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Evidence for paternal imprinting of the beta-3 adrenergic receptor gene and a variant association with reduced twinning rate in New Zealand Merino sheep (*Ovis aries* L.)

A dissertation submitted in partial fulfilment of the requirements for the degree

of

Bachelor of Agricultural Science with Honours

at

Lincoln University

By

Erin Waller

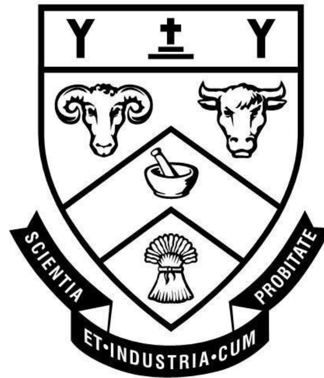
Lincoln University

2017

EVIDENCE FOR PATERNAL IMPRINTING OF THE BETA-3 ADRENERGIC RECEPTOR GENE AND A VARIANT ASSOCIATION WITH REDUCED TWINNING RATE IN NEW ZEALAND MERINO SHEEP (*Ovis aries* L.)

ERIN WALLER

A dissertation submitted in partial fulfilment of the requirements for the Degree of Bachelor of Agricultural Science with Honours.



LINCOLN UNIVERSITY
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E. Waller

ABSTRACT

Reproduction efficiency is key for the productivity and performance of New Zealand sheep farms. Thus improving reproduction efficiency and lamb survival is crucial for improving farm efficiency and profitability. Merino sheep are known to have lower scanning-rates than other sheep breeds within New Zealand.

The development of the Lincoln University Cold Tolerance Gene Marker (LUGMCTT) based on variants of the ovine beta-3 adrenergic receptor (β3-AR) gene has allowed sheep farmers to select for better cold tolerance and therefore improving lamb survival. Of the variants tested by the LUGMCTT, the D allele is only found in Merino sheep and is thought to have altered ligand binding. This is thought to impair receptor function due to amino acid changes in a highly conserved region of the gene. Recent research indicates the receptor may have a role in physiological functions during pregnancy in other tissues which indicates that the D allele may have adverse effects on scanning-rate. Thus the aim of this study is to identify whether the D allele of the ovine β3-AR gene is being selected against in New Zealand Merino populations and to identify whether the allele's presence has any effect on Merino scanning-rate.

Chi squared goodness of fit tests were performed to determine if the β3-AR genotypes of the sheep tested by the commercial LUGMCTT fit the Hardy-Weinberg equilibrium. Merino sheep were genotyped for the variants of the ovine β3-AR gene intron using the PCR-SSCP method of the Lincoln University Gene Marker Test Laboratory. 440 Merino ewes were mated to 9 rams, 5 of which were carrying the D allele of the β3-AR gene. Associations between dry rate and twinning rate at scanning and the presence of 0, 1 or 2 D alleles of the ewe and 0 or 1 D allele of the ram were accessed using Pearson Chi squared tests. A conditional forward stepwise logistic regression was used to determine if there was an interaction between sire and presence of D allele.

There were significant differences between observed and expected genotypes of the β3-AR gene alleles in all populations except one population. There was no association of the presence of ewe D allele on twinning rate. There was no association of presence of D allele from either the ewe or the ram on dry rate. Both sire (P = 0.054) and the presence of D allele (P = 0.000) had an effect on twinning rate. Sires with the D allele produced a reduced twinning rate (P = 0.003) of a large range of 7.10%-29.30%.

This study provides evidence that the D allele of the β3-AR gene reduces Merino scanning-rate, however the effect is complex with evidence of paternal imprinting and the presence of a sire effect. These results indicate that farmers could improve Merino scanning-rate by selecting rams that do not carry the D allele. Additionally selecting the rams without the D allele would be doubly beneficial for an increased scanning-rate due to paternal imprinting.

Key words: β3-adrenergic receptor, variant, allele, paternal imprinting, sheep, Merino, *Ovis aries,* scanning-rate, embryo survival, complex trait.

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TABLE OF CONTENTS

ABSTRACT i

ACKNOWLEDGEMENTS iii

TABLE OF CONTENTS v

LIST OF FIGURES vii

LIST OF TABLES viii

LITERATURE REVIEW 1

1.1 Introduction 1

1.2 Prenatal Survival 3

1.2.1 Embryogenesis 3
1.2.2 The sheep placenta 4
1.2.3 Prenatal Loss 7

1.3 Thermogenesis 10

1.3.1 Physiological Factors of Cold Survival: Thermogenesis 10
1.3.2 Adaptive Thermogenesis 13
1.3.3 Shivering Thermogenesis 13
1.3.4 Brown and White Adipose Tissue 13
1.3.5 Non Shivering Thermogenesis 14

1.4 The β3 Adrenergic Receptor 17

1.4.1 β3-AR effects on reproduction 18

1.5 The beta-3-adrenergic receptor gene 22

1.5.1 The β3-AR Gene Isolation 22
1.5.2 The β3-AR Gene Variants 24
1.5.3 Allele Associations 28
1.5.4 The D allele Frequencies 29
1.5.5 Allelic associations of the β3-AR gene in other traits and species 31

1.6 Perspectives and aims of this study 32

2 MATERIALS AND METHODS 34

2.1 Data collection 34

2.1.1 Population Data 34
2.1.2 Experimental Site 34
2.1.3 Animals 34

2.1.4 Scanning 34
2.1.5 DNA sampling 35
2.1.6 DNA Preparation and Genotyping 35

2.2 Statistical Analysis 35

3 RESULTS 36

3.1 Population Analyses 36

3.2 The D allele and scanning rate 37

3.2.1 Dry rate 37
3.2.2 Twinning rate 38

4 DISCUSSION 40

4.1.1 CONCLUSION 47

REFERENCES 49

APPENDICES 63

Appendix I – Proportion of Merino breeders that perform the Lincoln University Cold Tolerance Gene marker test 63

Appendix II – Genotype frequencies of the tested populations 63

Appendix III- Chi square goodness of fit outputs for each population 65

Appendix IV – Pearson chi squared results 69

Appendix V – DNA preparation method 78

LIST OF FIGURES

Figure 1: Diagram of ewe placentomes 4

Figure 2: Placentomal types (types A, B, C, and D) recovered from a single, d 78, nutrient-restricted Baggs, WY (Baggs) ewe, carrying a singleton fetus. Arrows depict cotyledonary (COT) and caruncular (CAR) tissues (Vonnahme et al 2006). 6

Figure 3: Schematic representation of the effect on sheep embryo development of transfer to a recipient ewe not in oestrus at the same time as the donor (asynchronous transfer) (Wilmut et al, 1985a.) 8

Figure 4: Diagrammatic representation of the relationship between metabolic rate (MR) and deep-body temperature (T_B) of a homeothermic animal in relation to environmental temperature. t_1 and t_4 define the thermoregulatory range of the animal and represent the lower and upper lethal temperatures, respectively. t_2 and t_3 are the lower and upper critical temperatures, respectively and they represent the limits of the animals thermoneutral zone. (Adapted from Pough et al., 1990 as cited by Forrest 2002).. 11

Figure 5: The components of metabolic rate at rest. The total amount of heat required to maintain body temperature depends on the environmental conditions. When basal heat production is insufficient, adaptive thermogenesis is activated. From Forrest, (2002). 12

Figure 6 - Control of non-shivering thermogenesis by the catecholamine noradrenaline, thyroid hormones and adenosine. From Forrest, (2002). 15

Figure 7: Structure of the human β3-adrenergic receptor. The sequence is represented in the one-letter code for amino acids. The single polypeptide chain is arranged according to the rhodopsin model. The disulphide bond essential for Cys^{110} and Cys^{189} activity is represented by ----. The two N-glycosylation sites in the amino-terminal portion of the protein are indicated by . The palmitoylated Cys^{360} residue in the N-terminus of the I4 loop is indicated by a . Residues in black circles are common to the three β3-adrenergic receptor subtypes. (From Strosberg & Gerhardt 2000 as cited by Forrest 2002)................................... 18

Figure 8: Influence of preeclampsia on the relaxing effects of SR 59119A on human placental arteries contracted with U46619 (10 nM). Results are expressed as the mean ± SEM (n = 7–20 arteries for normotensive and 6–8 for preeclamptic pregnancies, with each artery obtained from a single woman) (Rouget et al 2006).. 21

Figure 9: Structure of ovine β3-adrenergic receptor gene (ADRB3) (Yang 2014)......... 23

LIST OF TABLES

Table 1: Mean ± standard error of fetal weight, fetal:placentome weight ratio and placental parameters of placentomes of singleton and twin fetuses in a twin pair at Day 140 of pregnancy adapted from (van der Linden et al 2012). ... 6

Table 2: Amino acid changes from the putative sequence within the coding region of the variants of the ovine β3-AR gene within a PCR-SSCP of the 467bp region of the intron as identified by Forrest 2002. ... 26

Table 4: Phenotypic associations with the alleles A-F of the ovine β3-AR gene identified by a PCR-SSCP of the 467bp intron. ... 28

Table 5: Frequency of the D allele of the ovine β3-AR gene in different breeds ... 30

Table 6: The observed and expected numbers of sheep either heterozygous (DX) or homozygous (DD) for the D allele of the ovine beta-3-adrenergic receptor in different populations... 37

Table 7: The number and percentage of twins scanned from either ewes homozygous D (DD) ewes, heterozygous (Dx), or have no D (no D) or ewes that were mated to rams heterozygous D (Dx) or have no D (no D) of the beta-3-adrenergic receptor gene. ... 38

Table 8: The number and percentage of twins scanned in ewes that went to a sires that carry D allele (sire 1,4,5,8 and 6) and do not carry a D allele (sire 2,3,6,7) of the beta-3-adrenergic receptor. ... 39

LITERATURE REVIEW

1.1 Introduction

The reproduction efficiency of New Zealand sheep farms is crucial for the profitability of the farm. The production of both meat and wool produce is predominantly determined by the number of lambs that survive until weaning rather than on the individual production of each lamb (Sidwell et al 1962, Lax & Newton- Tuner 1965). Reduction of reproduction efficiency can occur through lower ovulation rates, fertilisation failure, embryonic mortality and perinatal mortality and have been shown to result and average of 25% less lambs (Kelly 1982). Thus improving lamb survival and reproduction efficiency is crucial for improving farm efficiency and profitability.

The beta-3-adrenergic receptor (β3-AR) is a catecholamine receptor that belongs to the R_7G superfamily of guanine-nucleotide binding protein (G-protein)-coupled receptors and are located in the membrane of adipose cells (Forrest 2002). The discovery of a low cold tolerance line of Scottish Blackface sheep that showed no thermogenic response to an injection of noradrenaline indicated that a single gene catecholamine pathway affects thermogenesis in sheep (Slee & Simpson 1991). In 2002 Forrest located and characterised 6 alleles (A-F) of the ovine β3-AR gene. Forrest (2002) and subsequent studies (Forrest et al 2006, Broughton 2006, Forrest et al 2007) provided evidence for associations between the β3-AR variants and important traits such as cold tolerance, birth weight and growth rate. Strong associations of the variants with varying levels of cold tolerance were noted throughout all of the studies and this has led to the development of the Lincoln University Gene Marker cold tolerance test. This allows producers to select for sheep that are at lower risk of dying due to cold exposure and therefore improving lamb survival.

Since the development of the Lincoln University Cold Tolerance Gene Marker increasing numbers of studies have provided evidence for the presence of β3-ARs in many other tissues other than adipose including the near-term human myometrium (reviewed by Ursino et al 2009) and the placental vessels of the human and sheep (Rouget et al 2006, Hynes et al 2008). Additionally there is evidence that stimulation of the β3-ARs in myometrium results in relaxation of myometrium contractions which could potentially prevent preterm labour (Bardou et al 2000, Dennedy et al 2001) and improve implantation success (Markiewicz & Jaroszewski 2016). Evidence that β3-AR stimulation within the placental vessels also results in vascular relaxation and the response of β3-AR in preeclamptic pregnancies to stimulus is attenuated (Rouget et al 2006).

Of the variants tested by the Lincoln University Gene Marker test the D variant has a very strong correlation with cold related mortality (Forrest et al 2006, Forrest et al 2007). This is thought to be due to the amino acid changes of the D variant protein being in the highly conserved section of the gene which is considered to be responsible for ligand binding (Forrest & Hickford 2000). The altered ligand binding is therefore likely to impair receptor function (Forrest & Hickford 2000).

To date the D allele has only been found in lines of Merino sheep. Merinos are known to have lower fertility and scanning-rates than other breeds with an average scanning-rate of 90-130% compared to 130-170% of New Zealand cross breeds (Geenty 1997). Considering that the β3-AR has been shown to affect placental blood flow and myometrium contractions during pregnancy in multiple species, it is likely that impaired function of the β3-AR could affect pregnancy. Thus the D variant has adverse effects on lamb survival by increasing cold related mortality and could potentially have adverse effects on lamb survival during pregnancy.

1.2 Prenatal Survival

1.2.1 Embryogenesis

Embryogenesis is the formation and development of an embryo. Embryogenesis begins with the zygote undergoing cleavage into two cells, which is the mitotic division without the accompanying cell growth. The early stage embryo begins to travel to the uterus during which the embryo continues to cleave into multiple cells identified as blastomeres which eventually from a cluster. Blastomeres are totipotent meaning that they are able to differentiate into any type of cell including embryo or placental cells (Mummery et al 2014). Before implantation different cell types begin to develop from the blastomeres. The outer blastomeres become the trophectoderm which consists of cells that will develop into the placenta the inner cell mass consists of cells that will become embryonic stem cells and therefore form the foetal tissue. These differentiated cells are classified as multipotent many they are able to differentiate into many but not all tissue cells of the body (Mummery et al 2014). At the stage where the blastomeres have differentiated into two types the embryo is classed as a blastocyst. At the blastocyst stage the embryo reaches the uterus. For sheep this is usually about 6 days after fertilization (Spencer et al 2004). At the uterus the embryo breaks out of the zona pellucida membrane surrounding the embryo and elongates. The trophectoderm then attaches to the uterine wall endometrium for implantation.

