Swedish University of Agricultural Sciences
Faculty of Veterinary Medicine and Animal Science

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PHENOTYPIC EFFECTS OF A FERTILITY MUTATION IN NORWEGIAN WHITE SHEEP

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Abstract

Norwegian White Sheep (NWS) is a synthetic breed that results from crosses between local Norwegian and foreign breeds. This breed is mainly kept for the meat production, and litter size is therefore a trait of large economic value. A mutation in the ovine *GDF9* gene (c.1111G>A) on chromosome 5 was recently found to be associated with increased litter size in daughters of AI rams of Norwegian White Sheep (NWS). This thesis aims at estimating the phenotypic effect of the (c.1111G>A) mutation in nearly 900 NWS-ewes'. Since litter size can only be directly observed at females, genotyping of the mother is considered to give a better phenotypic estimate of the allelic effect compared to estimates based on the EBVs of the rams. The information available for these ewes was number of lambs born at age 1 year and 2 year of age. A total of 853 NWS ewes were genotyped for the (GDF9 c.111G>A) mutation by the iPLEX Gold technology (SEQUENOM). The genotyping success rate was more than 90%. The average litter size for ewes at 1 and 2 year of age was 2.061±0.73 and 2.671±0.91, respectively, showing that the average litter size was lower at 1 year compared to those at 2 years. The phenotypic effect of being homozygous for the mutant allele at age 1 and 2 year was found to be 0.54 and 0.87 additional lamb per litter, respectively. The frequency of the c.1111A allele was 0.38 in NWS ewe population. As the experimental ewes were heavily selected for large litter size and strong association between c.1111A allele and litter size is known, it can be assumed that the selection has increased the frequency among these ewes. Handling of c.1111A allele in NWS will significantly influence the future litter size in this population. The Norwegian Association of Sheep and Goat Breeders will design how to exploit the rams carrying the c.1111A allele in Norwegian White Sheep breeding program.

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Abbreviations

AE buffer	Adams-Evans buffer
AI	Artificial insemination
BCO2	Beta-carotene oxygenase 2
BMP15	Bone morphogenic protein 15
BMPR1B	Bone morphogenetic protein 1B receptor
DNA	Deoxyribonucleic acid
EBV	Estimated breeding value
EDTA	Ethylenediaminetetraacetic acid
GDF9	Growth differentiation actor 9
GWAS	Genome wide association study
LS	Litter size
MALDI-TOF	Matrix assisted laser desorption/ ionization-time of flight
MC1R	Melanocortin 1 receptor
MS	Mass spectrometry
MSTN	Myoststin
NWS	Norwegian White Sheep
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait loci
rpm	Rotations per minute
SAP	Shrimp Alkaline Phosphatase
SNP	Single nucleotide polymorphism
NSG	Norwegian Sheep and Goat Association

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1. Introduction

1.1 Fertility in sheep

Fertility is the ability of an individual to produce live offspring and also the birth rate of a population refers to fertility (Frank, 2012; www.biology-online.org/dictionary/Fertility). In sheep the number of lambs born by a ewe or her litter size (LS) can be considered as fertility. Fertility is controlled by genetics and environmental factors (i.e. nutrition, season, herd, farm system etc.) Litter size is a complex trait with very low repeatability (Nichol et al., 2009) and the heritability is less than 10% (Notter, 2008). Number of lambs born is the ewe trait that is important in sheep breeding program (Steinheim et al., 2008). Litter size is an important economic trait in sheep production (Javanmard et al., 2011), mainly due to high economic value of meat (D'Arcy, 1990; Abdulkhaliq et al., 1989; Nitter, 1985; Well Gully, 2013). Increasing multiple births in sheep might improve meat production per ewe (Rajab et al., 1992).

1.2 Fertility in Norwegian White Sheep (NWS)

Norwegian White Sheep (NWS) is a synthetic breed resulting from cross-breeding between Norwegian and foreign breeds like Finnish Landrace (to improve fertility) and with East Friesian Dairy Sheep (to increase milking capacity) into 1970s and 1990s, respectively (Ådnøy, 1988; Larsgard and Standal, 1999). Since 2000-2001, this sheep breed has been considered as Norway's own breed and it is grouped among long-tailed breeds. This breed is now found throughout Norway and is used for meat and wool production. NWS is slightly smaller in size and the fertility is higher compared to the original Dala sheep (NORDGEN, 2013). The average number of lambs born alive (NLBA) was reported to be 2.06 in 2007 (Steinheim et al., 2008) and mean litter size (LS) of NWS at 1 year and 2 years of age was reported as be1.63 and 2.10, respectively (Eikje, 2008).

In Norwegian Sheep Breeding Scheme, litter size has been considered as one of the major traits with high economic value (about 12% of the National Sheep Breeding goal) (NGS, 2013). Selective breeding for this trait has been successful, and the number of lambs born or litter size (LS) in NWS has been increasing over the years.

1.3 Norwegian Sheep Breeding

For the genetic improvement of Norwegian sheep, the Norwegian Sheep and Goat Breeder's Association established the Norwegian Sheep Breeding Scheme in the 1960s. In early 1990s Norwegian sheep breeding had developed and merged into a single NWS breeding group. The Norwegian Sheep and Goat Breeder's Association (NSG) is taking care of the NWS recording

system and breeding program. The breeding stocks are kept in several small “ram-circles”, (breeding co-operative groups of 10-30 farmers) where the young rams are selected and progeny-tested. During the mating season, the tested rams are moved among different flocks. The ram lambs are selected based on their pedigree information and own performance; the ewes are selected following the selection index (for litter size, fleece weight, maternal effect on weaning weight, carcass weight and so on). By selection indices rams can also be selected elite rams for mating and some of the most promising (based on first year results) rams can be selected for artificial insemination (AI), to increase the genetic gain (Eikje et al., 2008). The semen is collected for long time storage from two AI-stations in Norway (Valdez-Nava, 2011).

