosen because cDNA sequence of *ITM2A* had been obtained in either human, mouse or pig.

A fragment from exon 9 to 3' UTR of the cattle *PMEL* gene was run on skin and ear cartilage samples as a control and the expected product size was obtained (Appendix B). This confirmed that the cDNA samples were of good quality.

```
          1                                                  2
Cattle    MVKTAFNTPTAVQKXEAQQDVEALTSHTVQAQIXTCKELXVATKEKAGSSGRCMLTLXGL
Human     MVKIAFNTPTAVQKEEARQDVEALLSRTVRTQILTGKELRVATQEKEGSSGRCMLTLLGL
Mouse     MVKIAFNTPTAVQKEEARQDVEALVSRTVRAQILTGKELRVVPQEKDGSSGRCMLTLLGL
Pig       MVKIAFNTPAAVQKEEVQQDVEALLSHT---------ELQVATKEKEGFSGRCMLTLVGL

                                  3
Cattle    SFILAGLIVGGACIYKYFMLKSTFYHGEMCFFDSEAPANSLQGEEPYFLPVMEEADIRED
Human     SFILAGLIVGGACIYKYFMPKSTIYRGEMCFFDSEDPANSLRGGEPNFLPVTEEADIRED
Mouse     SFILAGLIVGGACIYKYFMPKSTIYHGEMCFFDSEDPVNSIPGGEPYFLPVTEEADIRED
Pig       SFILAGLIVGGACIYKYFMPKSTIYHGEMCFFDSADPANSLQGGEPYFLPVMEEADIRED

                                            4
Cattle    DNIANHXCACPQILXXXPCS-NSXLQKGMIAYLDLLLGNCCLMPLNTSIVMPPKNLVELF
Human     DNIAIIDVPVPSFSDSDPAAIIHDFEKGMTAYLDLLLGNCYLMPLNTSIVMPPKNLVELF
Mouse     DNIAIIDVPVPSFSDSDPAAIIHDFEKGMTAYLDLLLGNCYLMPLNTSIVMTPKNLVELF
Pig       DNIAIIDVPVPSFSDSDPAAIIHDFEKGMTAYLDLLLGNCYLMPLNTSIVMPPKYLLELF

              5                                                        6
Cattle    GKLASGKYLLHTXVVHEDLVAVEEIRDVSNLGIFIYQLCNKLKSFHLHRRDLLLGFNKHA
Human     GKLASGRYLPQTYVVREDLVAVEEIRDVSNLGIFIYQLCNNRKSFRLRRRDLLLGFNKRA
Mouse     GKLASGKYLPHTYVVREDLVAVEEIRDVSNLGIFIYQLCNNRKSFRLRRRDLLLGFNKRA
Pig       GKLARGKYLPHTYVVHEDLVAVEEIRDVSNLGIFIYQLCNNRKSFRLRRRDLLLGFNKRA

Cattle    IDKCWKIRHPTNLLLRPTFVKNKRQQEMSIFSNRIRDLLVDFNIKVVGILKIFIHAFTLW
Human     IDKCWKIRHFPNEFIVETKICQD.
Mouse     IDKCWKIRHFPNEFIVETKICQE.
Pig       IDKCWKIRHFPNEFIVETKICQE.

Cattle    LIFFKGGKNLLTTSSFCQFLI.
```

Figure 4.3: ITM2A amino acid alignment of cattle, human (GenBank CAG33156.1), mouse (GenBank NP_032435.2), and pig (GenBank NP_001026968.1). Terminations are indicated by an X and highlighted in red. Beginning of each exon is indicated in blue numbers. Stop codons were only present in cattle sequence and the cattle exon 6 did not stop where it stops in other species (Obtained from http://www.ch.embnet.org/software/ClustalW.html).

4.4 Discussion

Twenty Angus and Simmental beef heifers were used for sequencing in this study in order that a 5% or higher allele frequency in the population would be detected. However, we did not observe any SNPs in the sequences obtained from these twenty heifers.

In developing scaffold sequences of large genomes that contain long stretches of contiguous sequences of millions of base pairs, the genome must be first fragmented, sequenced in smaller overlapping pieces called sequence reads, assembled into contigs based on the sequence similarity of sequence reads and finally merged into scaffold sequences (http://www.ncbi.nlm.nih.gov/assembly/basics/). However, these scaffold sequences contain captured gaps, where the sequence within the gap is not known, thus represented with a string of 'Ns' (Figure 4.4). The level of genome coverage in each genome assembly largely affects the number of gaps presented in the assembled scaffold sequence.

Two genome assemblies obtained by Sanger sequencing were available for *Bos taurus*: UMD 3.1.1 sequence submitted by the Center for Bioinformatics and Computational Biology, University of Maryland and Btau 4.6.1 sequence submitted by the Cattle Genome Sequencing International Consortium (http://www.ncbi.nlm.nih.gov), located in Texas. Both assemblies were based on the same set of genomic sequence reads from an adult Hereford cow, with approximately 9X coverage.

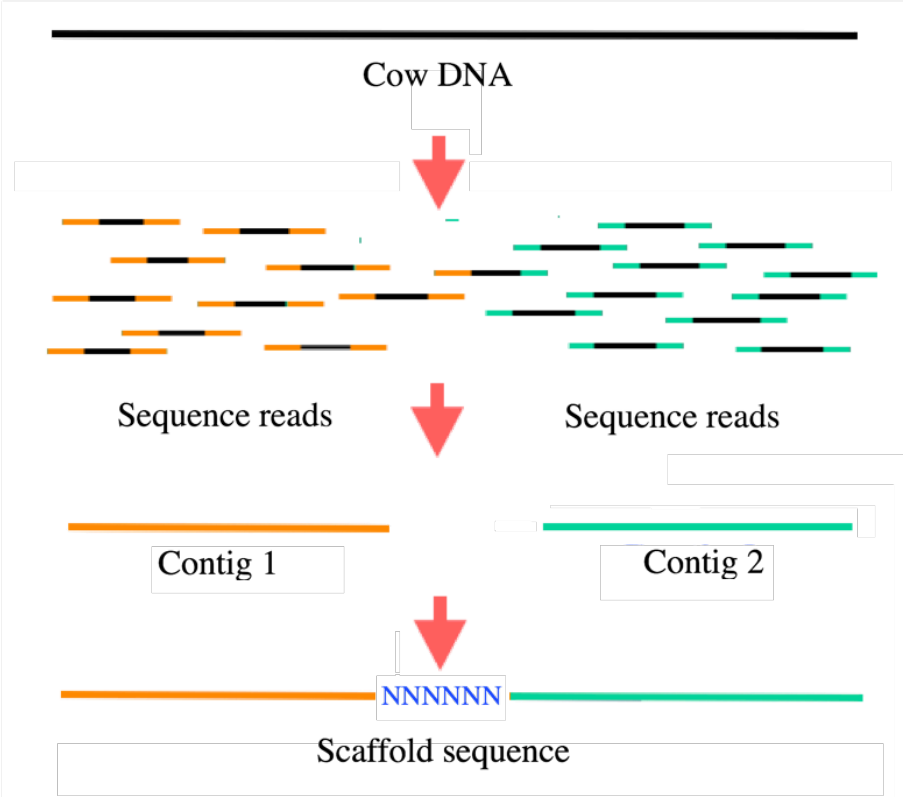


Figure 4.4: A schematic of genome sequencing. Overlapping sequence reads are indicated in orange and green. These sequences contain captured gaps and they are represented as a string of Ns due to sequence ambiguity (Adapted from http://www.ncbi.nlm.nih.gov/assembly/basics/).