At implantation and under the influence of continuous exposure to progesterone, the endometrium downregulates progesterone receptors in the epithelia. This results in the endometrium reducing cell surface mucin and the production of proteins for adhesion (Spencer et al 2004). The epithelia of the endometrium is considered to be important for secretions that regulate the elongation of the embryo (Spencer et al 2004).

After implantation the inner cell mass cells begin to differentiate into three cell types which are the ectoderm, epithelium and mesoderm. Ectoderm cells ultimately form

skin, brain and nerve cells, parts of the eye, mouth and anus epithelium, pituitary gland and part of the adrenal glands (Mummery et al 2014). Mesoderm cells ultimately form skeletal muscle, heart, blood vessels, parts of the kidneys, urethra and gonads, bone marrow blood, bone, cartilage and fat (Mummery et al 2014). Endoderm cells form the lining of gastrointestinal tract, respiratory tracts, liver, thyroid, thymus and bladder (Mummery et al 2014).

1.2.2 The sheep placenta

The placenta is responsible for transporting nutrients, respiratory gases and wastes between the dam and the foetus. Sheep have a cotyledonary placenta which is the presence of multiple smaller placentae (placentomes) instead of one large area for contact between mother and foetus. Each placentome consists of the foetal cotyledon and the maternal caruncle (figure 1). Caruncles which sit in the uterine mucosa evolve through proliferation of subepithelial connective tissue. Cotyledons evolve from sections of chorioallantoic membrane developing villi that extend into crypts in the carnucular epithelium where nutrient exchange occurs (Bowen 2000).

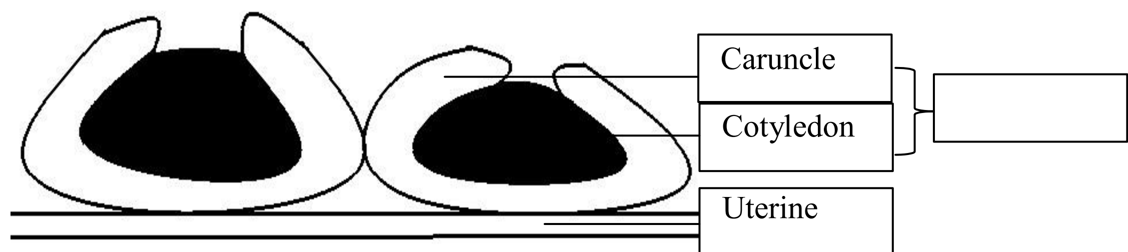

Figure 1: Diagram of ewe placentomes

Placentome efficiencies are affected by environmental conditions and genetics. Placentome structures become more efficient when feed is restricted during mid to late pregnancy and with increasing pregnancy ranks. Vonnahme et al (2006) studied the effect of restricted nutrition during early to mid-pregnancy on the foetus and placenta of ewes adapted to a nomadic environment and ewes adapted to a

sedentary lifestyle. Placentomes were sorted into four types A,B,C and D as shown in figure 2. Nutrient restricted ewes adapted to a sedentary lifestyle had lower foetal weights and blood glucose concentrations whilst there was no difference in foetal measurements between nomadic adapted nutrient restricted and well-fed ewes (Vonnahme et al 2006). Sedentary adapted ewes mainly possessed type A placentomes whilst nomadic adapted ewes possessed less type A placentomes and more type B, C and D placentomes. This indicates that B, C and D type placentomes are more efficient at providing nutrients for the foetus than A type placentomes and that in some ewes the placentomes are able to become more efficient when there is less feed available.

This efficiency adaptation is also reflected by van der Linden et al (2012) where there was a lower percentage of type A placentomes and a higher percentage of type B placentomes in twin bearing ewes. Additionally there was no difference in total cotyledonary weight or foetal:placentome weight ratio between twin bearing and single bearing ewes despite the lower number of placentomes in twin bearing ewes (van der Linden et al 2012) (table 1). Indicating that the placentomes adapt to be more efficient when the ewe is carrying more lambs.

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Figure 2: Placentomal types (types A, B, C, and D) recovered from a single, d 78, nutrient-restricted Baggs, WY (Baggs) ewe, carrying a singleton fetus. Arrows depict cotyledonary (COT) and caruncular (CAR) tissues (Vonnahme et al 2006).

Table 1: Mean ± standard error of fetal weight, fetal:placentome weight ratio and placental parameters of placentomes of singleton and twin fetuses in a twin pair at Day 140 of pregnancy adapted from (van der Linden et al 2012).

Measurement	**Between pregnancy rank**		
	Singleton	Twin	P value
Foetal weight (g)	4779 ± 140	4022 ± 17	0.006
Foetal:Placentome ratio	13.8 ± 1.1	16.2 ± 1.3	0.19
Total number of placentomes	88.4 ± 5.6	57.2 ± 5.4	0.001
Total placentone weight (g)	360.7 ± 25.5	258.7 ± 32.7	0.03
Total caruncular weight (g)	77.8 ± 3.9	52.0 ± 4.7	0.002
Total cotyledonary weight (g)	282.8 ± 24.3	206.7 ± 37.7	0.08

Increased placental vascularity has been associated with increased litter sizes. Placental blood flow is a crucial mechanism of increased transplacental exchange throughout gestation (reviewed by Reynolds et al 2006). The blood flow of the uterus increases during the length of the pregnancy to match the increasing demands of the foetus. In sheep the absolute rate of uterine blood flow has been shown to increase by 3 fold during the second half of the pregnancy (Meschia 1983). Decreased blood flow in the uterus is associated with foetal growth retardation (North et al 1994). Increased placental vascularity was seen in sheep (reviewed by Reynolds et al 2006) and pigs (Vonnahme & Ford 2004) that were selected for increased litter size long term. This indicates that increased blood flow is required to support larger litter sizes.

1.2.3 Prenatal Loss

Mated ewes have been shown to lose from 20-40% of lambs after ovulation and before birth (Edey 1979). These losses can include the loss of an individual in multiple bearing pregnancies without terminating the pregnancy (Schrick & Inskeep 1993). Embryonic and foetal losses can occur due to either the embryo or maternal environment. Embryonic causes of prenatal loss can be due to inherited defects, problems at meiosis or fertilisation or because of the environment affecting the embryo (Wilmut et al 1986).

1.2.3.1 Embryo causes

Mutations affecting the development of the embryo can cause prenatal loss. There have been multiple mutations associated with prenatal loss in mice including the yellow allele of the agouti locus (Pederson 1974) and the recessive mutant gene ‘siren’ (Schreiner & Hoornbeck 1973) both causing embryo loss. Translocations have been shown to increase prenatal loss in pigs (Akesson & Henricson 1972). There has been no reduction in ram fertility detected in several translocations in rams (Bruère 1975). Chromosomal abnormalities have been shown to be a major cause of

pregnancy loss in humans but contributes to lower proportions of aborted and still born animals in domestic animals including sheep (reviewed by Wilmut et al 1986).

1.2.3.2 Maternal environment

The maternal environment which can be affected by environmental factors must be adequate to support normal development of the embryo. This includes the timing and level of ovarian secretions present (Wimut et al 1986). Transfer of ovine embryos to ewes that expressed oestrus at a different time to the donor altered the rate of embryo development (Wilmut et al 1985).

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Figure 3: Schematic representation of the effect on sheep embryo development of transfer to a recipient ewe not in oestrus at the same time as the donor (asynchronous transfer) (Wilmut et al, 1985a.)

Embryos developed more quickly in ewes that expressed oestrus before the donor and more slowly in ewes that expressed oestrus after the donor (figure 3). When embryos were transferred to ewes who expressed oestrus at the time most deviated from the oestrus of the donor ewes, the embryos did not implant (figure 3).

Correct progesterone profiles at both oestrus and after mating is essential for the development and survival of embryos. At oestrus 4 phases are considered to be crucial for pregnancy establishment which includes a period of progesterone before mating, an oestradiol spike to match the progesterone levels that starts oestrus, a drop in progesterone levels during peri-ovulation and an increased level of progesterone after ovulation (Miller & Moore 1976, Moore 1985, Wilmut et al 1985a, Wilmut et al 1985b). The survival of embyros in ewes has been strongly associated with the lower progesterone concentrations present in ewe blood during the peri-ovulatory period (Ashworth 1985). Additionally when progesterone levels are too high can initiate the uterine functions too early (Wilmut et al 1985a). Progesterone levels later in pregnancy are also considered to be important for foetal development and survival. Cows that had progesterone intra-vaginally inserted were 2.4 times less likely to miscarry compared to the control (López-Gatius et al 2004). Progesterone presence during this pregnancy period is believed to reduce myometrium contractions (affecting preterm labour) (Csapo & Pulkkinen 1978) and improve the development of the placental caruncles (López-Gatius et al 2004). Additionally it is known that progesterone is important for maternal secretion of nutrients, growth factors and immunosuppressive agents (Ford 1980). Thus progesterone levels during pregnancy are a key influence on embryo and foetal survival.

Progesterone levels during oestrus and pregnancy are thought to be influenced by environmental factors such as nutrition and stress. It is thought nutrition after mating has an effect on progesterone concentration and that an intermediate level of nutrition is optimum (Wilmut et al 1986). Evidence suggests there is an inverse relationship between progesterone levels and food intake and a reduction in embryo survival is seen in both situations of underfeeding and over-feeding after mating (reviewed by Wilmut et al 1986). However multiple studies do not report a relationship between nutrition and embryo survival (reviewed by Wilmut et al 1986).

This could be affected by the ability of some ewes to change placental efficiency depending on environmental conditions as described previously.

There is strong evidence that feeding level, feeding type and body condition immediately prior to ovulation effect ovulation rate. Ovulation rate of the ewe increases by 2% with every increase 1kg increase in live weight (Smith 1988) with a maximum ovulation rate of 1.92 at 67.5kg (Rutherford et al 2003). Additionally feeding of ewes before mating will increase conception rates at both low and high live weights (Coop 1966). Furthermore ovine ovulation rate can be increased with increased rumen undegradable protein consumed. Flushing ewes with formaldehyde-treated casein (rumen undegradable protein) or lupin increased ovulation rates by 38 and 40% respectively (Teleni et al 1989). Lupin contains condensed tannins which at low concentrations bind to and reduce the degradation of forage proteins in the rumen effectively increasing the protein available to the animal (Min et al 2003).

Therefore both maternal and foetal factors affect the prenatal survival and reproduction efficiency. Additionally these factors and in particular the maternal factors interact heavily with the environment. Thus prenatal reproduction efficiency and survival is a complicated trait.

1.3 Thermogenesis

1.3.1 Physiological Factors of Cold Survival: Thermogenesis

Mammals maintain a constant core temperature through the means of balancing heat gain and loss again by the process of homeostasis if which the hypothalamus is of primary control. A key function in maintaining the homeothermy of the animal is metabolic rate.

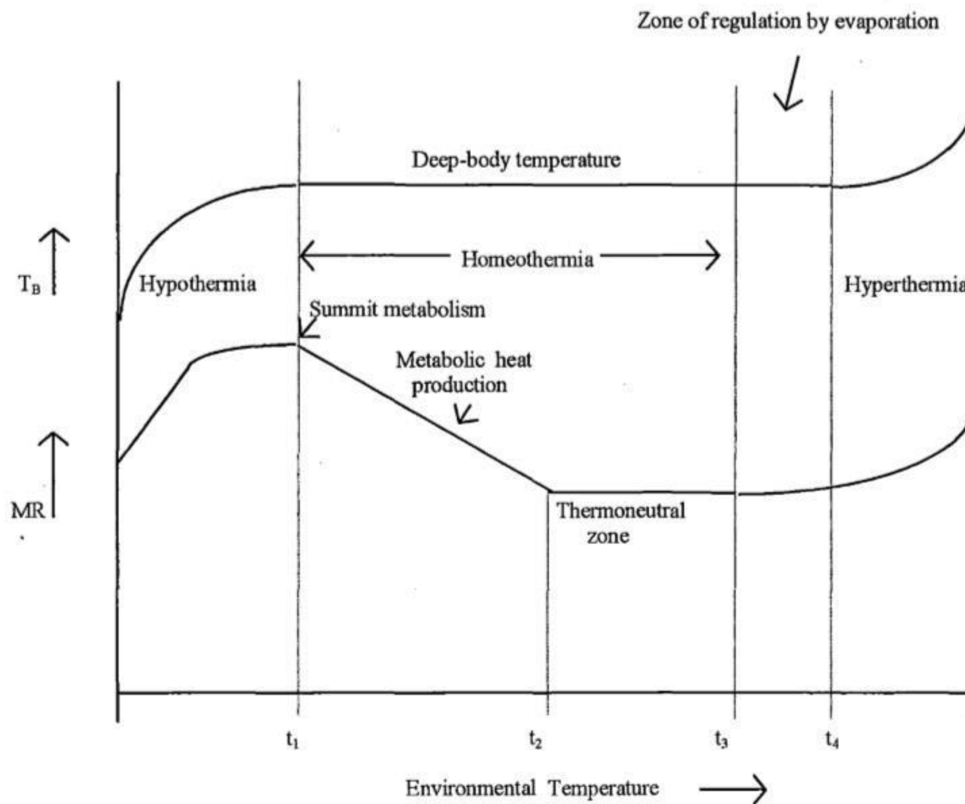

Figure 4: Diagrammatic representation of the relationship between metabolic rate (MR) and deep-body temperature (T_B) of a homeothermic animal in relation to environmental temperature. t_1 and t_4 define the thermoregulatory range of the animal and represent the lower and upper lethal temperatures, respectively. t_2 and t_3 are the lower and upper critical temperatures, respectively and they represent the limits of the animals thermoneutral zone. (Adapted from Pough et al., 1990 as cited by Forrest 2002).