1.4 Known genes for fertility in sheep and their functions

A large body of evidence supported that the oocyte plays a central role in regulating of ovarian follicle growth and development (Matzuk et al., 2002; Gilchrist et al., 2008; Su et al., 2009; Otsuka et al., 2011). In particular, the proteins secreted from oocytes play a vital role in ovulation rate with the follicular growth regulation (Juengel and McNatty, 2005; Moore et al., 2003). Booroola was the first gene to be identified as a major gene for prolificacy in sheep (Piper et al., 1985).

Litter size in sheep was recorded among different breeds and within breeds (Montgomery et al., 1992). Most of the domestic sheep breeds have one or two lambs per lambing, whereas only few breeds like the Booroola, Merino, Cambridge, D'Man, Finnish Landrace, and Romanov were proved more prolific (three or more lambs per ewe lambing) (Montgomery et al., 1992). From the genetic studies of prolificacy in sheep, it has been shown that litter size and ovulation rate can be influenced by segregation of a single gene (Piper and Bindon, 1982; Davis et al., 1982) and mutations in these genes have been associated with increased litter size and ovulation rate (Bindon et al., 1996).

Members of the transforming growth factor receptor beta (TGFβ) super family *BMP15*, *BMPR1*and *GDF9* genes has been shown to play important roles in regulating fertility in sheep (Knight and Glister, 2001; Chang et al., 2002; Durlinger et al., 2002). *BMP15*, *BMPR1*and *GDF9* genes are described below:

1.4.1 *BMPR1B*

Bone morphogenetic protein 1B receptor (*BMPR-1B*) gene or the *Booroola* gene (*FecB*) is situated on sheep chromosome 6 and is expressed in oocytes and granulosa cells (Piper et al., 1985; Wilson et al., 2001; Souza et al. 2001). *BMPR1B* was identified as the first major gene associated with prolificacy in sheep in 1980 (Davis et al., 1982). Mutations in this gene are associated with increased ovulation rate and litter size and have large effect on prolificacy in

ewes. In Merino Booroola sheep the Booroola variant ($FecB^B$) was found in the *BMPR1B* gene (Mulsant et al., 2001; Souza et al., 2001; Wilson et al., 2001) (Table 1.1).

1.4.2 *BMP15*

Bone morphogenetic protein 15 (BMP-15) is a growth factor and a member of the TGF*β* superfamily, expressed in oocytes during follicular development (Hanrahan et al., 2004; Galloway et al., 2000). The ovine *BMP-15* gene is situated on chromosome X. BMP15 is produced as a precursor protein that consists of 393 amino acids. This consists of a short signal peptide (1-25) followed by a propeptide (26-268) and finally the mature region of the protein (269-393) (Bodensteiner et al., 1999; Galloway et al., 2000).

In sheep, BMP15 is essential for activation of primordial follicle and subsequently in all stages of normal follicular development, maturation and ovulation (Bodensteiner et al., 1999; Eppig, 2001; Juengel et al., 2004; Mandon-Pepin et al., 2003). Mutations in the *BMP-15* gene have been found associated with increased ovulation rate and fertility (Hanrahan et al., 2004). Polymorphism in the *BMP15* gene was found to be associated with increased ovulation rate in Inverdale ($FecX^I$) and Hanna ($FecX^H$) sheep (Galloway et al., 2000) (Table 1.1).

1.4.3 Ovine *GDF9* gene

The ovine *GDF9* gene was mapped to ovine chromosome number 5, between the markers BM7247 and BMS2258 on the framework map (Figure 1.1) by linkage analysis (Maddox et al., 2001).

Ovine *GDF9* gene is 2.5 kb long, consists of two exons and one intron. Exon 1 and exon 2 is 397 bp and 968 bp, respectively and encodes for amino acids1–134 and 135–456, respectively (Hanrahan et al., 2004). Figure 1.2 showing the *GDF9* mRNA below. The *GDF9* mRNA is translated into a preprotein (Figure 1.3) having a signal peptide (27 aa), a propeptide (291 aa) and a small mature chain (135 aa) (McNatty et al., 2004).

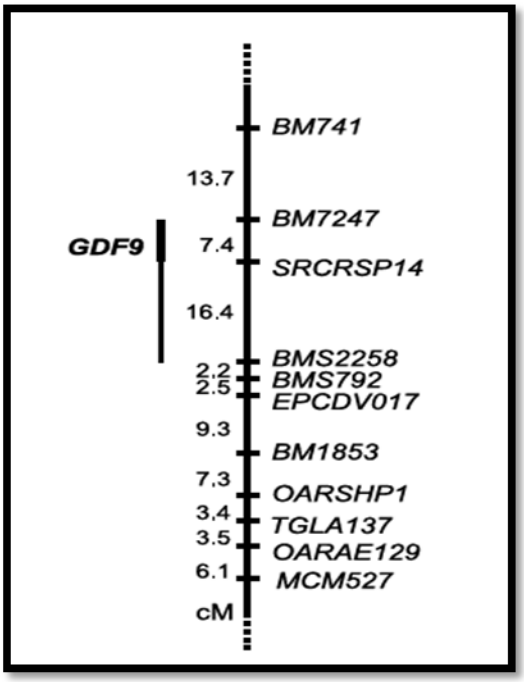

Figure 1.1 Linkage map position of *GDF9* in the central portion of sheep chromosome 5 (Maddox et al., 2001).