The Btau 4.6.1 X chromosome scaffold sequence contained two long ambiguous sequences of 1014 bp and 1791 bp (Ns), which disrupted the sequence. It is possible that these gaps contributed to a misalignment of the sequence in this area. The presence of stop codons within four of the predicted exons out of the total six exons in both the obtained and UMD reference sequences suggests that this is not the actual *ITM2A* gene.

We suggest that this sequence represents a pseudogene. Duplication of large fragments of DNA in the mammalian genome with a significant homology (>90%) to other regions of the genome is common during the development of complex genomes (Mighell *et al*. 2000; Zhang 2003). When genes that code for functional proteins are duplicated, pseudogenization of such genes occurs by introducing mutations such as premature stop codons within the coding sequence of the duplicated gene, as two or more functional copies of the same gene could lead to gene dosage in individuals (Zhang 2003).

It was reported that *ITM2A* mRNA was expressed in endocondral bone derived structures (Deleersnijder *et al*. 1996), skin (Tuckermann *et al*. 2000), lung, spleen and muscle (Liu *et al*. 2008). Our failed attempts to amplify the *ITM2A* cDNA from spleen, ear cartilage, cartilage of trachea, lung and skin tissue samples, with the primers designed using predicted mRNA sequence confirms that a mRNA was not transcribed from the predicted sequence. This further suggests that the captured sequence was likely from a pseudogene.

It has recently been reported, "40% of the cattle coding genes are missing, mis-assembled or have exons missing" (Jerry Taylor, personnel communication). In cattle, a comparison between the *Bos taurus* UMD 2 and Btau 4 genome assemblies shows that 136 Mbp are included in the UMD assembly of the X chromosome, whereas only 83 Mbp were included by the Btau assembly (Zimin *et al*. 2009). Therefore, assembly issues could be prevailing in the cattle X chromosome.

5.0 GENERAL DISCUSSION

5.1 Impact of DNA selection using the *PMCH* genotype on the beef cattle herds

The Canadian beef industry is comprised of both purebred and commercial beef breed operations. Most purebred operations market bulls and some heifers, which may or may not be bred as breeding animals, and some steers into the beef market. Cattle destined for the beef market are typically maintained in a three-phase system involving cow-calf operations, backgrounding on farm or at another facility, and finishing operations usually at a feedlot.

We suggest that the dominant effect of the *PMCH* genotype on calf early ADG observed in this study would be more useful in purebred cowherds, where the calves were born in early spring and depended primarily on milk for a longer period. But this effect may not particularly be beneficial to commercial cow-calf producers that calve in late spring and have a shorter period for the calf to depend mainly on milk.

In terms of lactational milk yield, we suggested that heifer dams having *PMCH A/T* and *T/T* genotypes are superior to dams having an *A/A* genotype (Table 3.4). However, even though calves from *A/T* or *T/T* dams had higher ADGs before they were sent to pasture, at the time of weaning, there was no difference in ADG between calves from heifer dams having *T/T, A/T* or *A/A* genotype (Table 3.4). It is possible that, once sent out to pasture, calves from *A/A* dams consumed more grass to compensate their nutritional requirements. In a cow-calf operation, heifers are selected based on their ability to produce a calf with higher

growth characteristics at weaning when the calves were sold for beef. Therefore in heifers, the effect of *PMCH* genotype on their increased milk yield may not be as advantageous when considering their calves' final ADG at weaning.

It has been repeatedly reported that a cow's milk yield increases until it peaks when they are 5 to 8 years of age (Boggs *et al*. 1980; Clutter & Nielsen 1987; Marston *et al*. 1992). It is therefore expected that the difference in milk yield among dams having the favorable *PMCH A/T* and *T/T* genotypes and *A/A* genotype would also increase. Whether this increase would exacerbate the difference in milk yield is not known. If this occurs, it could lead to a significant difference in calves' ADG at weaning and therefore the effect of the dam's *PMCH* genotype could become beneficial when the heifers get older.

The favorable *PMCH* genotype in terms of beef carcass characteristics has been reported to be *A/A*. The *A* allele had additively favored increased back fat in beef cattle (Helgeson & Schmutz 2008). Helgeson (2007) suggested that genetically selecting for cattle with *PMCH A/A* genotype would therefore be advantageous for beef cow-calf producers who sold their calves at weaning and also for feedlots who used sorting by genotype in their management system. In our study, it was the *T* allele that dominantly affected the increased calf's ADG in Simmental. Therefore, if a selection decision for replacement heifers were to be made based on their *PMCH* genotype, selecting for heifers with *A/T* genotype and breeding them to *A/A* bulls could still be beneficial to ranchers in improving the ADGs and carcass traits of their calves sold for beef.

Even though this effect of *PMCH* genotype on early lactation was observed in beef heifers, it is probable that this effect also occurs in dairy heifers. Because the milk yield can be directly measured in dairy cattle, a study of Holsteins would provide a better understanding about the actual yield differences in the two genotype groups. Selecting for heifers with higher lactational milk yields is the objective of a dairy farmer. Therefore, if this effect appears in dairy heifers, selection for heifers based on their *PMCH* genotype could benefit dairy farmers.

5.2 Future research

In this study, we only looked at the dam *PMCH* and *LEP* genotype effects on the performance of heifers and their calves. It would be worth examining whether the proposed dominant effect of the dam *PMCH* genotype on their calf's early ADG in Simmental heifers persists in their second calf and whether a significant difference would appear in the calves' ADG at weaning. This would confirm whether the selection for beef replacement heifers based on their *PMCH* genotype is a long-term advantage to the rancher or not.

As one possible reason for not observing the effect of dam *PMCH* genotype on calf ADGs in Angus, we stated that, these calves might not have stayed on their dam's milk long enough before having the opportunity to also graze on pasture to see the effect of the genotype. Therefore, it would be useful to evaluate other beef herds in which calving occurs

a longer period before grazing begins, and observe whether the effect prevails in other beef breeds.