The thermoregulatory range and it's relation to metabolic rate are shown in figure 4. The 'thermoneutral zone' is the range of ambient temperatures where the metabolic rate is at basal level and therefore body temperature regulation is maintained through altering the level of heat loss (Forrest 2002). The 'lower critical temperature' is the ambient temperature by which the animal must increase metabolic production to maintain homeothermy. As the ambient temperature decreases below the lower critical temperature the metabolic rate increases until 'summit metabolism'. The

summit metabolism is where the metabolic heat production has reached its maximum rate and this is also defined as the animal's lower lethal temperature. Temperatures below this are where the animal is unable to sustain homeothermy and thus are hypothermic. This results in a lowering core temperature which creates a positive feedback loop in which metabolic rate is reduced, producing less heat and allowing the core temperature to drop further resulting in death (Forrest 2002).

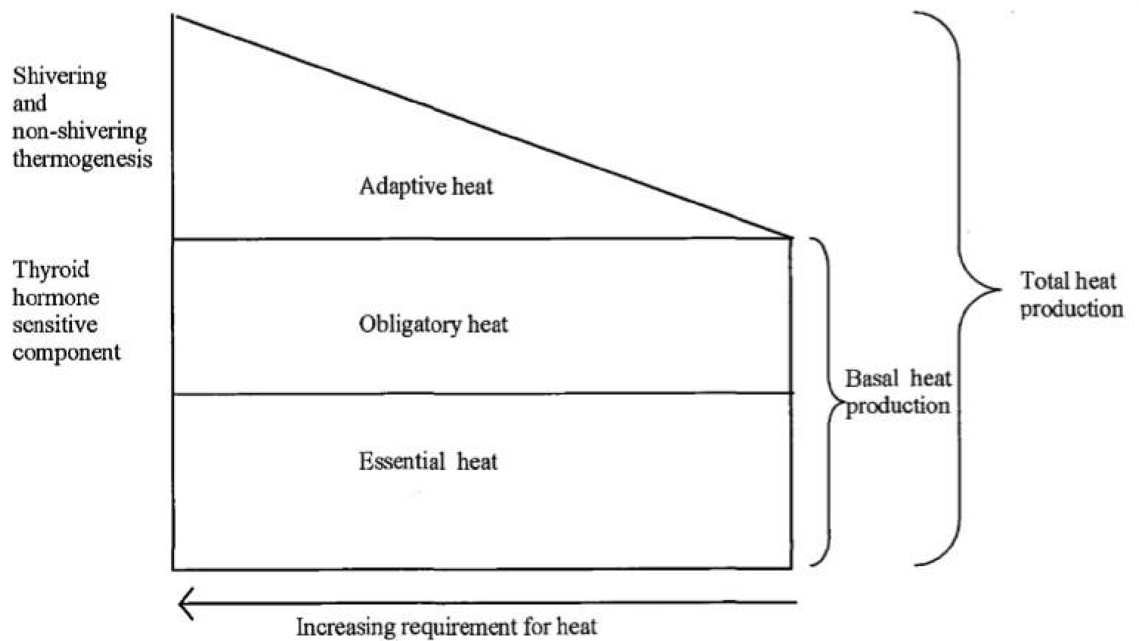

Figure 5: The components of metabolic rate at rest. The total amount of heat required to maintain body temperature depends on the environmental conditions. When basal heat production is insufficient, adaptive thermogenesis is activated. From Forrest, (2002).

The total metabolic heat production is made up of several components as shown in figure 5. Basal heat production consists of obligatory heat and essential heat. Essential heat is the heat produced from normal resting metabolic processes. The heat produced from essential heat is usually sufficient to maintain the animals required body temperature so the remaining required heat is produced through

obligatory heat (Forrest 2002). Obligatory heat is dependent on and regulated by thyroid hormones and increases when the animal is adapting to cold (reviewed by Shargo & Strielman 1987). The thyroid hormones include thyroxine (T_4) and tri-iodothyronine (T_3). These thyroids increase the rate of metabolism by essentially catalysing the metabolism of glucose by stimulation of Na^+/K^+-ATPase and enzymes used in glucose metabolism (Broughton 2006).

1.3.2 **Adaptive Thermogenesis**

When the environment conditions increase the requirement for heat adaptive heat production begins and increases with increasing environmental stimulus. The two forms of adaptive heat production are shivering thermogenesis and non-shivering thermogenesis (NST).

1.3.3 **Shivering Thermogenesis**

Shivering thermogenesis is a form of adaptive thermogenesis and is the involuntary unsynchronised contraction and relaxation of skeletal muscle motor units in high frequency rhythms (Broughton 2006). The sole purpose is to produce heat by converting ATP into heat energy. The body begins shivering thermogenesis when cold sensations stimulate thermoreceptors in the skin, spinal cord and hypothalamus to a threshold level (Jesson 1990). Shivering thermogenesis allows the production of heat without moving the body and therefore minimising convective heat loss (Broughton 2006).

1.3.4 **Brown and White Adipose Tissue**

White adipose tissue (WAT) mainly functions as a long term store of energy or triglycerides (Broughton 2006). WAT cells generally store lipid in large droplets and contain few mitochondria of which have loosely packed cristae (Broughton 2006).

In contrast brown adipose tissue (BAT) cells generally store lipid in varying droplet sizes and contain many mitochondria with densely packed cristae (Himms-Hagen 2000). This reflects the role of BAT as the site of non-shivering thermogenesis which requires energy and is closely linked with respiration. BAT also contains a dense capillary network which supplies oxygen and is able to move heat throughout the body along with extensive connections with nervous networks compared with WAT (Broughton 2006). These extensive capillary networks and high numbers of mitochondria in combination with lower lipid contents compared to WAT give BAT it's brown colour (Broughton 2006).

1.3.5 **Non Shivering Thermogenesis**

Non shivering thermogenesis or NST is defined as a cold induced increase in heat production not associated with the muscle activity of shivering (Himms-Hagen 1984) and is a form of adaptive thermogenesis. The primary site of NST is in brown adipose tissue where it is initiated by the transmitter noradrenaline (Broughton 2006). The capacity of NST depends on the proportion of brown adipose tissue in the adipose tissue and the number of mitochondria within the BAT and the amount of thermogenin or Uncoupling protein 1s (UCP-1s) within the mitochondria (Broughton 2006).

The activity of UCP-1s is a key factor for the production of heat during NST. UCP-1s are found on the inner mitochondrial membrane of BAT (Nedergaard 2001). The primary purpose of UCP-1 is to uncouple respiration from oxidative phosphorylation in respiration (Nicholls and Locke 1984). This is completed by allowing protons that have been extruded from the mitochondria through the electron transport chain to flow back through UCP-1 dissipating the energy from the electrochemical gradient as heat (Nicholls and Locke 1984). In respiration when no extra heat production is required UCP-1s are blocked by nucleotides such as ATP, GDP and GTP (Broughton 2006) and the protons flow through ATP synthase using the energy produced from the electrochemical gradient to convert ADP to ATP (Forrest 2002).

It has been clearly shown that thermogenesis stimulated by noradrenaline within brown adipocytes cannot be completed without the presence of UCP-1 (Matthias et al 2000). UCP-1 knock out models in cold sensitive mice demonstrated this soundly when measuring the oxygen consumption of brown adipocytes (and therefore thermogenesis) of mice exposed to noradrenaline with and without UCP-1s present. The oxygen consumption of brown adipocytes of mice significantly increased in mice with UCP-1 present compared to little change in the oxygen consumption of brown adipocytes of mice without UCP-1s present (Matthias et al 2000).

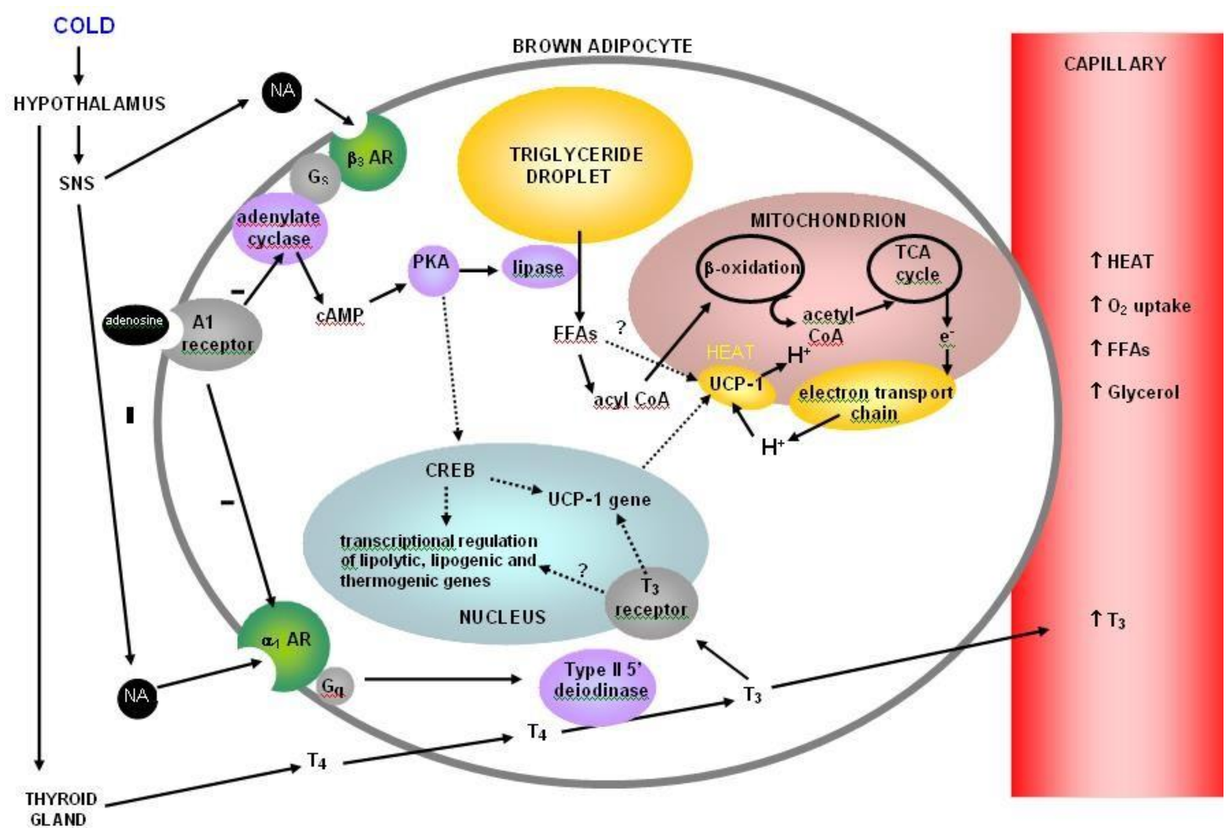

Figure 6 - Control of non-shivering thermogenesis by the catecholamine noradrenaline, thyroid hormones and adenosine. From Forrest, (2002).

Non-shivering thermogenesis begins by the release of noradrenaline from the sympathetic nervous system in response to cold which then travels to the BAT (Broughton 2006). Once thermogenesis begins blood flow rapidly increases (Ricquier 2006) as blood vessels dilate allowing the warmer blood leaving BAT to

travel to organs more quickly. The function control of non-shivering thermogenesis is shown in figure 6. At the BAT the noradrenaline influences β3, α_2 and α_1 adrenergic receptors (Broughton 2006).

The β3 adrenergic receptor that sits in the membrane of BAT cells (Figure 6) provides stimulus for the main thermogenesis pathway by coupling positively to G_s-proteins (Broughton 2006). This in turn stimulates adenylate cyclase producing a signal through cyclic adenosine 3'5 monophosphate (cAMP) which proceeds to signal cAMP dependant protein kinase A (PKA) (Broughton 2006).

Once activated PKA begins lipolysis by activating hormone sensitive lipase (HSL) and deactivating perilipin (Broughton 2006). The protein perilipin surrounds fat droplets and protects them from the activity of HSL and once deactivated HSL is able to translocate the fat droplet and catalyse triglyceride breakdown (Martinez- Botas et al 2000). Triglyceride break down releases free fatty acids which are then used as fuel for respiration and stimulation of UCP-1 in BAT (Nedergaard et al 2001). PKA is also considered to stimulate UCP-1 gene expression (Broughton 2006) through phosphorylating the CREB transcription factor (Thonberg et al 2002) and activating the p38 MAP kinase pathway.

The β_1 adrenergic receptor also plays a role in the stimulation of NST. The $_1$ adrenergic receptor couples with G_q-proteins which in turn increases the tri-iodothyronine (T_3) within the brown adipocyte cell (Broughton 2006). The T_3 then binds to T_3 receptors with a requirement of 70% of T_3 receptors to be saturated in order to activate UCP-1 (Bianco & Silva 1987).

The β_2 adrenergic receptors which couple to G_i- and G_o-proteins have an inhibitory effect on thermogenesis (Broughton 2006). This is achieved by the inhibition of adenylate cyclase and calcium channels which inhibits lipolysis and NST (reviewed in Lafontan & Berlan 1993).