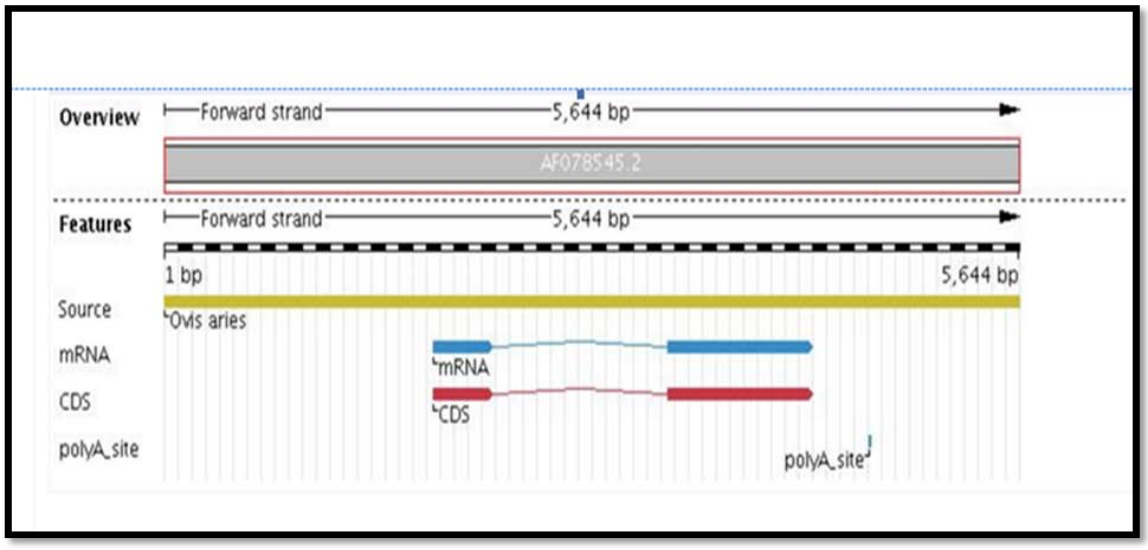

Figure 1.2: *GDF9* mRNA (EMBL-EBI)

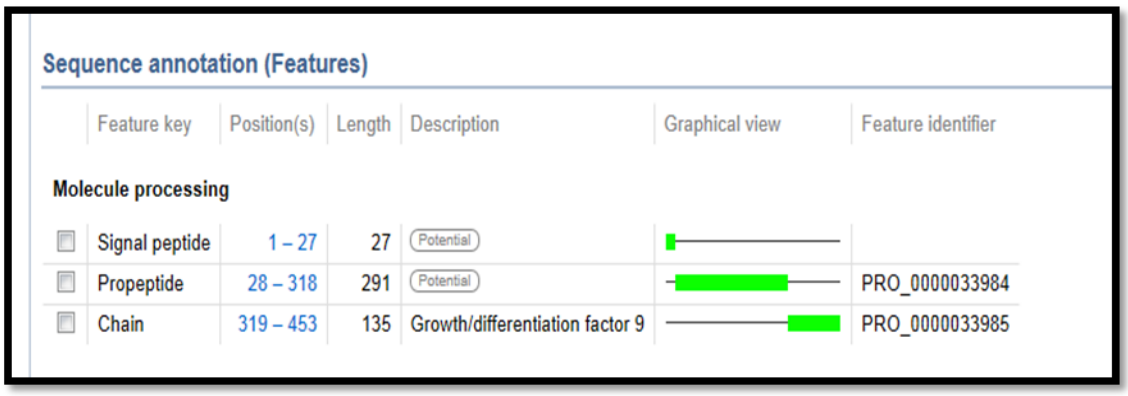

Figure 1.3: GDF9 amino acid sequence (SWISS-PROT; UNIPROT)

1.4.4 Probable functions of *GDF9* gene

GDF9 was the first gene found to be essential for follicular development in sheep (Dong et al., 1996). The protein encoded by the *GDF9* gene is involved in cumulus expansion, oocyte maturation, ovulation (Elvin et al., 1999, 2000), cumulus and granulosa cell proliferation (Hayashi et al., 1999). The changes in the concentration of *GDF9 in vivo* also exhibited increased ovulation rate in sheep (Galloway et al., 2000). *GDF9* was the latest gene found to be strongly association with prolificacy in Cambridge and F700-Belclare sheep, animals heterozygous for the $FecG^{H}$ mutation were fertile while homozygous animals were sterile. The same phenomenon (sterile homozygous animals) was also observed for the *BMP15* mutations (Hanrahan et al., 2004) (Table 1.1).

Table 1.1 Mutation in different fertility genes in sheep with their affect

Gene	Variant	Breed	Base change	Coding base position	Amino acid change	Codingamino acid position	Mature peptide position	Phenotype		Ref.
								Heterozyg.	Homozyg.	
GDF9	$FecG^{H}$	Bel./Cam.	C>T	1184	Ser(S)→Phe(F)	395	77	IOR	Sterile	1
	FecTT	Thoka	A>C	1279	Ser(S)→Arg(R)	427	109	IF	Sterile	2
	$FecG^{E}$	Santa Inês	T>G	1034	Phe((F)→Cys(C)	345	27	IOR	IOR	3
BMP15	$FecX^{H}$	Hanna	C>T	871	Gln(Q)→Stop	291	23	IOR	Sterile	4
	$FecX^{I}$	Inverdale	T>A	896	Val(V)→Asp(D)	299	31	IOR	Sterile	4
	$FecX^{G}$	Bel./Cam.	C>T	718	Gln(Q)→Stop	239	-	IOR	Sterile	1
	$FecX^{B}$	Bel./Cam.	G>T	1100	Ser(S)→Ile(I)	367	99	IOR	Sterile	1
	$FecX^{L}$	Lacaune	G>A	962	Cys(C)→Tyr(Y)	321	53	IOR	Sterile	5
	$FecX^{R}$	R.aragonesa	deletion	525_541		-	-	IF	Sterile	6,7
BMPR1B	$FecB^{B}$	Booroola	A>G	746	Gln(Q)→Arg(R)	249	-	IOR	IOR	8,9,10