We speculated that heifers had a low amount of body fat deposition at the age of two years. Therefore, the difference in body fat levels between dams having *LEP T/T* and *C/C* genotypes could be too low to observe an effect of the *LEP* genotype on milk production in heifers. However, it is possible that this effect could appear in these heifers in their later lactations. Hence, I propose that the additive effect of the dam *LEP* genotype on their second calves' ADG should also be evaluated. If an effect appears in their second lactation, ranchers might still benefit from selecting their heifers based on their *LEP* genotype, even though it did not appear to be beneficial in their first lactation.

Although we hypothesized that the favorable *LEP T/T* (Buchanan *et al*. 2002) and *PMCH A/A* (Helgeson & Schmutz 2008) genotypes that were reported to lead to higher body fat deposition would have had an effect on heifer conception and calving success, such an association was not seen in either the Angus or Simmental herds studied. During the first lactation, when heifer energy demand was high for continued growth and milk production, heifers might start depositing more fat, and a difference in body fat reserves could then appear between the genotypes. Therefore, evaluating the rebreeding success of heifer dams of different *LEP* and *PMCH* genotypes may provide useful findings that are important in heifer selection.

Since a polymorphism near the *ITM2A* gene was reported to be associated with stature in humans (Tukiainen *et al*. 2014), this could be a potential candidate gene for frame

size in cattle. A decision to make a new cattle genome assembly was just reached in September 2015 (Personnel communication Van Eenennan 2015). If an actual *ITM2A* gene is present in cattle based on this improved assembly, it is worth trying to characterize the gene again and then to identify any polymorphisms that might affect the hip height of beef heifers and thereby their reproductive performance.

5.3 Conclusions

The *T* allele of the *PMCH* -134A>T SNP, when present in Simmental heifers, showed a dominant effect on their calf's ADG before they were sent to pasture. It was speculated that this was caused by the *PMCH T* allele having a favorable effect on heifer lactational milk production. In support of this, the association was not observed once the cow-calf pairs were sent to pasture, suggesting the effect was more prominent when calved relied primarily on lactation. The association was not observed in Angus heifers probably due to their low *T* allele frequency and their limited time spent primarily on the dam's milk.

The dam *LEP* genotype did not show a significant association with their calves' ADG in either Simmental or Angus heifers. A possible reason could be that heifers have low fat deposition by the time of their first calving. Because leptin is expressed in fat tissues, they would express lesser amounts.

We attempted to characterize the cattle *ITM2A* gene to determine if it might affect frame size in cattle. We appear to have captured a pseudogene with stop codons within the predicted exons but not the actual cattle *ITM2A* gene.

6.0 LITERATURE CITED

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7.0 APPENDICES

Appendix A: DNA extraction method (Montgomery & Sise 1990)

Blood samples were transferred into labeled 50 ml centrifuge tubes and white blood cells were collected from whole blood following lysis of the red blood cells in 10 ml of cold sterile lysing solution (150 mM NH_4Cl, 10 mM KCl, and 0.1 mM EDTA). The solutions were mixed well and held on ice 1-2 min, until the mixture becomes very dark in color. White cells were then harvested after centrifugation at 3500 rpm for 10 min at 4 °C. The lysed red blood cells were decanted carefully and the pelleted white cells were resuspended and washed in 4 ml of Tris buffered saline (140 mM NaCl, 0.5 mM KCl, 0.25 mM Tris HCl pH 7.4). The sampled were spun at 1500 rpm for 5 min.

Tris buffered solution was decanted and white blood cells were completely resuspended by vigorous vortexing in 7.2 ml of TE (0.1 mM EDTA, 10 mM Tris-HCl pH 8.0) so that no cell clumps remained. 30µl of proteinase K (20 mg/ml) and 450 µl 0.5 M EDTA, pH 8.0 were added and mixed. 500 µl of 10% SDS was added while gently swirling the tubes. The tubes were then incubated at 60 °C in a water bath with occasional mixing for 2-3 h.

Following proteinase K digestion, 3 ml of a saturated NaCl solution was added, the tubes were shaken vigorously for 30-60 s, and spun at 2500 rpm for 10 min. The supernatant

containing DNA was transferred to 50 ml glass tubes and 2 volumes of 95% ethanol were added. The tubes were sealed with parafilm and inverted gently 2-3 times. The DNA appeared as a white strand. The DNA was spooled out using a sealed pasteur pipette, washed in 70% ethanol and blot dried on a clean paper towel. The DNA was transferred to labeled 1.5 ml microcentrifuge tubes and resuspended in 200-400 µl of TE. The tubes containing DNA in TE were placed at room temperature for at least 24 h to ensure resuspension was complete. The concentration of DNA was measured in a NanoDrop™ Lite spectrophotometer.

Appendix B: Birth year effect of heifers on their calves' ADG

Year effect of calf ADG to pasture in Simmental heifers

Year		Calf ADG on lactation[1] (kg/d)	Letter group
2009	23	0.83 ± 0.036	b
2011	56	0.77 ± 0.029	b
2013	32	1.03 ± 0.030	a

There was a significant difference between the calves' ADG on lactation ($P<0.0001$) for heifers born in 2009 and 2011 versus those born in 2013. The rancher believed this difference is likely due to the quality of pasture.

The effect of dam *PMCH* genotype on their calf ADG was evaluated for 2013 heifers separately and combining 2009 and 2011 heifers.

Subsequently, the calf's ADG was evaluated for 2013 heifers versus 2009 and 2011 heifers combined, and only a trend was observed ($P=0.08$). Therefore all three cohorts were analyzed as one group.

For Simmental, the calf ADG through pasture data was only available for 2009 and 2011 heifers. There was no year effect observed between these two cohorts ($P=0.43$). Two cohorts of Angus heifers were used from year 2012 and 2013. The calf ADG on lactation was not affected by the birth year of their dams ($P=0.92$).