1.4 The 3 Adrenergic Receptor

The beta-3-adrenergic receptor is a catecholamine receptor that belongs to the R_7G superfamily of guanine-nucleotide binding protein (G-protein)-coupled receptors, which are characterised by seven transmembrane (TM) domains, an extra-cellular glycosylated N-terminal region and an intra-cellular C-terminal region (Figure 7) (Forrest 2002). They are one of 3 β adrenergic receptor subtypes (ie β_1, β_2 and β_3). The activation of G-proteins, due to ligand binding, stimulates or inhibits effectors such as adenylate cyclase, phospholipase C, other enzymes (e.g. mitogen-activated protein kinase) or even ion channels (Forrest 2002).

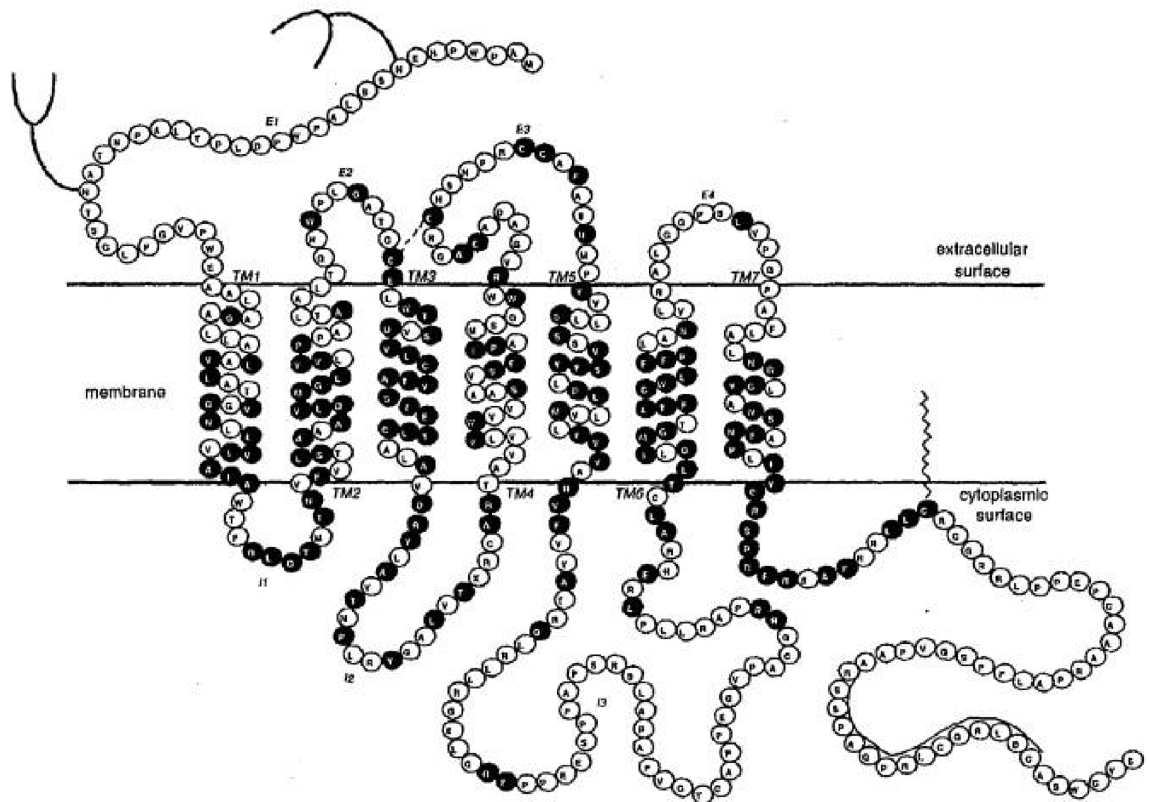

Figure 7: Structure of the human β3-adrenergic receptor. The sequence is represented in the one-letter code for amino acids. The single polypeptide chain is arranged according to the rhodopsin model. The disulphide bond essential for Cys^{110} and Cys^{189} activity is represented by ----. The two N-glycosylation sites in the amino-terminal portion of the protein are indicated by . The palmitoylated Cys^{360} residue in the N-terminus of the I4 loop is indicated by a . Residues in black circles are common to the three β3-adrenergic receptor subtypes. (From Strosberg & Gerhardt 2000 as cited by Forrest 2002).

1.4.1 β3-AR effects on reproduction

β3-ARs are not only found on the surface of adipose but are also found to be expressed in the human heart, gall bladder, gastrointestinal tract, prostate, urinary bladder detrusor, in the near-term myometrium (reviewed by Ursino et al 2009) and placental arteries (Rouget et al 2006, Hynes et al 2008). Specifically in sheep there is

also evidence that the β3-AR is found in both adipose tissue and non-adipose tissue such as the heart, liver, spleen, lung, kidney (Wu et al 2011) and placental vessels (Gao et al 2017).

In late pregnancy, stimulus of the β3-AR results in relaxation of myometrium contractions therefore preventing parturition and in particular pre-mature parturition. Bardou et al (2000) and later Dennedy et al (2001) investigated the function of β3- ARs in the myometrium of near term pregnant woman undergoing caesarean section. In both studies a concentration dependent relaxation of the myometrial spontaneous contractions was detected when the women were administered selective agonists of β3-AR. When stimulated by agonists, β3-ARs have higher inhibition of spontaneous contractions of the myometrium than in non-pregnant women and more of the receptors are likely to be present later in pregnancy with a 2 fold increase in up- regulation of β3-AR mRNA in later pregnancy (Rouget et al 2005). Therefore variation in the β3-AR gene may affect gestation length in humans and other species.

There is evidence that β3-ARs may also play an important role in inhibiting myometrium contractions during the peri-implantation period. It is suspected that uterine contractions may cause implantation failure (Lesny et al 1998). Markiewicz & Jaroszewski (2016) investigated the role of β3-AR during the peri-implantation period by administering β3-AR agonists to pregnant pigs. Both agonists reduced the tension, amplitude and frequency of myometrium contractions (Markiewicz & Jaroszewski 2016). Thus variation in the β3-AR gene could have an effect on the implantation success of the embryo in pigs and other species.

There is also evidence that β3-ARs are present in human placental arteries (Rouget et al 2006, Hynes et al 2008). In addition there is evidence that the β3-AR has a vasorelaxant effect in other vessels of other animals including the dog and the rat (Berlan et al 1994, Trochu et al 1999). This has led research into the potential effect the β3-AR has on pre-eclampsia. Pre-eclampsia is characterised as an abnormal

vascular response to placentation associated with increased systemic vascular resistance, enhanced platelet aggregation, activation of the coagulation system and endothelial cell dysfunction (Rouget et al 2006). The mechanisms underlying preeclampsia are unclear and it is one of the leading causes of neonatal morbidity and mortality due to reduced oxygen and nutrients being supplied to the fetus (Rouget et al 2006). Rouget et al (2006) studied the presence and effect of stimulation of $_2$-AR and β3-AR of the arteries in the chorionic plate of placentae obtained from women with uncomplicated and pre-eclamptic. β3-AR transcripts were found in both uncomplicated and pre-eclamptic arteries with no significant difference in the amount of transcripts between the two types of pregnancies. Additionally, in both uncomplicated and pre-eclamptic pregnancies stimulation of
β3-AR with SR 59119A resulted in relaxation of placental artery rings, although the pre-eclamptic response was attenuated (Figure 8) and the introduction of an β3-AR blockade antagonised the relaxation effect (Rouget et al 2006). The attenuated relaxation effect is likely due to posttranscriptional down-regulation of β3-AR as pre- eclampsia had no effect on the amount of β3-AR mRNA but the density of β3-AR proteins was decreased in the pre-elamptic arteries (Rouget et al 2006).

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Figure 8: Influence of preeclampsia on the relaxing effects of SR 59119A on human placental arteries contracted with U46619 (10 nM). Results are expressed as the mean ± SEM (n = 7–20 arteries for normotensive and 6–8 for preeclamptic pregnancies, with each artery obtained from a single woman) (Rouget et al 2006).

Dennedy et al (2002) observed similar results with the stimulation of β3-AR in the umbilical artery. The β3-AR agonist BRL 3734 used in the experiment by Dennedy et al (2002) reduced the contractility of human umbilical arterial rings but not to the same extent of relaxation as that of Rouget et al (2006). Rouget et al (2006) explained that this lesser extent of relaxation is likely due to BRL 37344 being a weaker agonist to β3-AR compared to SR 59119A (Bardou et al 1998 as cited by Rouget et al) and due to the experiments testing the relaxation in different arteries (umbilical arteries for Dennedy et al. and placental arteries for Rouget et al 2006). Hynes et al (2008) also found pre-eclampsia did not influence the amount of mRNA in placental vessels. However as a discrepancy to Rouget et al (2006), Hynes et al (2008) did not find pre-eclampsia had an effect on the amount of β3-AR in placental vessels. Hynes et al (2008) put this discrepancy down to the proteins being analysed from different regions of the placenta. Rouget et al (2006) studied the chorionic plate of the placenta and Hynes et al (2008) studied the basal plate of the placenta) as there is evidence that the β3-AR signalling pathway shows variability depending on which vascular bed it has come from. Hynes et al (2008) also concluded that

aberrations in the signalling system of β3-AR of the basal placenta vessels may occur in preeclampsia rather than down-regulation of the receptors.

Thus there is evidence that the β3-AR affects the contraction of multiple different vessels of the placenta and umbilical cord in humans and therefore is likely to affect the occurrence of pre-eclampsia and the nutrient supply to the foetus. Additionally, there is evidence that the β3-AR may operate differently in different vessels. Therefore impaired function of the β3-AR gene is likely to affect the ability of β3-AR as a vasorelaxant and therefore affecting the nutrient supply to the foetus although the effect is likely to be variable throughout different tissues.

1.5 The beta-3-adrenergic receptor gene

1.5.1 The β3-AR Gene Isolation

Variation in cold tolerance between sheep breeds has been noted for many years (Sykes et al 1976) (Samson & Slee 1981) (Wolff et al 1987). This indicates that the cold tolerance trait is under genetic control however there are many physical and physiological factors affecting the trait indicating it is under the control of multiple genes (Gudex 2001) (Forrest 2002). It was not until the observations of cold tolerance variation within breeds and the establishment of two lines of Scottish Blackface sheep with low and high cold tolerance by Slee in 1985 and further research, that the theory of a single gene having a prominent effect on sheep neonate cold tolerance came about. The low cold tolerance line showed no thermogenic response to an injection of noradrenaline (Slee et al 1987) which usually produces a response of an increase of 200-250% of thermogenesis in neonatal lambs (Alexander & Williams 1968). Physiological research showed that the BAT and the mitochondria within were present at normal levels in the lambs with low cold tolerance. Following matings indicated that the difference in noradrenaline response was due to an individual gene that was inherited in a Mendelian fashion (Simpson & Slee 1988). From this it was established that a single gene probably in the

catecholamine pathway affects thermogenesis in sheep (Slee & Simpson 1991). It is likely that variation in the β3-adrenergic receptor is responsible for this variation.

Later the ovine β3-AR gene was sequenced and characterised by Forrest and Hickford in 2000 through the means of a PCR cloning strategy and the genomic and cDNA libraries of the gene from the human, mouse, rat, dog, monkey and guinea pig β3-AR gene. The characterised ovine β3-AR gene was revealed by Forrest in 2002 to have a 2 exon structure (figure 9) with an intron size of 724bp being similar to the dog β3-AR gene of 704bp (Lenzen et al 1998). Homology of the ovine β3-AR gene of >85% was shown with the cow, goat, human and dog (Forrest 2002) which is notably higher than the 40-50% homology shown between different β3-AR subtypes of the same species (Strosberg 1997). This high conservation of sequences indicates the importance of the β3-AR for energy balance within all these species (Forrest 2002).

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Figure 9: Structure of ovine β3-adrenergic receptor gene (ADRB3) (Yang 2014)

Homology is high in the TM domains with several TM amino acid residues being conserved in all β3-AR subtypes (Strosberg 1997). These include (Asp_{83}, Asp_{117}, Ser_{169}, Ser_{209}, Ser_{212}, Phe_{309}, Asn_{312}, Tyr_{336}) which have been shown to be essiential for ligand binding and signal transduction of the receptors as reviewed in (Strosberg 1997).

1.5.2 The β3-AR Gene Variants

In 2002 Forrest identified 6 alleles (A-F) of the ovine β3-AR gene that segregated in a Mendelian fashion using a using polymerase chain reaction single strand conformational polymorphism (PCR-SSCP) of the 467bp region within the intron. The allelic differences between the 6 alleles are shown in table 3. Codon changes that cause amino acid changes within the coding region are shown in table 2. The alleles B and F both encode the same protein sequence. Alleles C and E also encode the same protein sequence however at position 270 the valine is an alanine. Allele A encodes a different putative protein sequence with amino acid changes at position 376 (table 3) and the D allele protein sequence differs again with amino acid changes with valine to alanine at position 52 and leucine to valine at position 322. The allelic changes of the D allele are considered to be conservative as the substituted amino acids are chemically similar to the original amino acids (Forrest & Hickford 2000). However the amino acid changes in the D allele occur in regions of the gene that are highly conserved in all three -adrenergic receptor subtypes. This region is thought to be involved in ligand binding (reviewed in Strosberg & Gerhardt 2000). It is thought that even a small change in amino acids could affect ligand binding, therefore it is thought that the D allele of the β3-AR gene has altered ligand binding affecting the function of the receptor therefore increasing the risk of cold related mortality (Forrest & Hickford 2000). The amino acid change within allele A is within the C-terminal region of the protein which is one of the protein regions responsible for G-protein-receptor coupling (Strogsberg 1997).