IOR=Increased ovulation rate, IF=Increased fertility

1) Hanrahan et al., (2004) Biol. Reprod , 70(4):900-909.
2) Nicol et al., (2009) Reproduction, 138(6):921-933.
3) Silva et al., (2011) Anim. Genet, 42(1):89-92.
4) Galloway et al., (2000) Nat. Genet, 25(3):279-283.
5) Bodin et al., (2007) Endocrinology, 148(1):393-400.
6) Martinez-Royo et al., (2008) Anim. Genet, 39(3):294-297.
7) Monteagudo et al., (2009) Anim. Reprod. Sci 2009, 110(1-2):139-146.
8) Mulsant et al., (2001) Proc. Natl. Acad. Sci. USA 2001, 98(9):5104-5109.
9) Souza et al., (2001) J. Endocrinol, 169(2):R1-6.
10) Wilson et al., (2001) Biol. Reprod, 64(4):1225-1235

1.5 Mutations recently found in the *GDF9* gene

In the Santa Inês (SI) sheep, a *GDF9* allele ($FecG^{E}$) was recently shown to result in increased ovulation rate and prolificacy, also in ewes homozygous for this mutation. This mutation caused an amino acid substitution from phenylalanine to cysteine at position 345 (F345C) (Silva et al., 2011).

In NWS, a Genome-Wide Association study for fertility was performed by using the Illumina 50K SNP array. AI rams progeny tested for the phenotypic trait “number of lambs born” were genotyped. SNPs located close to the growth and differentiation factor-9 (*GDF9*) gene at chromosome 5 showed strong association to this trait. Sequencing of the *GDF9* gene revealed a *GDF9* c.1111G>A mutation (Våge et al., 2013).

The c.1111G>A polymorphism caused an amino acid change in the mature region (the bioactive part) of the GDF9 protein. In this case, the nonpolar amino acid (Valine) is substituted by another nonpolar amino acid (Methionine), which is considered to be a conservative change in biochemical terms. Valine was also found in the same position in GDF9 protein in 6 totally different mammalian species (sheep, cattle, pig, cat, human and mouse).

1.6 SNP genotyping by MassArray platform method with iPLEX assay

iPLEX assay involves a locus-specific PCR reaction, followed by single base primer extension reaction with mass-modified dideoxynucleotide terminators of an oligonucleotide primer which anneals immediately upstream of the polymorphic site of interest. Based on matrix assisted laser desorption/ ionization-time of flight (MALDI-TOF) mass spectrometry (MS), the distinct mass of the extended primer identifies the SNP allele for multiplexed genotyping (ACRF Facility, 2013; Gabriel et al., 2009; Boman, 2009; Oeth et al., 2006).

According to the iPLEX® Gold Application Guide (SEQUENOME®, www.sequenome.com) SNP genotyping by MassARRAY include the following steps: *i.* DNA isolation and quantification *ii.* DNA amplification iii. Preparation of iPLEX Gold reaction products *iv.* Transfer of iPLEX Gold reaction products to SpectroCHIP® Arrays *v.* Defining the setup of assays and plates in the MassARRAY database *vi.* Acquisition of spectra using mass spectrometer *vii.* Analysis of spectral data. Different steps (Figure 1.4) in MassARRAY platform are shortly described below.

To detect single nucleotide polymorphism (SNP) the isolated DNA should be of high purity. Genomic DNA is used to amplify the surrounding areas of the genetic variation (s) of interest. The genomic DNA should be amplified using PCR method. The PCR conditions for the iPLEX assay have been optimized for amplification of multiplexed reactions (upto 40 plexes). The SAP (Shrimp alkaline phosphatase) enzyme treatment is applied to neutralize unincorporated dNTPs in the PCR amplicons and convert them into dNDPs by cleaving. The conditioning or cleaning of extension products with Clean Resin is very important to optimize the mass spectrometry analysis of the iPLEX Gold Reaction products (Oeth et al., 2006). By using MALDI-TOF mass spectrometry the mass of the extended primer in the spectrum acquired thereby identifying the sequence and alleles at the polymorphic site of interest.