Appendix C: *LEP* and *PMCH* genotypic and trait data for Simmental heifer dams

Tag	*LEP*	*PMCH*	Pregnancy status October	Calving status	Calf ADG on lactation[1] (kg/d)	Calf ADG through[2] pasture (kg/d)
188W	-	*T/T*	-	-	0.94	-
642W	*C/C*	*A/T*	-	-	0.67	0.83
643W	*C/T*	*A/T*	-	-	0.85	0.91
648W	*C/T*	*A/T*	-	-	0.81	1.28
652W	*C/C*	*A/T*	-	-	1.07	0.96
656W	*C/T*	*A/A*	-	-	1.01	1.02
657W	*C/C*	*T/T*	-	-	0.72	1.01
665W	*C/T*	*A/A*	-	-	0.43	1.00
668W	*C/T*	*A/T*	-	-	0.89	0.9
673W	*C/C*	*A/A*	-	-	0.98	1.04
674W	*C/C*	*A/T*	-	-	1.00	0.91
675W	*C/T*	*A/T*	-	-	1.03	1.02
677W	*C/T*	*A/A*	-	-	0.55	0.88
679W	*C/C*	*A/T*	-	-	-	0.97
680W	*C/T*	*A/A*	-	-	-	1.41
681W	*C/T*	*A/T*	-	-	0.9	1.01
687W	*C/T*	*A/T*	-	-	0.91	1.03
695W	*C/T*	*A/A*	-	-	0.97	0.98
700W	*C/T*	*A/T*	-	-	-	0.91
701W	*C/T*	*A/T*	-	-	0.6	0.89
704W	*C/T*	*T/T*	-	-	1.10	1.07
705W	*T/T*	*A/A*	-	-	0.68	1.00
709W	*C/T*	*A/A*	-	-	0.44	1.04
807Y	*C/T*	*A/A*	Open	Sold	-	-
810Y	*C/C*	*A/T*	Pregnant	Calved	0.86	1.15
812Y	*C/T*	*T/T*	Pregnant	Calved	0.66	1.04
814Y	*C/T*	*A/T*	Pregnant	Calved	0.71	1.20
815Y	*C/C*	*A/T*	Pregnant	Calved	0.95	1.17
817Y	*C/C*	*A/T*	Pregnant	Calved	0.86	1.16
818Y	*C/T*	*A/T*	Open	Sold	-	-
819Y	*C/C*	*A/T*	Pregnant	Calved	0.72	1.05
820Y	*C/T*	*T/T*	Pregnant	Calved	-	-

Tag	*LEP*	*PMCH*	Pregnancy status October	Calving status	Calf ADG on lactation[1] (kg/d)	Calf ADG through[2] pasture (kg/d)
821Y	*C/C*	*A/T*	Open	Sold	-	-
827Y	*C/T*	*A/T*	Pregnant	Calved	0.84	1.13
828Y	*C/C*	*A/T*	Pregnant	Calved	-	-
830Y	*C/C*	*A/A*	Pregnant	Calved	0.77	1.12
831Y	*C/T*	*A/T*	Pregnant	Calved	0.73	1.13
832Y	*C/C*	*A/A*	Pregnant	Calved	0.67	1.09
834Y	*C/T*	*A/T*	Open	Sold	-	-
835Y	*C/C*	*A/A*	Pregnant	Calved	0.8	1.12
837Y	*C/C*	*A/A*	Pregnant	Calved	0.67	1.21
838Y	*C/C*	*A/T*	Pregnant	Calved	0.64	1.05
840Y	*C/C*	*A/T*	Pregnant	Calved	0.63	1.14
841Y	*C/C*	*A/T*	Open	Sold	-	-
842Y	*C/C*	*A/T*	Pregnant	Calved	0.67	1.03
843Y	*C/C*	*A/T*	Open	Sold	-	-
845Y	*C/T*	*A/A*	Pregnant	Calved	0.77	1.23
847Y	*C/T*	*T/T*	Open	Sold	0.66	1.15
848Y	*C/T*	*A/T*	Open	Sold	-	-
849Y	*C/T*	*A/A*	Pregnant	Calved	0.77	1.20
850Y	*T/T*	*A/T*	Open	Sold	-	-
851Y	*C/C*	*A/A*	Pregnant	Calved	0.73	1.06
853Y	*C/T*	*T/T*	Pregnant	Calved	0.83	1.16
854Y	*C/C*	*A/A*	Open	Sold	-	-
856Y	*C/T*	*A/T*	Pregnant	Calved	-	-
858Y	*C/C*	*A/A*	Open	Sold	-	-
859Y	*T/T*	*A/T*	Pregnant	Calved	0.90	1.21
860Y	*C/C*	*A/T*	Open	Sold	-	-
861Y	*C/C*	*A/T*	Open	Sold	-	-
862Y	*C/T*	*A/A*	Open	Sold	-	-
863Y	*C/C*	*A/T*	Pregnant	Calved	0.85	1.11
865Y	*C/T*	*T/T*	Pregnant	Calved	-	-
868Y	*C/T*	*A/T*	Open	Sold	-	-
871Y	*C/C*	*A/A*	Open	Sold	-	-
872Y	*C/T*	*A/T*	Pregnant	Calved	0.66	1.22
874Y	*C/T*	*A/T*	Pregnant	Calved	0.77	1.21

Tag	*LEP*	*PMCH*	Pregnancy status October	Calving status	Calf ADG on lactation[1] (kg/d)	Calf ADG through[2] pasture (kg/d)
876Y	*C/C*	*A/T*	Open	Sold	-	-
877Y	*C/T*	*A/T*	Open	Sold	-	-
879Y	*C/C*	*A/T*	Open	Sold	-	-
881Y	*C/C*	*A/T*	Pregnant	Calved	0.69	1.19
882Y	*T/T*	*A/T*	Pregnant	Calved	0.70	1.19
883Y	*C/T*	*A/T*	Pregnant	Calved	0.62	1.12
885Y	*C/T*	*A/A*	Pregnant	Calved	0.72	1.09
886Y	*C/T*	*A/T*	Open	Sold	-	-
887Y	*C/C*	*A/T*	Open	Sold	-	-
889Y	*C/T*	*A/A*	Pregnant	Calved	0.81	1.17
1A	*C/T*	*T/T*	Open	Sold	-	-
2A	*C/T*	*T/T*	Pregnant	Calved	1.07	-
6A	*T/T*	*A/T*	Pregnant	Calved	1.36	-
9A	*C/C*	*T/T*	Pregnant		1.03	-
10A	*C/T*	*A/T*	Pregnant	Calved	1.09	-
12A	*C/T*	*A/T*	Pregnant	Calved	1.12	-
15A	*C/T*	*A/T*	Pregnant	Calved	0.95	-
16A	*T/T*	*A/T*	Pregnant	Calved	1.16	-
17A	*T/T*	*A/T*	Pregnant	Calved	1.13	-
20A	*C/T*	*A/A*	Pregnant	Calved	1.12	-
21A	*T/T*	*T/T*	Pregnant	Calved	1.35	-
27A	*T/T*	*T/T*	Pregnant	Calved	1.03	-
28A	*C/C*	*A/T*	Pregnant	Calved	0.98	-
30A	*C/C*	*A/T*	Pregnant	Calved	1.27	-
32A	*C/C*	*A/T*	Pregnant	Calved	1.10	-
34A	*C/T*	*A/T*	Pregnant	Calved	1.17	-
36A	*T/T*	*A/A*	Pregnant	Calved	1.01	-
37A	*T/T*	*A/T*	Pregnant	Calved	1.10	-
41A	*C/C*	*A/T*	Pregnant	Calved	1.25	-
43A	*C/C*	*A/A*	Pregnant	Calved	0.65	-
44A	*C/C*	*A/T*	Pregnant	Calved	1.13	-
47A	*C/T*	*T/T*	Pregnant	Calved	1.13	-
49A	*C/T*	*A/T*	Pregnant	Calved	0.93	-
50A	*C/T*	*A/T*	Pregnant	Calved	1.07	-

Tag	*LEP*	*PMCH*	Pregnancy status October	Calving status	Calf ADG on lactation[1] (kg/d)	Calf ADG through[2] pasture (kg/d)
51A	*C/C*	*A/T*	Pregnant	Calved	1.58	-
53A	*T/T*	*T/T*	Pregnant	Calved	-	-
55A	*C/T*	*A/T*	Pregnant	Calved	1.03	-
56A	*C/C*	*A/T*	Pregnant	Calved	1.36	-
57A	*C/C*	*T/T*	Open	Sold	1.07	-
5A	*C/C*	*A/A*	Pregnant	Calved	1.19	-
60A	*C/C*	*T/T*	Pregnant	Aborted		-
61A	*C/T*	*A/T*	Open	Sold		-

[1, 2] Calf ADGs were sex corrected to heifers. The correction factors were 1.1 for bulls and 1.05 for steers.