Most of the variation between the alleles occurs in non-coding sequences including within the intron, 5' region and 3'region of the β3-AR gene (table 3). Variation within these gene regions are likely to also affect gene function due to possible regulatory roles. Forrest (2002) highlighted the fact that intron regions and other non-coding regions can play important roles in ensuring that the gene functions normally and can have an effect on the gene protein and phenotype. Examples included were mutations within gene splice sites being associated with growth

hormone deficiency (Binder & Ranke 1995) and a mutation within intron four of the lecithin:cholestorel acyltransferase gene causes fish eye disease (Kuivenhoven et al 1996).

Although since the publication of Forrest (2002) the effect of non-coding variation on phenotype has been debated (Graur et al 2013) there has since been evidence for intron variation effect on phenotype. More recent examples of the effect of intronic variances affecting phenotype include an insertion of a thymidine in intron 4 of the adenomatous polyposis coli (APC) gene in humans causing colon cancer (Neklason et al 2002) and the adaptive trait of the pea-comb in chickens is caused by a large amplification of a duplicated sequence in intron 1 of the gene (Wright et al 2009). Forrest (2002) speculated that variation in the non-coding sequences of the β3-AR gene could affect genotype. Homology between rat β3-AR gene intron sequences and sequences that bind transcription factors involved in tissue specific expression (Granneman et al 1992) suggests that variation within the β3-AR gene intron may affect gene expression and therefore possibly receptor function. Forrest (2002) highlighted the different mRNA lengths the β3-AR gene ha such as in rodents having varying mRNA lengths due to differing promotor positions (Granneman & Lahners
1994) and the use of alternative splicing (Evans et al 1999, van Spronsen et al 1993). Since this evidence has arisen that changes in the 3'UTR and intron sequences of the sheep β3-AR gene affects phenotype. Haplotypes derived from variation in the β3- AR 3'UTR region and the full length of the intron have also shown associations with variation in both carcass and growth traits (Horrell et al 2009, Yang 2014). These included effects on the post weaning growth rates, weaning weights and fat depths. For example 3'UTR variant *b* alone was associated with a lower weaning weight and the intron variant C alone was associated with a higher weaning weight (Yang 2014). This indicates that variation within sequences of many areas of the β3-AR gene can have significant effects on the phenotype including non-coding regions and that the gene is pleiotropic.

Since the characterisation and first discovery of the ovine β3-AR gene variants two more alleles (G & H) have been discovered (Forrest et al 2007) using the PCR-SSCP of the 467bp region within the intron. Additionally during the characterisation of the ovine gene ambiguities in the PCR-SSCP of the 467bp region of the intron obtained for allele A suggested that greater variation existed elsewhere in the sequence (Forrest 2002). This was further confirmed by Horrell et al (2009) and Yang (2014) by which reported further variation within the 3'UTR regions of the β3-AR gene variants. Additionally 46 ovine β3-AR gene variants were sequenced in 2012 by Wu et al by sequencing and identifying SNPs of the whole gene.

Table 2: Amino acid changes from the putative sequence within the coding region of the variants of the ovine β3-AR gene as identified by Forrest 2002.

Allele	Amino Acid Changes
A	Ala270->Val and Arg376->Gln
B	Ala270->Val
C	-
D	Val52->Ala and Leu322->Val
E	-
F	Ala270->Val

Table 3: Allelic differences identified at the beta -3- adrenergic receptor gene locus. The positions listed are based on the sequence of the ovine beta -3- adrenergic receptor gene (GenBank accession AF109928). The putative protein is encoded by two exons (560 .. 1755,2479 .. 2500). Where sequencing has given ambiguous results, K represents T or G, S represents C or G, W represents A or T and Y represents C or T. UTR= untranslated region. Allelic differences occurring in the 5'UTR and intron are shaded. GenBank accession numbers for alleles A through Fare AF314200 through AF314205, respectively.

Position & base AF1099289		Base present in allele A	B	C	D	E	F	Comment
374	**T**	T	T	A	T	T	T	5'UTR
399	**C**	A	C	A	A	C	A	5'UTR
400	**T**	K	G	G	G	T	G	5'UTR
472	**T**	C	C	C	C	C	C	5'UTR
588	**T**	C	C	C	C	C	C	coding region, the T→C base change translates to a Phe10→Ser substitution forming an N-linked glycosylation site in the first extracellular loop (the amino-terminal region).
689	**C**	Y	T	T	T	C	T	no amino acid change
714	**T**	T	T	T	C	T	T	base substitution in D results in an amino acid change from Val52→Ala in the first transmembrane domain.
853	**G**	S	G	C	G	G	C	no amino acid change
1138	**G**	G	G	T	G	G	G	no amino acid change
1251	**A**	C	C	C	C	C	C	the A→C base change translates to a Asp231→Ala substitution in intracellular loop three.
1368	**C**	T	T	C	C	C	T	base substitution in A, B and F results in an amino acid change from Ala270→Val in intracellular loop three.
1523	**C**	C	C	C	G	C	C	base substitution in D results in an amino acid change from Leu322→Val in the seventh transmembrane domain.
1534	**C**	Y	C	C	C	C	C	no amino acid change
1686	**G**	A	G	G	G	G	G	base substitution in A results in an amino acid change from Arg376→Gln in the fourth intracellular loop (the carboxyl terminal region).
1808	**A**	A	A	A	C	A	A	intron
1809	**T**	.	.	.	T	T	.	intron
1810	**T**	T	T	T	T	T	.	intron
1860	**A**	G	A	G	G	A	A	intron
1976	**C**	A	A	A	A	A	A	intron
2005	**G**	G	G	G	A	G	G	intron
2087	**G**	G	A	G	G	G	G	intron
2153	**T**	C	C	C	C	T	C	intron
2176	**C**	T	T	T	C	T	T	intron
2182	**C**	C	C	C	C	C	T	intron
2202	**A**	A	A	A	C	A	A	intron
2291	**A**	G	A	A	C	A	A	intron
2307	**T**	C	T	T	C	T	T	intron
2322	**C**	C	A	C	C	C	C	intron
2324	**T**	C	T	T	C	T	T	intron
2542	**G**	G	S	G	G	G	G	3' UTR
2555	**C**	A	A	A	A	A	A	3' UTR
2733	**A**	C	C	A	A	A	A	3' UTR
2857	**A**	W	A	A	T	A	A	3' UTR
2978	**T**	Y	C	T	C	T	C	3' UTR
3034	**T**	T	T	K	T	T	T	3' UTR
3045	**C**	C	C	T	C	C	C	3' UTR

1.5.3 Allele Associations

It is clear that the ovine β3-AR gene variants have variable effects on sheep phenotype and in particular cold survival (table 4). Multiple studies have reported the association of the allele E and A with increased cold survival and increased cold mortality when sheep carried alleles C and F. The strongest association of cold mortality and overall mortality has been reported with allele D (Forrest et al 2006, Forrest et al 2007). This is thought to be caused by impaired function of the β3-AR receptor ligand binding due to the substituted amino acids as described previously.

Table 4: Phenotypic associations with the alleles A-F of the ovine β3-AR gene identified by a PCR-SSCP of the 467bp intron.

	A	**C**	**D**	**E**	**F**
Cold Survival	Increased cold survival (Broughton 2006) (Forrest et al 2007)	Decreased cold survival (Broughton 2006) (Forrest et al 2007)	Decreased cold survival (Forrest 2002) (Forrest et al 2006)(Forrest et al 2007)	Increased cold survival (Forrest 2002) (Forrest et al 2006) (Forrest et al 2007)	Decreased cold survival (Forrest et al 2007)
Birth weight	Increased birth weight (Broughton 2006)	Increased birth weight (Forrest 2002) Decreased birth weight (Broughton 2006)			
Growth rate and weaning weight	Increased growth rate (Forrest 2002[a])				
Carcass traits			Higher muscle to fat ratio (Forrest 2002)	Heavier carcass weights (Forrest 2002)	

[a] dependant on birth rank or gender.

The discovery of the β3-AR 467bp intronic gene variant associations with cold mortality have enabled the development of a cold tolerance gene test by the Lincoln

University Gene Marker Laboratory. The gene test is based on a PCR-SSCP of the 467bp intronic region as performed by (Forrest et al 2003) (Forrest et al 2006) (Forrest et al 2007). A score based on the risk of dying due to cold exposure in association with each allele (figure 10). This allows breeders to accurately select more cold tolerant animals.

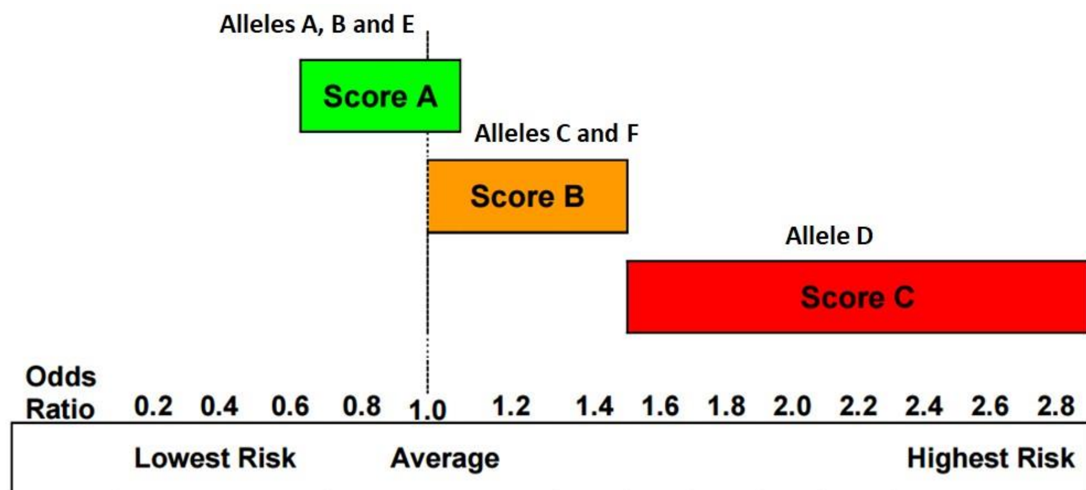

Figure 10: The graph shows the 95% confidence intervals for the risk of cold-related mortality (expressed as an odds ratio) for each of the three scores A, B and C. Currently Score A represents alleles A, B and E, Score B represents alleles C and F, and Score C represents allele D. Adapted from (Forrest & Hickford 2006).

1.5.4 The D allele Frequencies

Previous research indicates that the high risk of cold related mortality D allele is only present in commercial Merino populations and in small proportions (Table 5). Each of the studies were completed with New Zealand sheep breeds of which the D allele was only present when Merinos were sampled.

Table 5: Frequency of the D allele of the ovine β3-AR gene in different breeds

Breed	Frequency of D allele	Reference
Suffolk	0.0%	Yang 2014
Romney	0.0%	Broughton 2006
Feral Merino	0.0%	Mckenzie et al 2010
Various (including Merino)	2.4%	Forrest et al 2007
Merino	6.3%	Forrest et al 2006
Merino, Borderdale, Coopworth	6.7%	Forrest 2002

Mckenzie et al (2010) investigated the allele frequencies of the β3-AR gene in 10 feral Merino populations none of which displayed a D allele whilst the reference (commercial) Merino flock displayed a D allele frequency of 0.06%. It is hypothesised that the D allele is not found in the feral Merino sheep by either the founder effect or balancing selection. Founder effect could explain the absence as the populations are thought to be founded on 50 or less sheep and the D allele is considered rare in farmed NZ sheep. Balancing selection could explain the absence of the D allele due to it not providing any selective advantage (Mckenzie et al 2010). It is also hypothesised that due to the feral populations experiencing cold D allele carrying animals would not survive.

1.5.5 Allelic associations of the β3-AR gene in other traits and species

The β3-AR gene is pleiotropic affecting many traits within sheep and has been shown to affect many traits across other species. This is expected as BAT is not only important for NST but it also important for regulating energy intake and body fat stores (Forrest 2002). In Duroc pigs an insertion/deletion of a thymine in exon 2 of the β3-AR gene has been associated with variation in loin Eye-Muscle Area (Hirose et al 2009). Variation in the β3-AR gene of cattle have been discovered and in a comparative analysis of the human chromosome 8 and bovine chromosome 27 indicates that bovine the β3-AR gene is likely to affect partitioning body fat deposition (Connor et al 2006). In sheep the β3-AR gene variants have been associated with wool traits such as mean staple strength (MSS) and yield. In Merino sheep allele C was associated with increased MSS and yield and alleles A and E were associated with negatively affecting these traits (Forrest et al 2009). However it is not known whether this is due to a physiological link between the ovine β3-AR gene and wool production or it is because the β3-AR gene is serving as a marker for other close by wool production affecting genes (Forrest et al 2009). In humans a mutation in codon 64 (Trp64Arg)of the β3-AR gene resulting in the replacement of arginine by tryptophan within the protein has been associated with increased body weight, early development of type 2 diabetes mellitus and clinical features of insulin resistance (Sliver et al 1999). However these associations are not present in all populations with the Arg64 allele being associated with a higher Body Mass Index (BMI) in East Asians but not Europeans (Kurokawa et al 2008) and increased fasting insulin levels in Asians but not Caucasians (Zhan & Ho 2005).