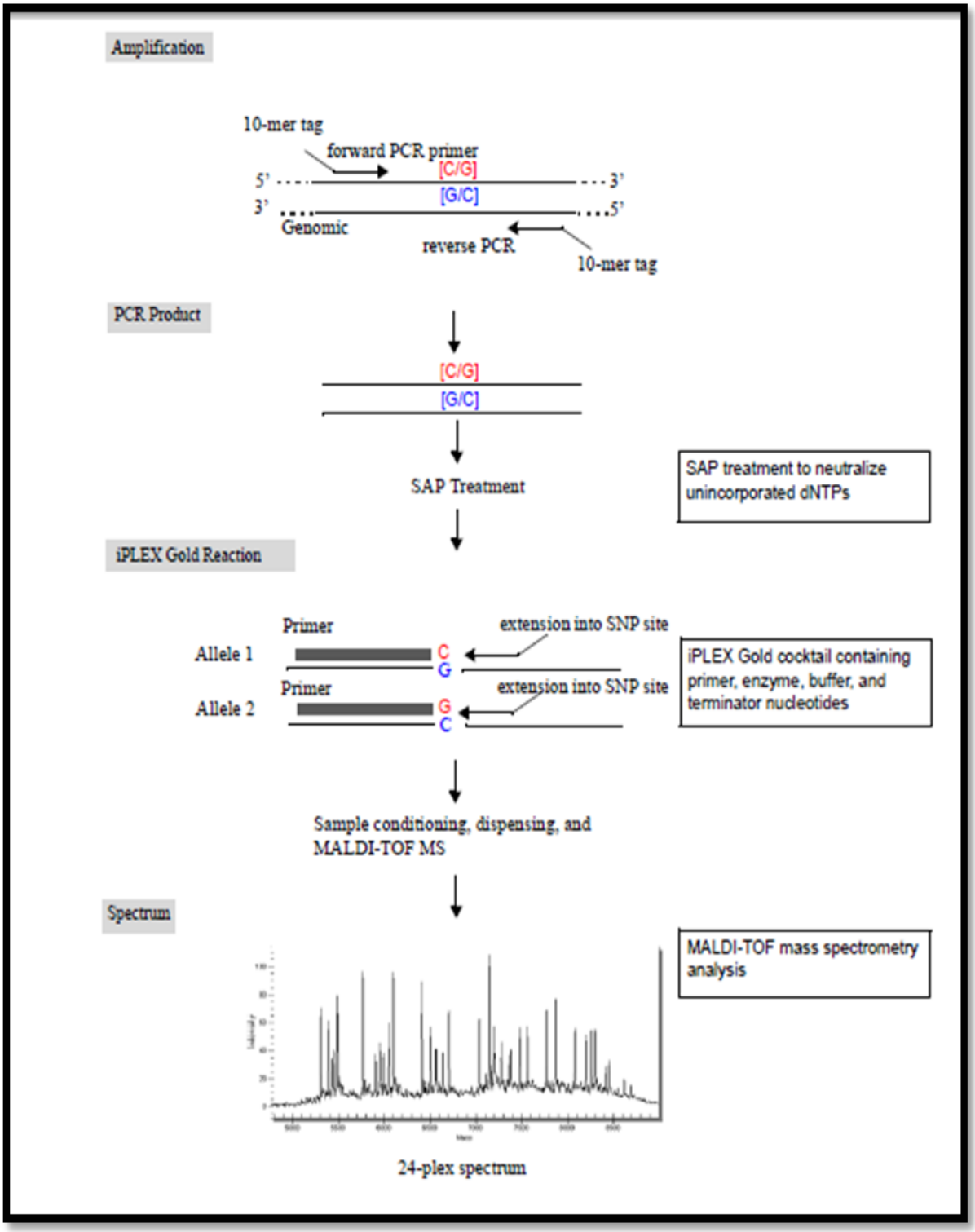

Figure 1.4: iPLEX Assay (The scheme depicts a single assay) (Gabriel et al., 2009; Oeth et al., 2006)

1.7 Aim of the Study

The EBVs for number of lambs born were estimated for the AI rams based on their daughters' performance. According to the EBVs, daughters of AI rams being homozygous for c.1111A gave birth to 0.46 - 0.57 additional lambs compared to daughters of c.1111G homozygous wild rams. When the frequency of the c.1111G>A allele in the ewe population is low, only the rams will contribute this allele, while the phenotypic effect will be "shared" by the dams when calculating EBVs, and the allele effect will therefore be underestimated. Since only females are showing this trait, it was decided to genotype a larger number of ewes to observe the direct allelic effect of c.1111G>A on the numbers of lambs born. Moreover, we wanted to verify that homozygous ewes are fertile as indicated in the report of Hanrahan et al., 2004, investigating Cambridge and Belclare sheep.

The aim of this thesis is to estimate the phenotypic effect of the (c.1111G>A) mutation in nearly 900 NWS-ewes. The information available for these ewes is number of lambs born at age 1 year, and for a subset of this population, also the number of lambs born at age 2 year is available.

2 Materials and Methods

This chapter describes the materials and methods used in this thesis. An introduction to different steps of method; composition of master mix, reagents used and the settings for specific PCR reactions are given bellow:

2.1 Animals

Only flocks (farms) participating in a NWS ram circle were considered for inclusion in this study (1036 flocks). The flocks were ranked according to flock-mean breeding values (best linear unbiased prediction (BLUP-solutions)) for litter size at age 1 and age 2 year (two separate rankings). The flocks were also ranked according to flock average phenotypic litter size at age 1 and age 2 year (two separate rankings). The flocks that ranked in the best third (345) for all four traits were invited to participate. After a second ranking among those that were willing to participate, 15 different farms contributed in total 884 individual blood samples to this study. The ewes were born in 2010 and 2011. The blood samples were collected by local veterinarians. About 5 ml blood were collected from the jugular vein of each experimental ewe in vacutainers containing EDTA as anticoagulant.