Appendix D: *LEP* and *PMCH* genotypic and trait data for Angus heifer dams

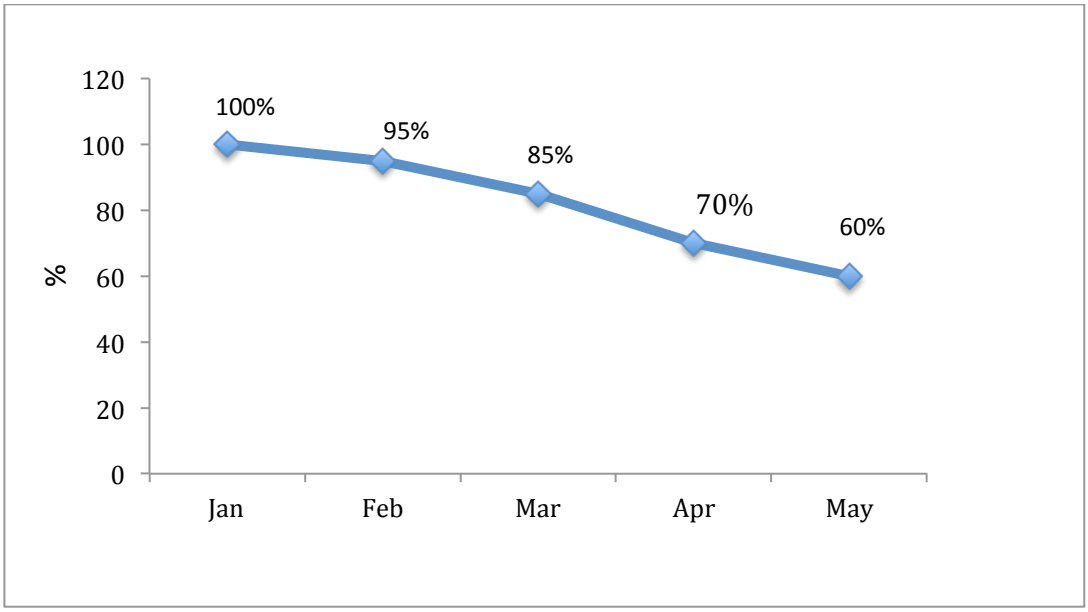
Tag	***LEP***	***PMCH***	**Pregnancy status October**	**Calving status**	**Calf ADG on lactation[1] (kg/d)**	**Calf ADG through pasture[2] (kg/d)**
564Z	*C/C*	*A/A*	Pregnant	Calved	0.60	0.71
565Z	*C/T*	*A/A*	Pregnant	Calved	0.96	0.99
570Z	*C/T*	*A/A*	Pregnant	Calved	0.77	-
574Z	*T/T*	*A/A*	Pregnant	Calved	0.86	0.84
577Z	*C/C*	*A/T*	Open	Sold	-	-
579Z	*T/T*	*A/A*	Open	Sold	-	-
580Z	*C/T*	*A/T*	Pregnant	Calved	1.09	1.07
583Z	*C/T*	*A/A*	Pregnant	Calved	0.96	0.99
584Z	*C/C*	*A/A*	Pregnant	Calved	1.10	1.07
588Z	*C/C*	*A/T*	Pregnant	Calved	0.91	0.98
590Z	*T/T*	*A/A*	Pregnant	Calved	1.14	1.17
596Z	*T/T*	*A/T*	Pregnant	Calved	0.80	0.90
598Z	*T/T*	*A/T*	Pregnant	Calved	0.94	0.99
599Z	*C/T*	*A/A*	Pregnant	Calved	1.16	1.20
600Z	*C/C*	*A/A*	Pregnant	Calved	0.79	0.89
602Z	*T/T*	*A/T*	Pregnant	Calved	1.03	0.99
603Z	*T/T*	*A/A*	Pregnant	Calved	1.10	1.01
604Z	*C/C*	*A/A*	Pregnant	Calved	1.06	1.01
606Z	*T/T*	*A/A*	Pregnant	Calved	1.20	1.19
609Z	*C/T*	*A/A*	Pregnant	Calved	1.17	1.21
614Z	*C/C*	*A/T*	Pregnant	Calved	1.05	1.03
618Z	*C/T*	*A/A*	Pregnant	Calved	1.13	1.14
619Z	*C/C*	*A/A*	Pregnant	Calved	0.99	1.01
620Z	*T/T*	*A/T*	Pregnant	Calved	1.12	1.01
621Z	*C/T*	*A/A*	Pregnant	Calved	1.08	1.03
623Z	*C/T*	*A/A*	Pregnant	Calved	1.11	1.09
625Z	*C/T*	*A/A*	Pregnant	Calved	1.10	1.07
628Z	*T/T*	*A/A*	Pregnant	Calved	-	-
629Z	*C/T*	*A/A*	Pregnant	Calved	0.94	1.01
630Z	*T/T*	*A/A*	Pregnant	Calved	1.01	1.08