1.6 Perspectives and aims of this study

Reproduction efficiency is key for the productivity performance of New Zealand sheep farms. The production of both meat and wool produce is predominantly determined on the number of lambs that survive until weaning than on the individual production of each lamb (Sidwell et al 1962, Lax & Newton-Tuner 1965). Thus improving lamb survival and reproduction efficiency is crucial for improving farm efficiency and profitability.

The development of the Lincoln University Cold Tolerance Gene Marker test based on variants of the β3-AR gene has allowed sheep farmers to select for better cold tolerance and therefore improving lamb survival. Of the variants tested by the Lincoln University Cold Tolerance Gene Marker test the D allele is thought to have altered ligand binding and therefore impaired receptor function due to amino acid changes in a highly conserved region of the gene.

It is suspected that the D allele of the ovine β3-AR gene severely impairs receptor function and has been proven to significantly decrease cold survival due to the allele's effect on thermogenesis. The D allele has only been identified in Merino sheep which are known to have lower fertility and scanning-rates of an average of 90-130% compared to 130-170% of other New Zealand sheep breeds (Geenty 1997). In addition variation in embryo survival between Merino flocks has also been observed suggesting genetic influence on the trait (Wilkins 1996). Recent research indicates the receptor has a role in physiological functions during pregnancy in various tissues which indicates that the D allele may have adverse effects on scanning-rate. Thus the aim of this study is to identify whether the D allele of the ovine β3-AR gene is being selected against in New Zealand Merino populations and to identify whether the allele's presence has any effect on Merino scanning rate.

If the D allele is proven to be selected against in Merino populations and to have adverse effects on merino scanning-rate, producers will be able to utilise the already

developed Cold Tolerance Gene Marker to select for improved scanning-rates. This study could therefore provide the opportunity for Merino producers to improve the production efficiency and profitability of their systems.

2 MATERIALS AND METHODS

2.1 Data collection

2.1.1 Population Data

Allele frequency data was collected from the commercial Lincoln University Gene-Marker Laboratory database records and included sheep that had been tested for cold tolerance between 2005 and 2017. The data had 18,415 samples and a range of sheep breeds were tested.

2.1.2 Experimental Site

The study was conducted on Mt Hay Merino stud of John Simpson in Tekapo, Mackenzie District, Canterbury, New Zealand. The farm is 10,500ha at altitudes ranging from 700m to 2000m with an average rainfall of 350mm per year.

2.1.3 Animals

The experiment was conducted with 9 Merino rams (5 of which were carrying the D allele of the β3-AR gene) and 440 Merino ewes. The sheep used in the experiment were polled and of Australian Peppin bloodlines. Each sheep was able to be identified by their plastic ear tags each with a unique code. The ram was put out on the 24th of May.

2.1.4 Scanning

Scanning was completed on the 24th if August (13 weeks after mating) using a SSD-500V portable veterinary Ultrasound System (Hitachi Aloka Medical, Ltd) with a 3.5MHz convex ultrasonic probe. Twin and single bearing ewes were identified.

2.1.5 DNA sampling

Blood was collected from each sheep by clipping a small notch at the tip of the ear and collecting blood drops on FTA cards (Whatman Bioscience, Middlesex, UK). Tag numbers were recorded with each corresponding sample.

2.1.6 DNA Preparation and Genotyping

All DNA preparation and genotyping was performed by Staff at the Lincoln University Gene-Marker Laboratory, Canterbury New Zealand. The method used for DNA preparation, genotyping and detection of polymorphism was completed as described by (Byun et al 2009, Appendix V).

2.2 Statistical Analysis

Chi squared goodness of fit tests were performed using Minitab version 17 to determine if the observed D allele frequencies within the population of gene tested sheep fit the Hardy-Weinberg equilibrium. Pearson chi square tests were performed on SPSS version 24.0 to determine the effect of presence of either 0,1 or 2 D alleles of the dam or the presence of 0 or 1 D alleles of the sire on twinning rate and dry rate. A conditional forward stepwise logistic regression was performed on SPSS version 24.0 to determine if there was an interaction between sire and presence of sire D allele.

3 RESULTS

3.1 Population Analyses

Animals with missing or unknown values were removed from the data set. The D allele was only present in Merino sheep. There was a significant difference between observed and expected genotype frequencies in all populations except the Simpson farm (table 6). In the all breeds, all breeds adjusted and Merino populations heterozygous D genotype counts were lower than expected. However these differences contributed only a small proportion to the overall chi squared value indicating that differences between expected and observed frequencies in other genotypes were mainly contributing to the significant P values (table 6). In the Merino adjusted population there was no difference in observed and expected counts for the heterozygous D genotypes.

In the all breeds, adjusted all breeds and Merino populations homozygous D was observed at higher numbers than expected whilst the adjusted Merino population had no homozygous D alleles which was as expected (table 6). This meant that the homozygous D genotypes of the adjusted Merino population had to be grouped into the heterozygous D counts in order to prevent the chi squared from being biased. The higher counts of observed homozygous D alleles in the populations had high contribution to chi square values in ratio in the chi square values indicating that these differences between observed and expected values are putting a large contribution into the significant P values (table 6).

There was no significant difference between the observed and expected genotypes within the Simpson farm population although the effect was not far from significant (P = 0.065) (table 6). The Homozygous D genotype appeared to be at a lower than expected count while heterozygous D alleles appeared to be at a higher than expected count (table 6).

Table 6: The observed and expected numbers of sheep either heterozygous (DX) or homozygous (DD) for the D allele of the ovine beta-3-adrenergic receptor in different populations

Population	Genotype	Observed (n)	Expected[b] (n)	Contribution to Chi Square[d]	Chi square value[c]	P value[c]
All breeds	**Dx**	375	422.04	5.244	1159.88	0.000
	DD	26	2.48	223.282		
All breeds[a]	**Dx**	138	165.07	4.439	1241.15	0.000
	DD	14	0.47	393.486		
Merino	**Dx**	371	406.7	3.130	213.634	0.000
	DD	26	8.145	39.141		
Merino[a]	**Dx/DD**	18	17.943	0.0002	86.0281	0.000
Simpson Farm	**DD**	7	14.48	3.86		
	Dx	146	131.04	1.70	20.12	0.065

[a] Adjusted population whereas sheep from farms with high D frequencies were removed
[b] Calculated using chi square goodness of fit of all genotypes tested in the population
[c] Based on the chi square goodness of fit all genotypes found in that population
[d] Represents how much difference the observed and expected of that comparison contributes to the chi square P value when compared with the chi square value.

3.2 The D allele and scanning rate

3.2.1 Dry rate

There was no significant effect of the presence of 0 or 1 D alleles from either the sire or the dam on the number of dry ewes (Appendix IV). The sample size (n=13) was

too small for the effect of dam homozygous D allele on the number of dry ewes scanned to be interpreted.

3.2.2 Twinning rate

There was no significant effect of the dam carrying 0,1 or 2 D alleles on the number of twins scanned (table 7). Dams that were mated to sires carrying a D allele had a lower twinning rate than dams mated to sires not carrying the D allele (P = 0.003) (table 7). A conditional forward stepwise logistic regression revealed that both the presence of a sire D allele (P=0.000) and sire (P=0.054) had an effect on the number of twins scanned. Sire only had an effect when the sire carried a D allele (Table 8). Within the D allele sires there was a large range of twinning rates of 7.10%-29.30% (table 8).

Table 7: The number and percentage of twins scanned from either ewes homozygous D (DD) ewes, heterozygous (Dx), or have no D (no D) or ewes that were mated to rams heterozygous D (Dx) or have no D (no D) of the beta-3-adrenergic receptor gene.

	Sire Genotype		**Dam Genotype**		
	Dx	**no D**	**DD**	**Dx**	**no D**
Twins (n)	48	52	2	29	69
Twins (%)	19.5%	32.7%	28.6%	22.0%	25.9%
P value[a]		0.003			0.668

[a] P value from Cross tabulation chi squares determining whether the presence of sire or dam D has a significant effect on scanned twin rate.

Table 8: The number and percentage of twins scanned in ewes that went to a sires that carry D allele (sire 1,4,5,8 and 6) and do not carry a D allele (sire 2,3,6,7) of the beta-3-adrenergic receptor.

Sire with D	twins (n)	twin (%)	Sire effect Significance
1	9	22%	0.048
4	12	17.90%	
5	11	26.80%	
8	12	29.30%	
9	4	7.10%	
Sire without D			
2	10	25%	0.146
3	14	34.10%	
6	11	26.20%	
7	17	47.20%	

4 DISCUSSION

The objective of this study was to determine if the presence of the D allele of the ovine β3-AR gene in either the sire or the dam had an effect on scanning-rate and whether the D allele is being selected against in New Zealand Merino populations.

The D allele only has reduces fecundity when it is carried by the sire of the mating (table 7). This indicates that β3-AR gene is under the influence of genomic imprinting, in particular paternal imprinting. Genomic Imprinting is when genes are differently expressed when inherited from either the maternal or paternal parent due to modification of the parental genomes during gametogenesis (Rance et al 2005). This can occur through differential DNA methylation in the egg and sperm resulting in the gene from each gamete being expressed differently (Reik & Walter 2001) or through DNA-protein and DNA-RNA interactions (Killian 2005). This differential gene expression is therefore implemented without altering the primary DNA sequence (Killian 2005) although the methylation remains throughout the organisms life and it is usually removed in primordial germ cells (reviewed by Wood and Oakey 2006). An example of a human imprinted gene includes the retinoblastoma (RB1) gene where a CpG island (cluster of cytosine-phosphate–guanine in a DNA strand sequence) within intron 2 that acts as a promotor and is methylated in the maternal chromosome whilst in the paternal chromosome it acts as a weak promotor for an alternative RB1 transcript (Kanber et al 2009). This results in a higher expression of the maternal RB1 gene than the paternal gene (Kanber et al 2009).

In agreement with these results, there is evidence suggesting that the β3-AR gene may be under the influence of genomic imprinting in other species. Rance et al (2005) provided evidence that a Quantitative Trait Loci (QTL) for Body Mass Index (BMI) on chromosome 8 of mice is paternally imprinted. No candidate genes within the QTL of chromosome 8 had been identified as imprinted genes but β3-AR was suggested as a possible imprinted gene although this is yet to be identified (Rance et al 2005). CpG islands are sites associated with DNA methylation and a higher

proportion of imprinted genes have CpG islands (88%) compared to 47% of normal genes (Reik and Walter 2001). Additionally many imprinted genes also have clustered direct base repeats near the CpG islands (Rance et al 2005). Screening of the β3-AR revealed a CpG island within exon 1 but no clustered direct repeats (Rance et al 2005). However DNA methylation is not the only mechanism that can inflict genomic imprinting with other mechanisms including histone and higher order chromatin structure modification (MacDonald 2012). In mammals and in particular mice, extensive non-coding RNA sequences have been found in many imprinted loci indicating non-coding RNA may play a role in genomic imprinting (Babak et al 2008, Gabory et al 2010). Therefore the β3-AR gene could be imprinted through mechanisms other than DNA methylation.

There is evidence that β3-AR is epigenetically mediated in a non-imprinting manner. These include evidence supporting folates from the diet that reduce methylation of the β3-AR gene in the blood of overweight women (Lima et al 2017) and evidence that increased DNA methylation in blood β3-AR is associated with dyslipidaemia in familial hypercholesterolaeimia, higher blood pressure and a lower waist to hip ratio in men (Guay et al 2014).

This study therefore provides further evidence for genomic imprinting of the β3-AR gene. Further research is needed to uncover the mechanism behind the effect and which tissues this plays a role in. Knowledge surrounding this paternal imprinting effect of the β3-AR gene would be very useful for farmers as they would only need to remove negative alleles from the sire to see an improvement in phenotype, depending on which tissues the imprinting takes effect on.

In addition to the D allele reducing twinning rate, there was an effect of sire on twinning rate when the sire carried a D allele (table 8). This was reflected in the large range of the scanned twinning rates of the ewes that were mated to D allele rams with the highest and lowest sire scanned twin rate being 29.3% (sire 5) and 7.1% (sire 9) respectively (table 8). This indicates that twinning rate is a complex

trait with the effects of β3-AR are likely to be affected by other genes and their variants present.

Complexity of the β3-AR traits has been observed in other species. For example the effects of the Trp64Arg variant of the β3-AR gene in on body mass index and fasting insulin levels has been shown to vary across populations. Kurokawa et al (2008) reported an association between the Trp64Arg variant and higher BMI in East Asians but no association was found in Europeans. Furthermore, Zhan and Ho (2005) reported increased fasting insulin levels in Asians but not Caucasians. Additionally the Trp64Arg has been found to interact with variants of other genes such as the lipoprotein lipase gene and peroxisome proliferator-activated receptor gene to increase the effect of obesity (Hsueh et al 2001, Ochoa et al 2004, Corella et al 2008). In addition multiple studies have found an epistatic link between variants of the β3-AR and β_2-AR genes affecting human obesity. Park et al (2005) provided evidence that the 1053G/C variant of the β_2-AR gene and the Trp64Arg variant of the β3-AR gene additively affected BMI, percentage of body fat in Korean adolescents. Lee and Park (2007) reported either increased or decreased risk of obesity depending on varying combinations of β3-AR and β_2-AR variants carried by Korean adolescents. Therefore an explanation for the large variation of effect of the D allele on scanning-rate could be due to other gene variants interacting to either increase or decrease the effect the D allele is having. These other interacting gene variants could be passed on to the foetus from the sire also carrying the D allele or from the dam.

It is most likely that the effect on twinning rate the D allele of the β3-AR gene has on scanning-rate is due to the D allele inherited from the foetus. This is indicated by the lack of dam D allele presence effect on scanning-rate indicating that the dam D allele is not directly impacting scanning-rate by the genetics of the dam altering the maternal environment.