2.2 SNP Genotyping by iPLEX MassARRY MALDI-TOF MS method

2.2.1 DNA isolation

Genomic DNA was extracted from 884 blood samples using DNeasy®96 Blood and Tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. 100 µl blood sample was transferred into each microtube of 96-well plate from the EDTA mixed vacutainer. 96-Well-Register was used to maintain the identification of each sample. In each 96-well plate one well was filled with dionized water as "negative control", while other 95 wells were filled with blood. 20 µl enzyme (proteinase K) and 100 µl of phosphate buffered saline (PBS) were added to each collection microtube. Then, 200 µl of cell lysis solution (Buffer AL) (without ethanol) was added to each microtube in the plate and the microtubes were sealed with caps; each plate was covered with clear cover, shacked vigorously up and down for 15 seconds and centrifuged at 3000 rpm for 1-2 minute in the SIGMA 4-15 C Centrifuge machine (LABEX, France). The plate was then incubated at 56˚ C for 10 minutes in Termaks incubator (Termaks, Norway). The caps and cover were remove carefully and 200 µl of ethanol (96-100%) was added to each blood samples, microtubes were sealed with caps and covered with a clear cover, shacked up and down vigorously for 15 seconds and centrifuged again for about 1-2 minutes at 3000 rpm. Each DNeasy 96 plate was placed on top of an S-Block. The caps and cover were removed and the total lysis mixture (around 620 µl) from each microtube of a 96-well plate was carefully transferred to well of each DNeasy 96 plate. Each DNeasy 96 plate was sealed with a AirPore Tape Sheet and centrifuged at 6000 rpm for 4 minutes. Plates were centrifuged several times at same speed until the lytate mixture completely passed through the membrane in each well of the

DNeasy 96 plates. 500 µl of first washing buffer (AW1) was added to each well and was ensured that ethanol was added according to the manufacturer's instruction; each DNeasy 96 plate was sealed with AirPore Tape Sheet and centrifuged at 6000 rpm for 2 minutes. 500 µl of second washing buffer (AW2) was added to each sample and centrifuged for 15 minutes at 6000 rpm without sealing the DNeasy 96 plate. In elution step the DNeasy 96 plate was placed to a new rack of elution Microtubes RS. Total of 100 µl elution buffer (AE) was added to each sample by adding of 50 µl AE buffer, kept in room temperature for 1 minute and centrifuge at 6000 rpm for 4 min (twice). During each step of transfer of sample, buffer and so on multichannel pipettes were used. The final isolated DNA samples were stored in refrigerator at (-20) ˚C until the next step starts. Reagents used for DNA extraction from blood samples are given in Table 2.1.

Table 2.1 Reagents used in DNA extraction from blood in ewe

Reagents	Volume (µl) per well
Proteinase K enzyme (lytate)	20
PBS buffer	100
AL buffer (without ethanol)	200
Ethanol (96-100%)	200
AW1 buffer (washing buffer)	500
AW1 buffer (washing buffer)	500
AE buffer (elution buffer)	100
Blood sample	100

2.2.2 DNA quantification

The concentration and purity of isolated DNA was measured using NanoDrop 8000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA) by Absorbance method. About 1.5 µl isolated DNA was used for measuring the concentration and purity in each spectrophotometer well. The absorbance reading was recorded at 260 (A_{260}) and 280 (A_{280}) nm. Then the DNA concentration (ng/µl) and DNA purity ($A_{260/280}$) was calculated. Good quality DNA should have $A_{260/280}$ ratio of 1.8-2.2.

2.2.3 DNA amplification

2.2.3.1 Primer design

For genotyping the GDF9 c.1111G>A SNP the Sequenom MassARRAY Workstation 4.O was used. The MassARRAY®Design software (www.sequenome.com) was used for designing both PCR primers and Mass EXTEND® primers for the multiplexed assay. Mass EXTEND® is a primer extension process designed to detect sequence differences at the single nucleotide level. A10-mer tag (5′-ACGTTGGATG-3′) was added on the 5′- end of each PCR primer for allowing greater flexibility in their masses. Table 2.2 shows the sequences of forward, reverse and extension primer pairs for c1111G>A SNP in ovine *GDF9* gene in ewes at chromosome 5. In addition, 5 SNPs routinely tested in NWS AI rams were included in the same multiplex (multiplex 6). These are MSTN_c.120insA, MSTN_c.969delG, MSTN_c*1232G>A, BCO2_c.196C>T, MC1R_c361G>A. The primers of the above mentioned SNPs are shown in Table 2.2.

Table 2.2 Primers for GDF9_c1111G>A

SNP	Primer sequences		
	Forward	Reverse	Extension
MSTN_c120insA	ACGTTGGATGCGAGC AGAAGGAAAATGTGG	ACGTTGGATGTATGGCTTCT AGTCTTGAGG	CTCCACAAGCATGC ATT
MC1R_c361G_A	ACGTTGGATGTGGTA CAGCAGCTGGACAAT	ACGTTGGATGAAGCAGAGG CTGGACACCAT	GCTGCAGATGAGCA CGT
BCO2_c196C_T	ACGTTGGATGTTCTA ACCACGGTGGAAGAG	ACGTTGGATGATAGCCATTG AGCCACTCAG	CGGTGGAAGAGACT CTG
MSTN_c960delG	ACGTTGGATGTAAGG CCAATTACTGCTCTG	ACGTTGGATGTTGCTTGGTG CACAAGATGG	CAAGATGGGTATGA GGATA
MSTN_cS1232G_A	ACGTTGGATGGGTTT ACTGTCATTGTATTC	ACGTTGGATGGTTAAATCAT TTTGGTTTGC	GTCATTGTATTCAAA TCTCAAC
GDF9_c1111G_A	ACGTTGGATGGCTTT AGTCAGCTGAAGTGG	ACGTTGGATGCAGTCCCCTT TACAGTATCG	TGAAGTGGGACAAC TGGATT

2.2.3.2 Primer dilution

Both PCR primers and Extend primers were transferred from stock plates into 96-well Sarstedt plates by Beckman®FX (Beckman Coulture, CA, USA) pipetting robot. The concentration for PCR primers (forward and reverse) and Extend primers (without dilution) was 50 μM each and 500 μM, respectively for the PCR protocols.