Tag	*LEP*	*PMCH*	Pregnancy status October	Calving status	Calf ADG on lactation[1] (kg/d)	Calf ADG through pasture[2] (kg/d)
635Z	*T/T*	*A/A*	Pregnant	Calved	-	-
640Z	*C/T*	*A/A*	Pregnant	Calved	1.14	1.18
644Z	*C/T*	*A/A*	Pregnant	Calved	0.88	1.05
645Z	*C/T*	*A/A*	Pregnant	Calved	0.95	0.99
646Z	*C/T*	*A/A*	Pregnant	Calved	1.08	1.08
647Z	*C/C*	*A/A*	Open	Sold	-	-
649Z	*T/T*	*A/A*	Pregnant	Aborted	-	-
662Z	*T/T*	*A/A*	Pregnant	Calved	-	-
667Z	*C/T*	*A/A*	Pregnant	Calved	0.89	1.07
668Z	*C/T*	*A/A*	Pregnant	Calved	1.02	0.94
692Z	*C/T*	*A/A*	Pregnant	Sold	-	-
696Z	*C/T*	*A/A*	Pregnant	Calved	0.85	0.99
739A	*T/T*	*A/A*	Pregnant	Calved	0.87	-
740A	*T/T*	*A/A*	Pregnant	Calved	1.24	-
745A	*C/T*	*A/A*	Pregnant	Culled	-	-
746A	*C/T*	*A/A*	Pregnant	Calved	0.81	-
749A	*C/T*	*A/A*	Pregnant	Calved	1.01	-
753A	*T/T*	*A/T*	Pregnant	Calved	0.98	-
754A	*C/C*	*A/A*	Pregnant	Culled	-	-
760A	*C/T*	*A/A*	Pregnant	Calved	1.29	-
761A	*T/T*	*A/A*	Pregnant	Calved	1.11	-
762A	*C/T*	*A/A*	Pregnant	Calved	0.94	-
763A	*C/T*	*A/A*	Pregnant	Calved	1.26	-
766A	*T/T*	*A/A*	Pregnant	Calved	0.98	-
770A	*C/C*	*A/A*	Pregnant	Calved	1.15	-
771A	*C/T*	*A/A*	Pregnant	Did not calve	-	-
772A	*T/T*	*A/T*	Open	Sold	-	-
773A	*C/T*	*A/A*	Pregnant	Calved	1.14	-
774A	*C/T*	*A/T*	Pregnant	Calved	0.61	-
776A	*T/T*	*A/A*	Pregnant	Calved	1.06	-
778A	*C/C*	*A/T*	Pregnant	Calved	1.20	-
780A	*C/T*	*A/A*	Pregnant	Calved	0.89	-

Tag	*LEP*	*PMCH*	Pregnancy status October	Calving status	Calf ADG on lactation[1] (kg/d)	Calf ADG through pasture[2] (kg/d)
785A	*C/C*	*A/A*	Pregnant	Calved	0.92	-
792A	*C/T*	*A/A*	Pregnant	Calved	0.78	-
796A	*C/C*	*A/A*	Pregnant	Calved	0.74	-
797A	*C/T*	*A/A*	Pregnant	Calved	1.07	-
800A	*C/T*	*A/A*	Pregnant	Calved	0.73	-
801A	*C/T*	*A/A*	Pregnant	Calved	1.06	-
806A	*C/T*	*A/A*	Pregnant	Calved	1.01	-
807A	*C/T*	*A/A*	Pregnant	Calved	1.05	-
810A	*T/T*	*A/A*	Pregnant	Calved	1.58	-
819A	*T/T*	*A/A*	Open	Sold	-	-
821A	*C/C*	*A/A*	Pregnant	Calved	0.95	-
826A	*T/T*	*A/A*	Pregnant	Calved	1.11	-
827A	*T/T*	*A/A*	Pregnant	Calved	0.72	-
828A	*C/C*	*A/A*	Pregnant	Calved	0.94	-
829A	*C/T*	*A/A*	Pregnant	Calved	-	-
831A	*T/T*	*A/A*	Pregnant	Calved	0.9	-
832A	*T/T*	*A/A*	Pregnant	Calved	1.07	-
834A	*C/T*	*A/A*	Pregnant	Calved	0.92	-
839A	*T/T*	*A/A*	Pregnant	Calved	1.16	-
842A	*C/T*	*A/A*	Pregnant	Calved	0.96	-
844A	*C/T*	*A/A*	Pregnant	Calved	1.08	-
847A	*C/C*	*A/A*	Pregnant	Not calved	-	-
850A	*T/T*	*A/T*	Pregnant	Calved	0.80	-
851A	*C/T*	*A/A*	Pregnant	Calved	1.20	-
852A	*C/T*	*A/A*	Pregnant	Calved	0.94	-
856A	*T/T*	*A/A*	Pregnant	Calved	1.14	-
860A	*C/T*	*A/T*	Pregnant	Calved	0.92	-
861A	*T/T*	*A/A*	Open	Sold	-	-
876A	*C/T*	*A/A*	Pregnant	Calved	1.09	-
878A	*T/T*	*A/A*	Pregnant	Culled	0.60	-

[1, 2] Calf ADGs were sex corrected to heifers. The correction factors were 1.1 for bulls and 1.05 for steers.

Appendix E: Calf milk intake estimated by the rancher at the Braithwaite Simmental ranch



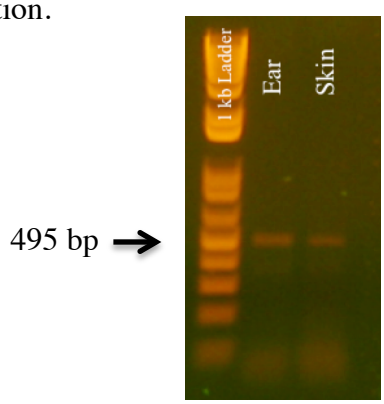
Calves depended primarily on their dam's milk from birth (in January/February) to late May, however in decreasing amounts. From late February, cows were fed hay and calves were creep feed. Cow-calf pairs were sent to pasture in May. By the time calves were weaned in October, dependency on milk was estimated to be 10%.

Appendix F: PCR protocol for *PMEL* gene exon 9 to 3' UTR

Exon 9 to 3'UTR of the PMEL gene was run on skin and ear samples obtained from Holstein steer (Lab no. 15-001) as a control to check the quality of cDNA. The protocol used was developed previously in our lab (Schmutz & Dreger 2013). To amplify the *ITM2A* gene, 2 µl of genomic DNA was added to each 14 µl reaction containing 1.5 ml 10X PCR buffer with $(NH_4)_2SO_4$, 10 mM dNTP, 1.5 mM $MgCl_2$, 0.5 U of *Taq* Polymerase (Fermentas Co., Carlsbad, CA, USA), 10 pmol of each primer (Cow PMEL Ex9-For and Cow PMEL 3'UTR-Rev) and 9.2 µl of deionized water (dH_2O).

The reaction was carried out in a BioRad T100™ thermo cycler. Initial denaturation was 4 min at 95 °C, followed by 34 cycles of: denaturation for 50 s at 95 °C, annealing for 50 s at 62 °C and 1 min extension at 72 °C with a final extension of 10 min at 72 °C. The PCR products were run on a 1% agarose gel.

The cDNA product size was 495 bp and the genomic product size was 1216 bp. Only the 495 bp cDNA product was amplified indicating our cDNA was of good quality and did not contain genomic DNA contamination.