Additionally there is no effect of the presence of the D allele from either the sire or the D on the number of dry ewes. This suggests that the D allele is not having an

impact on the fertility of the sire or the dam or, their ability to conceive. Therefore the effect is most likely due to the D allele inherited by the embryo, or foetus either directly affecting the development of the foetus, or creating an environment that inhibits development meaning less embryos or foetuses are able to be supported or survive. In support of genetics contributing to Merino embryo survival, evidence has shown that twin embryo survival is variable between Merino flocks, with treatments such as different feeding regimes and Fecundin immunisation having no effect on the variability between flocks (Wilkins 1996). The D allele of the β3-AR gene which to date has only been found in Merino populations could be contributing to the variable twinning rate between Merino flocks.

There is evidence that the β3-AR gene may affect foetal growth in other species with both maternal and foetal β3-AR stimulation affecting placental vessel relaxation and pre-eclampsia in humans (Rouget et al 2006, Hynes et al 2008). Effect on foetal growth is also supported by evidence that a β3-AR polymorphism in the foetus is a factor by which contributes to reduced foetal growth and insulin resistance in humans (Jaquet et al 2002). Therefore reduced reception of the β3-AR could lead to less nutrients going to the foetuses affecting growth and development. In support of this there is evidence that variation in the β3-AR gene of lambs affects birth weight (Forrest 2002, Broughton 2006) however results were not consistent (Horrell et al 2008). Forrest (2002) presented that lambs that inherited the D allele in one sire line were lighter than lambs which inherited the C allele in the same line whilst in another sire line ewe lambs carrying the D allele were heavier than lambs carrying the C allele. It would be expected that if the mechanism behind the highly significant decrease in scanning-rate was due to the D allele reduced chorionic placental vessel relaxation that birthweights would reflect this. It is likely that the β3-AR D allele of the foetus could be affecting some other aspect of foetal or embryonic development.

There is evidence that catecholamines play a role in embryogenesis and deficiencies can even cause embryo death. Therefore β3-AR is likely to effect the reception of the catecholamines and could therefore affect embryogenesis. A lack of noradrenaline

has been shown to cause embryonic death at mid-gestation (Thomas et al 1995, Zhou et al 1995, Lim et al 2000). Additionally there is evidence that it is not just the lack of noradrenaline that can cause the embryo death. Lim et al (2000) investigated the effect of an ineffective gata3 gene (required for the production of noradrenaline) in mice embryos. All embryos with the ineffective gene died, however catecholamine feeding of dams with embryos with the ineffective gene produced some surviving embryos. This indicates that the lack of noradrenaline is not the only factor causing the embryo death and that the ineffective gata3 gene is causing death through other means. This indicates the importance of multiple stages of the catecholamine pathway for embryo survival highlighting the likely role of the β3-AR for embryo survival.

The embryo death due to lack of noradrenaline in Lim et al (2000) may be a consequence of reduced cell differentiation. Rowe et al (1993) found that inhibiting noradrenaline production in *Xenopus laevis* embryos decreased cell differentiation in the brain however antagonising β3-AR made no difference in cell differentiation. More recently it has been found that catecholamines regulate the emergence of Hematopoietic Stem Cells (HSC) in the aorta-gonads-mesonephros (AGM) region of mouse embryos (Fitch et al 2012). The HSC cells of the AGM region are responsible for forming the blood and blood derived cells in the adult (Medvinsky & Dzierzak 1998). Furthermore Fitch et al (2012) noted the presence of both β_2-ARs and β3-AR transcripts developing HSCs although the expression of β_2-ARs was notably the higher. A later study indicated that emerging HSC in the presence of noradrenaline were not affected by β_2-AR antagonists indicating that another adrenergic receptor subtype mediates the HSC emergence (Guibentif et al 2014). β3-ARs have also been shown to be expressed in HSCs in adult bone marrow with evidence that β3-AR stimulation results in lower Stromal cell-derived factor 1 (cxcl12) mRNA expression leading to the mobilisation of HSCs in the blood (Méndez-Ferrer et al 2008). Stromal cell-derived factor 1 is also considered essential for embryogenesis with expression occurring in the embryonic brain, liver heart and

bone marrow cells (Nagasawa et al 1996) and gene knockouts of Stromal cell-derived factor 1, result in death of all neonates (Nagasawa et al 1998).

There is evidence that β3-ARs interact with multiple key hormones and signalling proteins that are key for embryo development. Therefore the D allele of the β3-AR receptor of the embryo may be reducing the effectiveness of the receptor which could be contributing to reduced embryo development. More research into the role of β3- AR in embryo development is needed to determine the nature of the effect.

It would be expected that there would be a dramatically lower than expected frequency of the β3-AR gene D allele in sheep populations due to the proven negative effect of D allele on cold tolerance and scanning-rate. It would be expected that if animals with the D allele were less likely to make it to sexual maturity, then the frequency of the D allele would reduce in the population. Therefore it is expected that populations would not be in Hardy-Weinberg Equilibrium due to selection occurring on the D allele. In this study heterozygous D genotypes present were lower than expected in all populations except for the Merino adjusted population and the Simpson farm however this difference only contributed a low proportion of difference to the chi squared value (table 6). Additionally homozygous D allele frequencies were present at much higher rates than expected in all populations except for the adjusted Merino and Simpson farm populations (table 6).

There are a number of factors that were likely to be contributing to these results. First of all a large proportion of the populations were under the effect of non-random mating. It is assumed that a population has non-random mating when performing the Hardy-Weinberg equilibrium and random mating causes a population to be out of Hardy-Weinberg Equilibrium. Most of the populations were under the effect of non- random mating as they were from farms using selective breeding practices, the exception being that some of the Merinos were from wild populations. Non-random mating could be causing the frequencies to not be in Hardy-Weinberg Equilibrium due to selection for production traits that may have loci close to the β3-AR gene and

having a low proportion of rams mated to ewes. It was attempted to remove some of this effect by removing sheep from farms that had high numbers of D allele carrying sheep and thus creating the adjusted populations (table 6). It was interesting to note that the Merino adjusted population had no difference between observed and expected D allele genotypes with no homozygous D allele genotypes observed when over half of the population consisted of wild Merinos. The population also had no homozygous D sheep observed. This could be a reflection of less non-random mating occurring in the population and could indicate that the high level of homozygous D genotypes in other populations is due to non-random mating.

The effect of selection against the D allele of the β3-AR gene may also be diluted by the fact that the traits it affects are likely to be complex. Forrest (2002) indicated that thermogenesis is a complex trait and the variation of D allele effect between sires in this study indicate that the reasons for the reduced twinning rate trait are also complex. The complexity of the traits can reduce selection pressure. For example D allele carrier sire 9 produced an average twinning rate of 7.10% whilst D allele carrier sire 8 produced an average twinning rate of 29.30% (table 6). Therefore selection against the D allele in sire 8 will be less intensive than in sire 9, and presumably due to other variants in other related genes present also interacting with the effect β3-AR has on twinning-rate.

The reduced selection pressure in some circumstances may reduce the deviation from the Hardy-Weinberg Equilibrium. This could explain the little difference we see in observed and expected D allele frequencies in the adjusted Merino population. Additionally the effect of non-random mating in the smaller half of the adjusted Merino population could also balance out the effect of selection against the D allele.

Paternal imprinting could also affect the intensity of selection and therefore the level that the population genotype frequencies deviate from the Hardy-Weinberg Equilibrium. It is thought when an allele is at low frequencies in a population and is under low selection pressure the presence of paternal imprinting increases selection

pressure on the allele (Patten & Haig 2008). The D genotype frequencies in the populations in this study were low (appendix II) and the selection pressure on the D allele could be considered low as the presence of the D allele does not guarantee death. Thus the presence of paternal imprinting is likely to be increasing the selection pressure on the D allele. However it is not known if paternal imprinting of the β3-AR gene is occurring in all tissues. Thus selection pressure increase due to paternal imprinting may only be occurring in the embryo or foetal tissues that are having an effect on scanning-rate but not in the adipose tissues where thermogenesis occurs therefore affecting the intensity of cold tolerance selection. Overall then, paternal imprinting may be increasing selection pressure on the D allele but the extent of this is unknown.

Overall most of the populations of sheep frequencies of the D allele of the β3-AR gene deviate from the Hardy-Weinberg Equilibrium. It cannot be confirmed in this study that some of this is due to selection against the D allele due to other pressures present in the populations such as non-random mating. However there is evidence in other studies to suggest that in the absence of non-random mating that the D allele is being selected against. McKenzie et al (2010) reported a frequency of 0.06 for the D allele of the β3-AR gene in a commercial Merino flock and no presence of the D allele in multiple wild Merino flocks. This could be due to negative selection on the D allele however it was also hypothesised that it could have been due to the founder effect (McKenzie et al 2010).

4.1.1 **CONCLUSION**

This study provides evidence that the D allele of the β3-AR gene reduces Merino scanning-rate, however the effect is complex. Whilst the scanning-rate is reduced by the presence of the D allele, the dry rate is not reduced. In addition there is evidence that the β3-AR gene is paternally imprinted in some form. The effect is likely to be due to the D allele being expressed in the embryo or foetus.

It is not what clear physiological process the D allele of the β3-AR gene is affecting on the foetus or embryo to reduce scanning rate. There is evidence to suggest that the β3-AR may be involved in embryo cell differentiation regulation and the reception of noradrenaline which is considered crucial for embryo development. However more research is needed as no research was found of β3-ARs directly affecting embryo development.

From this study it was not clear if the D allele was being selected against in New Zealand Merino populations due to the presence of non-random mating in the population study. Based on other research it is likely that it is being selected against.

The results from this study indicate that farmers could improve Merino scanning-rate by selecting rams that do not carry the D allele. Additionally selecting the rams without the D allele would be doubly beneficial for an increased scanning-rate due to the paternal imprinting effect. Research is needed to determine if the gene is paternally imprinted in other tissues. More research into the effect of the other β3- AR gene allele effects on scanning-rate could also be beneficial as other alleles may have effects on scanning-rate and be beneficial for selection.

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APPENDICES

Appendix I – Proportion of Merino breeders that perform the Lincoln University Cold Tolerance Gene marker test

	Perform ADRB3 cold tolerance gene test	Do not perform ADRB3 cold tolerance gene test
No. of breeders	18%	82%
Flock size	22%	78%

Appendix II – Genotype frequencies of the tested populations

Genotype	All breeds	Merino only	Adjusted all breeds	Adjusted Merino
AA	0.24	0.14	0.14	0.09
AB	0.08	0.03	0.03	0.03
AC	0.16	0.17	0.17	0.16
AD	0.01	0.03	0.03	0.00
AE	0.09	0.08	0.08	0.13
AF	0.07	0.14	0.14	0.05
AG	0.02	0.00	0.00	0.00
AH	0.00	0.01	0.01	0.00
BB	0.02	0.00	0.00	0.01
BC	0.04	0.02	0.02	0.03
BD	0.00	0.00	0.00	0.00
BE	0.03	0.01	0.01	0.01
BF	0.02	0.01	0.01	0.01

BG	0.00	0.00	0.00	0.00
BH	0.00	0.00	0.00	0.00
CC	0.04	0.05	0.05	0.07
CD	0.00	0.02	0.02	0.01
CE	0.05	0.07	0.07	0.14
CF	0.03	0.06	0.06	0.05
CG	0.01	0.00	0.00	0.00
CH	0.00	0.00	0.00	0.00
DD	0.00	0.00	0.00	0.00
DE	0.00	0.02	0.02	0.00
DF	0.00	0.01	0.01	0.00
DG	0.00	0.00	0.00	0.00
DH	0.00	0.00	0.00	0.00
EE	0.03	0.04	0.04	0.09
EF	0.03	0.04	0.04	0.08
EG	0.00	0.00	0.00	0.00
EH	0.00	0.00	0.00	0.00
FF	0.02	0.04	0.04	0.04
FG	0.00	0.00	0.00	0.00
FH	0.00	0.00	0.00	0.00
GG	0.00	0.00	0.00	0.00
GH	0.00	0.00	0.00	0.00
HH	0.00	0.00	0.00	0.00

Appendix III- Chi square goodness of fit outputs for each population

Chi-Square Goodness-of-Fit Test for Observed Counts in Variable: no.individuals – all breeds population

Using category names in Genotype

Category	Observed	Historical Counts	Test Proportion	Expected	Contribution to Chi-Sq
AA	4358	0.206322	0.206322	3795.29	83.430
AB	1482	0.094253	0.094253	1733.78	36.564
AC	2953	0.169616	0.169616	3120.08	8.947
AE	1622	0.117464	0.117464	2160.76	134.332
AF	1336	0.084919	0.084919	1562.09	32.722
AG	425	0.017631	0.017631	324.32	31.256
AH	34	0.001383	0.001383	25.44	2.883
BB	350	0.010764	0.010764	198.01	116.668
BC	645	0.038742	0.038742	712.67	6.425
BE	606	0.026830	0.026830	493.54	25.624
BF	315	0.019397	0.019397	356.80	4.897
BGBH	50	0.004343	0.004343	79.89	11.182
CC	765	0.034860	0.034860	641.25	23.882
CE	893	0.048283	0.048283	888.17	0.026
CF	639	0.034906	0.034906	642.09	0.015
CG	109	0.007247	0.007247	133.31	4.433
CH	12	0.000568	0.000568	10.46	0.228
DD	26	0.000135	0.000135	2.48	223.282
Other D genotypes	375	0.022943	0.022943	422.04	5.244
EE	518	0.016719	0.016719	307.54	144.018
EF	470	0.024173	0.024173	444.67	1.443
EGEH	46	0.005412	0.005412	99.56	28.815
FF	305	0.008738	0.008738	160.73	129.488
FGFH	28	0.003913	0.003913	71.98	26.869
GGGHHH	33	0.000438	0.000438	8.06	77.205

N DF Chi-Sq P-Value

18395   24   1159.88   0.000

1 cell(s) (4.00%) with expected value(s) less than 5.