2.2.3.3 Polymerase Chain Reaction (PCR)

The cocktail mix for the first PCR in SEQUENOME iPLEX reaction in MassARRAY method was calculated automatically using the "HotStar" PCR-program. Reactions were performed in 384 formats by liquid handling process. PCR master mix was made in Ultraviolet Sterilizing PCR Station using the iPLEX Multiplexed PCR cocktail mix (same multiplexed assay, different DNA) kit protocol (SEQUENOME, San Diego, CA, USA) and the set up for PCR reaction in a 384-well PCR plate was by Beckman®FX (Beckman Coulture, CA, USA) pipetting robot. The plates were the sealed and the "HotStar" program was run in Veriti 384-Well Thermal Cycler (Applied Biosystems, Foster city, CA, USA) in a specific settings. Table 2.3 and Table 2.4 showing the composition of cocktail mix for PCR reaction and PCR cycle, respectively.

Table 2.3: Master mix for PCR to obtain ovine specific c1111G>A amplicon

Reagent	Final Conc.	Volume 1x
dH_2O	NA	0.750
Hotstar Taq PCR buffer (10x) Containing 15 mM $MgCl_2$	1.875 mM $MgCl_2$	0.625
dNTP mix (25 mM)	500 µM each	0.100
Primer mix (500 nM each)	100 nM each	1.000
$MgCl_2$ (25 mM)	1.625 mM	0.325
HotStar Taq (5U/µl)	1 U/rxn	0.200
Total	3.00 µl	
Genomic DNA (5-10 ng/µl)	2.00 µl	2-10ng/rxn
Total	5.00 µl	

Table 2.4: PCR cycle adjusted for the amplification of c1111G>A in ewe

Reactions	Temperature °C	Time	Repeats
Initial denaturation	95	5 min	1 cycle
Denaturation	94	30 sec.	40 cycles
Annealing	56	30 sec.	
Extension	72	1 min	
Final extension	72	3 min	1 cycle
Hold	4	Forever	Final step

2.2.4 Dephosphorylation of dNTPs (SAP Reaction)

SAP enzyme cocktail mix was mixed with the genomic DNA on 384-well micro pipetter following SEQUENOME SAP enzyme cocktail mix protocol by Biomek® FX (Beckman Coulture, CA, USA) robot and was set into Veriti 384-Well Thermal Cycler. No primer was added in this step. The SAP cocktail mix and the thermal cycle are given in Table 2.5 and Table 2.6 below:

Table 2.5: Master mix for SAP enzyme cocktail mix

Reagent	Volume 1x
dH_20	1.53 µl
SAP buffer (10x)	0.17µl
Shrimp alkaline phosphatase (SAP)	0.30 µl
Total	2.00 µl

Table 2.6: **Thermal cycle for SAP program**

Temperature ° C	Time	Repeats
37	40 min	1 cycle
85	5 min	1 cycle
4	forever	Final step

2.2.5 iPLEX Gold Reaction /Extension Reaction

To optimize the extension primers, the masses of primers were divided into low mass group and high mass group and the master mix for iPLEX Gold Reaction cocktail was mixed with the SAP treated PCR products by Biomek® FX pipetting (Beckman Coulture, CA, USA) robot and set for extension PCR reaction into Veriti 384-Well Thermal Cycler. All primers in the high mass group were doubled in concentration with respect to the low mass group. Composition of iPLEX Gold Reaction cocktail (Table 2.7) and the thermal cycles (Table 2.8) are shown below.

Table 2.7: Master mix for iPLEX Gold Reaction cocktail

Reagent	Final Conc.	Volume 1x (µl)
dH20	NA	0.619
iPLEX Plus buffer (10x)	0.222x	0.20
iPLEX termination mix	1x	0.20
Primer mix (7µM, 9µM,11µM,14µM)	-	0.940
iPLEX enzyme	1x	0.041
Total		2.00 µl

Table 2.8: Thermal cycle for iPLEX Gold Reaction

Steps	Temperature° C	Time	Repeats	
Initial denaturation	94	30 sec	1 cycle	
Denaturation)	94	5 sec		40 cycles
Annealing	52	5 sec	5 cycles	
Extension	80	5 sec		
Final extension	72	3 sec	1 cycle	
Hold	4	Forever		

2.2.6 Conditioning the iPLEX Gold Reaction products/ Desalting

6 mg of Clean Resin was added into each well of a 384 dimple plate according to manufacturer's instruction (SEQUENOM, CA, USA) and was kept for 20 min at room temperature. iPLEX Gold Reaction products in 384-microplate was diluted with 16 µl nanopure water in each well using the robot by the "hME_4x_Wash_Water_Add" method and thereafter Resin was added , sealed with cover and was rotated at room temperature for minimum 5 min. Sealed 384-microplate was centrifuged for 5 min. at 1600 rpm. iPLEX Gold Reaction products were ready for dispensing to SpectroCHIP®Array.

2.2.7 Dispensing of iPLEX Gold reaction products to SpectroCHIP® Arrays

To dispense the iPLEX Gold reaction products to a 384-well SpectroCHIP® Array in SEQUONOME MassARRAY™Nanodispenser (SEQUENOME) was used. By MassARRAY

nanodispenser small amount of samples (~15 nl) were transferred onto 384 SpectroCHIP® array from the 384-microplates and for the calibration the calibrant was transferred from calibration well onto the calibrant patches of the 384 chip. This step was done by using SpectroPint program.

2.2.8 Defining the setup of assays and plates in the MassARRAY

After the completion of nanodispension onto the SpectroCHIP®, the assay and plates were designed in the MassARRAY database using Assay Editor 4.0 and Plate Editor 4.0 software.