Appendix G: The Manuscript submitted to Journal of Heredity- A Missense Mutation in *SLC45A2* is Associated with Albinism in Several Small Long Haired Dog Breeds

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Original Article

A Missense Mutation in *SLC45A2* Is Associated with Albinism in Several Small Long Haired Dog Breeds

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Abstract

Homozygosity for a large deletion in the solute carrier family 45, member 2 (*SLC45A2*) gene causes oculocutaneous albinism (OCA) in the Doberman Pinscher breed. An albino Lhasa Apso did not have this g.27 141_31 223del (CanFam2) deletion in her *SLC45A2* sequence. Therefore, *SLC45A2* was investigated in this female Lhasa Apso to search for other possible variants that caused her albinism.The albino Lhasa Apso was homozygous for a nonsynonymous substitution in the seventh exon, a c.1478G>A base change that resulted in a glycine to aspartic acid substitution (p.G493D). This mutation was not found in a wolf, a coyote, or any of the 15 other Lhasa Apso dogs or 32 other dogs of breeds related to the Lhasa Apso. However, an albino Pekingese, 2 albino Pomeranians, and an albino mixed breed dog that was small and long haired were also homozygous for the 493D allele. The colored puppies of the albino Lhasa Apso and the colored dam of the 2 albino Pomeranians were heterozygous for this allele. However, an albino Pug was homozygous for the 493G allele and therefore although we suggest the 493D allele causes albinism when homozygous in several small, long haired dog breeds, it does not explain all albinism in dogs. A variant effect prediction for the albino Lhasa Apso confirms that p.G493D is a deleterious substitution, and a topology prediction for SLC45A2 suggests that the 11th transmembrane domain where the 493rd amino acid was located, has an altered structure.

Subject areas: Gene action, regulation and transmission
Key words: albino, Lhasa Apso, oculocutaneous albinism, Pekingese, Pomeranian, Pug

Introduction

An albino dog does not produce any pigment and appears white with pink skin, nose leather, paw pads, lips and either pink or red irises. Albinism in dogs has been rarely observed (Little 1957), in breeds other than the Doberman Pinscher. In several species including mice (Yokoyama et al. 1990), humans (Oetting and King 1994; Fukai et al. 1995), rabbits (Aigner et al. 2000), cattle (Schmutz et al. 2004), and cats (Schmidt-Küntzel et al. 2005; Imes et al. 2006), mutations in the tyrosinase (*TYR*) gene are responsible for causing albinism. However, an albino Lhasa Apso dog did not have any mutations in its complementary DNA (cDNA) *TYR* sequence (Schmutz and Berryere 2007a).

Solute carrier family 45, member 2 (*SLC45A2*) is one of the recently identified genes involved in the pigmentation pathway, and it

has been found that mutations in *SLC45A2* are associated with reduction of melanin synthesis in varying degrees, resulting in phenotypes such as oculocutaneous albinism type 4 (OCA 4) in humans (Newton et al. (2001), underwhite in mice (Du and Fisher 2002), palomino in horses (Mariat et al. 2003), white/pale cream in white tigers (Xu et al. 2013) and Western Lowland Gorillas (Prado-Martinez et al. 2013), gold color in lower vertebrates such as Medaka fish (Fukamachi et al. 2001) and silver in chicken (Gunnarsson et al. 2007).

Very recently, Winkler et al. (2014) found that homozygosity for a 4081 base-pair deletion in the *SLC45A2* gene, including the last 163 bp of the last exon (exon 7; g.27,141_31,223del [CanFam2]) causes OCA in the Doberman Pinscher breed. Based on their finding, we hypothesized that other mutations in *SLC45A2* could be associated with other forms of albinism in dogs.

Materials and Methods

Dogs

cDNA prepared from skin biopsies was available from an albino Lhasa Apso, a coyote, and a gray wolf. Cheek brush genomic DNA samples (Epicentre, Madison, WI) were obtained from breeders and owners for 15 Lhasa Apso dogs of other coat colors, an albino Pekingese, an albino long haired small dog of mixed breed, 2 albino Pomeranian siblings and their colored dam and an albino Pug. In addition, genomic DNA from 4 colored puppies of the albino Lhasa Apso was available. Thirty-two dogs (that were colored and/or white, but not albino) from 10 other related breeds (Parker et al. 2004) including Pekingese (2), Japanese Chin (5), Chow Chow (2), Shar Pei (4), Akita (2), Saluki (3), Afghan Hound (4), Siberian Husky (6), Alaskan Malamute (3), and Samoyed (1) that were available from previous coat color studies were selected as controls.

DNA Extraction, Amplification, and Sequencing

Genomic DNA from cheek brushes was extracted using the Epicentre BuccalAmp DNA Extraction Kit protocol. Primers (see Supplementary Table 1 online) were designed using primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/) and Oligoanalyzer (https://www.idtdna.com/analyzer/Applications/OligoAnalyzer/).

The PCR was carried out as a 15-µL reaction, which contained 50–100 ng of DNA template, 1.5-µL of 10× PCR buffer with $(NH_4)_2SO_4$, 1.5 mM of $MgCl_2$, 10 mM of dNTP, 0.5 U of *Taq* DNA polymerase (Fermentas Co., Carlsbad, CA), and 10 pmols of each primer. An initial denaturation step at 95°C for 4 min was followed by 35 cycles of 50 s at 95°C, 50 s at the specific annealing temperature (see Supplementary Table 1 online), 1 min at 72°C, and a final extension step at 72°C for 10 min.

After extracting the PCR products from a 1% agarose gel using the Omega Biotech Gel Extraction Kit protocol, they were sent to the National Research Council of Canada Plant Biotechnology Institute in Saskatoon for Sanger dideoxy sequencing on an Applied Biosystems Sequencer. The sequences were analyzed using Sequencher 4.8 software (Gene Codes, Ann Arbor, MI).

We have deposited the primary data underlying these analyses and the Supplementary Material online with Dryad.

Results

A female Lhasa Apso who exhibited classical characteristics of albinism (Figure 1a) did not have the large deletion found in Doberman Pinscher dogs, nor did any of the other albino dogs in this study. Therefore, to search for other possible variants that could explain her albinism, the entire *SLC45A2* coding sequence of this albino Lhasa

Figure 1. Photographs of the albino dogs tested in this study: **a**) Lhasa Apso, **b**) Pekingese, **c**) and **d**) Pomeranian siblings, **e**) mixed breed dog, and **f**) Pug. All the dogs are homozygous for the p.G493D substitution except for the Pug, which is assumed to have a different mutation in *SLC45A2* or another locus.

Apso (GenBank KM222822, KM222823, KM222824) was obtained in 4 parts: exons 3–4 and 5–7 from cDNA, and exons 1 and 2 from genomic DNA, due to the limited amount of cDNA available. This sequence was compared with previous cDNA sequences obtained from 3 pigmented dogs that had identical sequence: a cream Poodle (GenBank DQ302162), a black and white Large Munsterlander, and a red and white Brittany Spaniel. We also sequenced a wolf (GenBank KJ944376) and a coyote (GenBank KM222820), as the canid ancestors of the dog to verify the "wild-type" alleles. The comparison revealed that the albino Lhasa Apso was homozygous for an allele of each of the 4 single nucleotide polymorphisms (SNPs) in exons 3, 4, 6, and 7. c.1023C>T in exon 4 and c.1362G>A in exon 6 were silent variants and therefore excluded from further analysis.