Chi-Square Goodness-of-Fit Test for Observed Counts in Variable: Count – All breeds adjusted

Using category names in Genotype

Category	Observed	Historical Counts	Test Proportion	Expected	Contribution to Chi-Sq
AA	3733	0.214496	0.214496	3174.11	98.409
AB	1373	0.110417	0.110417	1633.95	41.674
AC	2290	0.166188	0.166188	2459.26	11.649
AE	1448	0.132294	0.132294	1957.68	132.695
AF	660	0.060811	0.060811	899.88	63.942
AG,AH	425	0.022378	0.022378	331.14	26.602
BB	340	0.014210	0.014210	210.28	80.026
BC	575	0.042775	0.042775	632.98	5.311
BE	575	0.034051	0.034051	503.88	10.038
BF	267	0.015652	0.015652	231.62	5.406
BG,BH	48	0.005760	0.005760	85.23	16.264
CC	586	0.032190	0.032190	476.35	25.240
CE	773	0.051250	0.051250	758.39	0.281
CF	356	0.023558	0.023558	348.61	0.157
CG,CH	110	0.008669	0.008669	128.28	2.606
DD	14	0.000031	0.000031	0.47	393.486
All D genotypes	138	0.011155	0.011155	165.07	4.439
EE	483	0.020399	0.020399	301.86	108.703
EF	378	0.018753	0.018753	277.51	36.392
EG,EH	43	0.006901	0.006901	102.12	34.225
FF	127	0.004310	0.004310	63.78	62.666
FG,FH	23	0.003172	0.003172	46.94	12.210
GG, GH, HH	33	0.000584	0.000584	8.64	68.726

N	DF	Chi-Sq	P-Value
14798	22	1241.15	0.000

WARNING: 1 cell(s) (4.35%) with expected value(s) less than 1. Chi-Square approximation

probably invalid.

1 cell(s) (4.35%) with expected value(s) less than 5.

Chi-Square Goodness-of-Fit Test for Observed Counts in Variable: count – merino only

Using category names in genotype

Category	Observed	Historical Counts	Test Proportion	Expected	Contribution to Chi-Sq
AA	785	0.136491	0.136491	749.607	1.6711
AB	163	0.031819	0.031819	174.748	0.7898
AC	947	0.167300	0.167300	918.813	0.8647
AE	440	0.105950	0.105950	581.878	34.5939
AF,AG	761	0.128822	0.128822	707.490	4.0472
AH	34	0.003565	0.003565	19.581	10.6185
BB	18	0.001854	0.001854	10.184	5.9979
BC	113	0.019501	0.019501	107.097	0.3254
BE	70	0.012350	0.012350	67.824	0.0698
BF,BG,BH	73	0.015431	0.015431	84.747	1.6284
CC	296	0.051266	0.051266	281.554	0.7412
CE	380	0.064933	0.064933	356.612	1.5339
CF,CG,CH	367	0.081135	0.081135	445.595	13.8628
DD	26	0.001483	0.001483	8.145	39.1409
Other D genotypes	371	0.074055	0.074055	406.710	3.1354
EE	193	0.020561	0.020561	112.920	56.7908
EF,EG,EH	216	0.051382	0.051382	282.192	15.5264
FF,FG,FH,GG,GH,HH	239	0.032102	0.032102	176.303	22.2964

N	DF	Chi-Sq	P-Value
5492	17	213.634	0.000

Chi-Square Goodness-of-Fit Test for Observed Counts in Variable: count – merino only adjusted

Using category names in Genotype

Category	Observed	Historical Counts	Test Proportion	Expected	Contribution to Chi-Sq
AA	124	0.075404	0.075404	108.055	2.3530
AB	46	0.026828	0.026828	38.444	1.4852
AC	236	0.147360	0.147360	211.166	2.9205
AE	182	0.146593	0.146593	210.068	3.7503
AF,AG	68	0.074159	0.074159	106.270	13.7816
BB	8	0.002386	0.002386	3.419	6.1361
BC	37	0.026214	0.026214	37.565	0.0085
BE	19	0.026078	0.026078	37.369	9.0295
BF,BG	21	0.013192	0.013192	18.904	0.2323
CC	103	0.071995	0.071995	103.168	0.0003
CE	206	0.143240	0.143240	205.263	0.0026
CF,CG	76	0.072463	0.072463	103.839	7.4636
AD,BD,CD,ED,DF,DG	18	0.012522	0.012522	17.943	0.0002
EE	123	0.071248	0.071248	102.098	4.2792
EF,EG	110	0.072086	0.072086	103.299	0.4347
FF,FG,FH,GG	56	0.018233	0.018233	26.129	34.1504

N	DF	Chi-Sq	P-Value
1433	15	86.0281	0.000

1 cell(s) (6.25%) with expected value(s) less than 5.

Appendix IV – Pearson chi squared results

Crosstabs

EweDany * Dry

Crosstab

			Dry		
			N	Y	Total
EweDany	0	Count	259	7	266
		% within EweDany	97.4%	2.6%	100.0%
	1	Count	133	6	139
		% within EweDany	95.7%	4.3%	100.0%
Total		Count	392	13	405
		% within EweDany	96.8%	3.2%	100.0%

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.834[a]	1	.361		
Continuity Correction[b]	.380	1	.538		
Likelihood Ratio	.800	1	.371		
Fisher's Exact Test				.383	.263
N of Valid Cases	405				

a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 4.46.
b. Computed only for a 2x2 table

RamD * Dry

Crosstab

			Dry		Total
			N	Y	
RamD	0	Count	153	6	159
		% within RamD	96.2%	3.8%	100.0%
	1	Count	239	7	246
		% within RamD	97.2%	2.8%	100.0%
Total		Count	392	13	405
		% within RamD	96.8%	3.2%	100.0%

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.268[a]	1	.605		
Continuity Correction[b]	.052	1	.819		
Likelihood Ratio	.263	1	.608		
Fisher's Exact Test				.774	.403
N of Valid Cases	405				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 5.10.

b. Computed only for a 2x2 table

sire * Dry

Crosstab

			Dry		Total
			N	Y	
sire	CARL 200/15	Count	40	1	41
		% within sire	97.6%	2.4%	100.0%
	CLAY 264/14	Count	37	3	40
		% within sire	92.5%	7.5%	100.0%
	NEIL 381/14	Count	39	2	41
		% within sire	95.1%	4.9%	100.0%
	NEL 61/15	Count	65	1	66
		% within sire	98.5%	1.5%	100.0%
	NEL 61/15 or WANG 7/15	Count	1	0	1
		% within sire	100.0%	0.0%	100.0%
	NEL 63/15	Count	41	0	41
		% within sire	100.0%	0.0%	100.0%
	NEL 83/15	Count	42	0	42
		% within sire	100.0%	0.0%	100.0%
	ROD 290/13	Count	35	1	36
		% within sire	97.2%	2.8%	100.0%
	WANG 5/15	Count	40	1	41
		% within sire	97.6%	2.4%	100.0%
	WANG 7/15	Count	52	4	56
		% within sire	92.9%	7.1%	100.0%
Total		Count	392	13	405
		% within sire	96.8%	3.2%	100.0%

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	9.099[a]	9	.428
Likelihood Ratio	10.569	9	.306
N of Valid Cases	405		

a. 11 cells (55.0%) have expected count less than 5. The minimum expected count is .03.

EweDany*twin
Crosstab

			twin		
			N	Y	Total
EweD	0	Count	197	69	266
		% within EweD	74.1%	25.9%	100.0%
	1	Count	103	29	132
		% within EweD	78.0%	22.0%	100.0%
	2	Count	5	2	7
		% within EweD	71.4%	28.6%	100.0%
Total		Count	305	100	405
		% within EweD	75.3%	24.7%	100.0%

Chi-Square Tests

	Value	df	p-value
Pearson Chi-Square	.805[a]	2	0.668

EweDany * twin

Crosstab

			twin		
			N	Y	Total
EweDany	0	Count	197	69	266
		% within EweDany	74.1%	25.9%	100.0%
	1	Count	108	31	139
		% within EweDany	77.7%	22.3%	100.0%
Total		Count	305	100	405
		% within EweDany	75.3%	24.7%	100.0%

Chi-Square Tests

	Value	df	p-value	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.650[a]	1	0.420		
N of Valid Cases	405				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 34.32.

b. Computed only for a 2x2 table

RamD * twin

Crosstab

			twin		
			N	Y	Total
RamD	0	Count	107	52	159
		% within RamD	67.3%	32.7%	100.0%
	1	Count	198	48	246
		% within RamD	80.5%	19.5%	100.0%
Total		Count	305	100	405
		% within RamD	75.3%	24.7%	100.0%

Chi-Square Tests

	Value	df	p-value	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	9.039[a]	1	0.003		
N of Valid Cases	405				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 39.26.

b. Computed only for a 2x2 table

Crosstabs

sire * twin Crosstabulation

			twin		
			N	Y	Total
sire	CARL 200/15	Count	32	9	41
		% within sire	78.0%	22.0%	100.0%
	CLAY 264/14	Count	30	10	40
		% within sire	75.0%	25.0%	100.0%
	NEIL 381/14	Count	27	14	41
		% within sire	65.9%	34.1%	100.0%
	NEL 61/15	Count	55	12	67
		% within sire	82.1%	17.9%	100.0%
	NEL 63/15	Count	30	11	41
		% within sire	73.2%	26.8%	100.0%
	NEL 83/15	Count	31	11	42
		% within sire	73.8%	26.2%	100.0%
	ROD 290/13	Count	19	17	36
		% within sire	52.8%	47.2%	100.0%
	WANG 5/15	Count	29	12	41
		% within sire	70.7%	29.3%	100.0%
	WANG 7/15	Count	52	4	56
		% within sire	92.9%	7.1%	100.0%
Total		Count	305	100	405
		% within sire	75.3%	24.7%	100.0%

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	23.511[a]	8	0.003

Crosstabs

RamD = 0

sire * twin Crosstabulation[a]

			twin		
			N	Y	Total
sire	CLAY 264/14	Count	30	10	40
		% within sire	75.0%	25.0%	100.0%
	NEIL 381/14	Count	27	14	41
		% within sire	65.9%	34.1%	100.0%
	NEL 83/15	Count	31	11	42
		% within sire	73.8%	26.2%	100.0%
	ROD 290/13	Count	19	17	36
		% within sire	52.8%	47.2%	100.0%
Total		Count	107	52	159
		% within sire	67.3%	32.7%	100.0%

a. RamD = 0

Chi-Square Tests[a]

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	5.375[b]	3	0.146

RamD = 1

sire * twin Crosstabulation[a]

			twin		
			N	Y	Total
sire	CARL 200/15	Count	32	9	41
		% within sire	78.0%	22.0%	100.0%
	NEL 61/15	Count	55	12	67
		% within sire	82.1%	17.9%	100.0%
	NEL 63/15	Count	30	11	41
		% within sire	73.2%	26.8%	100.0%
	WANG 5/15	Count	29	12	41
		% within sire	70.7%	29.3%	100.0%
	WANG 7/15	Count	52	4	56
		% within sire	92.9%	7.1%	100.0%
Total		Count	198	48	246
		% within sire	80.5%	19.5%	100.0%

a. RamD = 1

Chi-Square Tests[a]

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	9.603[b]	4	0.048

Appendix V – DNA preparation method

DNA preparation:

To extract DNA by the NaOH method, an aliquot of 200µL of 20mM NaOH solution was added to the tube containing the blood disk and it incubated for 30 min at room temperature. The tube was inverted occasionally during incubation. The solution was then discarded, and the disk was washed in 200µL TE-1 buffer (10mM Tris-HCl, 0.1mM EDTA, pH 8.0) for 5 min. After the removal of the TE-1, the disk was air-dried.

Genotyping using PCR-SSCP:

Source and description of primers :
Primers designed from the ovine ADRB3 sequence (Forrest & Hickford 2000; GenBank accession no. AF109928).

(Primers refer to the attached paper)

Detection of sequence variation (polymorphism)

Polymerase chain reaction–single strand conformational polymorphism (PCR–SSCP) was used to screen for polymorphism at the ovine ADRB3 locus. Amplifications were performed in a 15 μl reaction containing the purified genomic DNA on one punch of the FTA card, 0.25 μM of each primer, 150 μM of each dNTP (Bioline, London, UK), 1.5 mM of Mg^{2+}, 0.3 U of Taq DNA polymerase (Qiagen, Hilden, Germany) and 1 × reaction buffer supplied with the enzyme. The thermal profile consisted of 2 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 61 °C and 30 s at 72 °C, with a final extension of 5 min at 72 °C. Amplification was carried out in S1000 thermal cyclers (Bio-Rad, Hercules, CA, USA).

The amplicons produced were then subjected to SSCP analysis. A 0.7 μl aliquot of each amplicon was mixed with 7 μl of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol). After denaturation at 95 °C for 5 min, the samples were cooled rapidly on wet ice and loaded onto 16 cm × 18 cm, 14% acrylamide:bisacrylamide (37.5:1) (Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad) at 200v for 19 h in a 25°C room with 26 °C water circulating through the cell core. The gels were silver-stained by the method of Byun et al. [1].