The c.749C>T variant in exon 3 resulted in an amino acid change from proline to leucine (p.P250L). Five other albino dogs and the rest of the dogs of breeds related to the Lhasa Apso were genotyped using a PCR–restriction fragment length polymorphism (RFLP), with a purposeful mismatch primer designed to create a cut site for the *AciI* restriction enzyme. The PCR–RFLP revealed that the exon 3 SNP appeared to be a neutral variant as both homozygous genotypes were seen in several dogs. Winkler et al. (2014) also reported that p.P250L was seen in white Doberman Pinschers and was considered to have no phenotypic effect.

The c.1478G>A variant in exon 7 resulted in a glycine to aspartic acid change (p.G493D). However, all 51 colored and nonalbino white dogs, the wolf, and the coyote were homozygous for the 493G allele in exon 7. The 4 colored puppies of the albino Lhasa Apso were heterozygous for this SNP (Figure 2a). We suggest this 493D allele causes albinism when homozygous.

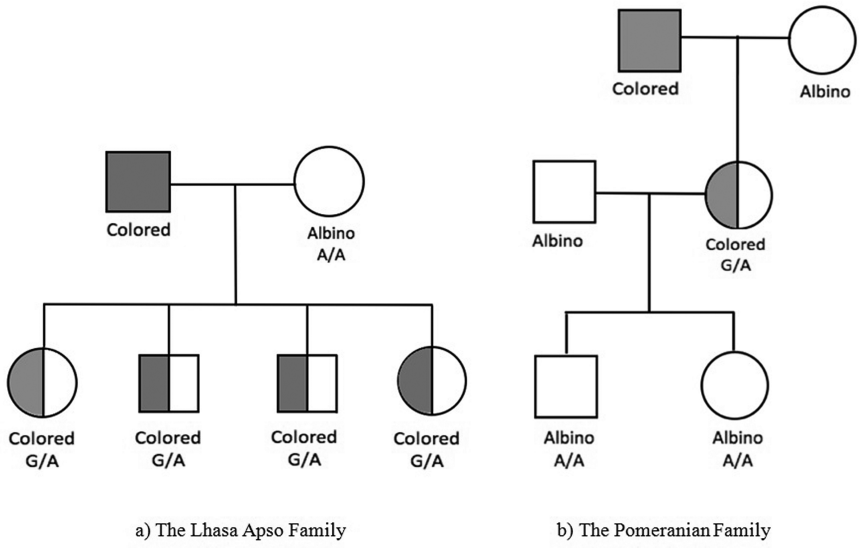

Figure 2. Diagrams of 2 albino dog families. (**a**) The Lhasa Apso family. (**b**) The Pomeranian family. The dams and offspring were genotyped.

In order to verify this, DNA from other albino dogs was genotyped: an albino Pekingese, 2 albino Pomeranians, an albino mixed breed dog that was small and long haired and an albino Pug (Figure 1). We were able to amplify the entire exon 7 from these dogs, suggesting that they do not carry the large deletion found in the albino Doberman Pinschers. All but the albino Pug were also homozygous for the 493D allele. The colored dam of the 2 albino Pomeranian siblings was heterozygous (Figure 2b).

All the albino dogs were also genotyped for *MC1R* for the *E* alleles (Newton et al. 2000; Schmutz and Berryere 2007b) because in the horse, the shades of "white" caused by *SLC45A2* mutations are slightly different depending on the *MC1R* genotype (Mariat et al. 2003). The mixed breed dog was *e/e*, the Lhasa Apso was E^M/E^M, and the others were *E/E* at this locus suggesting that the albino genotype at *SLC45A2* in these dogs was epistatic to the E^M, *E*, and *e* alleles of *MC1R*.

Discussion

SLC45A2 consists of 7 exons and was mapped to canine chromosome 4 (Schmutz and Berryere 2007a). *SLC45A2* codes for a transporter type protein called membrane-associated transporter protein (MATP) of 529 amino acids. The protein contains 12 hydrophobic transmembrane domains and shows sequence and structural similarity to plant sucrose transporters (Fukamachi et al. 2001) and H^+/sucrose symporters in *Drosophila* (Vitavska and Wieczorek 2013). MATP is known to play a role as a membrane transporter in melanosomes (Fukamachi et al. 2001) and helps to acidify the melanosomes, thus facilitating the melanogenesis by proper sorting of proteins and enzymes necessary for melanogenesis (Dooley et al. 2013). Mutations in the *SLC45A2* are believed to disrupt the sorting and trafficking of tyrosinase, tyrosinase-related protein 1, and to a certain extent Dopachrome tautomerase from the trans Golgi network to the melanosomes, preventing these enzymes from performing their tasks in the melanosomes (Hearing 2005).

The level of deleteriousness of the 2 amino acid substitutions (p.P250L and p.G493D) was analyzed using the Ensemble Variant Effect Predictor (http://www.ensembl.org/info/docs/tools/vep/index.html). The p.G493D substitution was given a SIFT (sorts intolerant from tolerant) score of 0.01 and a score of 0.33 for the p.P250L substitution. An amino acid substitution is predicted damaging when the SIFT score is ≤0.05 and tolerated if the score is >0.05 suggesting that the p.G493D is a deleterious substitution, whereas the p.P250L is tolerable.

Further a topology prediction for SLC45A2 of the albino Lhasa Apso was carried out using TMHMM (v. 2.0; http://www.cbs.dtu.dk/services/TMHMM-2.0/) and was compared with one obtained from a cream poodle (GenBank: DQ302162.1). Amino acid 493 is located in the 11th transmembrane domain and the topology predicted that the 11th transmembrane domain of the 493D alllele in albino Lhasa Apso has an altered structure (see Supplementary Figure 1 online). We suggest this alteration may lead to a malfunctioning MATP in this albino dog.

An amino acid alignment of *SLC45A2* was carried out with Clustal W (http://embnet.vital-it.ch/software/ClustalW.html) and revealed that glycine is conserved in several species including dog, human, cattle, pig, sheep, horse, cat, and mouse. Aspartic acid occurred only in the albino dogs.

All the albino dogs in this study with the 493D allele share certain characteristics such as a small body and long hair. It is possible that breeders of small, long haired dogs may have found the phenotype attractive and the allele might therefore have been spread to similar breeds.

The family studies (Figure 2) and homozygous genotypes of the long haired albino dogs confirm that this type of albinism is inherited as a recessive condition. Little (1957) postulated that albinism would be caused by a recessive allele at the C locus in dogs. Given that the albino Doberman Pinschers and their descendants were registered by the American Kennel Club on a "Z list," Winkler et al. have suggested the deletion in *SLC45A2*, which causes albinism in that breed be known as the c^{aZ} allele (Winkler PA, personal communication). We suggest that the p.G493D mutation found in this study be known as the c^{aL} allele as it was detected originally in a Lhasa Apso and subsequently in several long haired small dog breeds.

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