2.2.9 Acquisition and Analyzing of spectra using mass spectrometer

Genotype calling and results of SpectroCHIP®Array was placed into MALDI-TOF by mass spectrometer and by MassARRAY Analyzer 4. 0 (SEQUENOME) mass correlation genotype was determined in real time. The Call Rate and Extension Rate were calculated by Caller 4.0 software in Microsoft Excel 2010 (Microsoft Corp., www.microsoft.com) and peak areas were calculated by TYPER 4.0 software. The calculated rates and peaks were then transferred to Minitab for analysis. One-way analysis of variance (ANOVA) was conducted using Minitab software (Minitab Inc., www.minitab.com).

2.2.10 Statistical Analysis

Data were sorted as required for the model used. The main issue was to measure the effect of GDF9_c.1111G>A SNP genotypes on two differently correlated fertility traits; number of lambs born at age 1 year (LS1) and age 2 years (LS2) of aged Norwegian White ewes. However, the additional 5 SNPs in the multiplex were also tested to exclude any possible effect on fertility The dataset with genotypes of 6 SNPs was converted from excel to a plain text file for analysis. Traits were adjusted for fixed effects of genotype and flock year. Best fitted bivariate analyses in the linear model in GLM (generalized linear model) procedure were used to estimate the allelic effect on the phenotypes, LS1 and LS2 by SAS (Version 9.2) program (SAS Institute Inc., Cary, NC, USA).
The following model was used for multivariate comparison in litter size among three different genotypes

$$\boldsymbol{Y_{ij} = \mu + G_i + FY_j + e_{ij},}$$

where, $\boldsymbol{Y_{ij}}$ is the phenotypic value of number of lams born ; μ is the population mean; $\boldsymbol{G_i}$ is the fixed effect of the *i*th genotype (GG, GA and AA) ; $\boldsymbol{FY_j}$ is the fixed effect of the *j*th flock year (*j* = 1, 2…..,30) and $\boldsymbol{e_{ij}}$ is the residual effect of each observation. Flock year is the combination of farm number and birth year (2010-2011) of lambs. Least-square means procedure was applied for significant difference test (Cao et al., 2010).

3 Results

This chapter represents the results obtained from the SNP genotyping and statistical analysis. SNP genotyping was conducted using MassARRAY platform method. SAS 9.2 software was used for analyzing descriptive statistics and genotypic effect of GDF9_c.1111G>A on two differently closely linked traits i.e. number of lambs born at 1 year (LS1) and 2 years (LS2) of age of in Norwegian White ewes by GLM (generalized linear model) model. Snap shorts for SAS commands and results are given in Appendix.

3.1 DNA measurement

The concentration of extracted DNA was measured as ng/µl. Ideal range of $A_{260/280}$ ratio purity of DNA is 1.8-2.2. Not all samples had sufficiently high concentration and/or purity results. The variation for the $A_{260/280}$ among the samples was high and the DNA concentration for each sample was not high. The $A_{260/280}$ was not adjusted in this study and the quality of the samples was just routinely measured. The purity and DNA concentration of the samples indicated that they were suitable for the PCR step in SNP genotyping method.

3.2 Genotyping

The success rate of genotyping was more than 90%. Some animals received “no call”, which means that it is not possible to obtain a reliable genotype. The BCO2_c.196C>T SNP’s genotype quality was generally bad, and was deleted from the dataset. The final dataset consisted of genotypes for the 5 SNPs remaining SNPs for all experimental ewes.

3.3 Animals

Among the 884 initially genotyped ewes a total of 853 animals were found to have both a reliable genotype and phenotypic information available. Of these, 364 were born in 2010 and 489 in 2011. Number of lambs born of ewes at age 1 year (LS1) and age 2 year (LS2) were registered in the 15 different flocks included in the study during 2011 and 2012. Animals born on 2010 have data on LS1 and LS2, but animals born on 2011 only have information of LS1.

3.4 Descriptive Statistics of the GDF9 SNP

The overall allelic frequency was 0.623 for the GDF9_c.1111G allele and 0.377 for the c.1111A allele. The number of ewes in our study being homozygous (GG) for wild type allele (c.1111G), heterozygote (GA) and homozygote (AA) for mutant allele (c.1111A) were 337, 388 and 128, respectively (Table 3.1). Among the animals genotyped for the GDF9_c.1111G>A SNP , 364 ewes have LS1 data and 489 ewes have LS2 data, respectively. The number of ewes and number of lambs at 1 year and 2 years with each genotype are shown in Table 3.2.

Table 3.1: Genotype and allele frequency of c.1111G>A in Norwegian White Ewes

Genotype	Number of ewes	Frequency of Genotype
GG	337	0.40
GA	388	0.45
AA	128	0.15

Table 3.2: Number of ewes and lambs born (LS) per genotype

Phenotype	Number of Ewes	Number of Lambs born	Number of Lambs born for different genotypes		
			GG	GA	AA
LS1	364	743	298	332	113
LS2	489	359	146	169	44
Total			444	501	157

3.5 Statistical Analysis

The SAS package version 9.2 was used for analyzing the data. After genotyping 853 ewes for MRC1_c.361G>A, 788 ewes for MSTN_c.S1232G>A , 848 ewes for MSTN_c.960delG>A,845 ewes for MSTN_c.120insA and 853 ewes for GDF9_c.1111G>A, SNPs genotype were analyzed according to the given statistical model. As expected only the GDF9_c.1111G>A mutation showed any effect on number of lambs born alive at 1 year (LS1) and 2 years (LS2). Therefore, further statistical analysis was only conducted for the GDF9_c.1111G>A SNP.

The mean litter size at 1 year was 2.061±0.73 (mean±sd) and at 2 years it was 2.671±0.91. The genotype showed significant effect on litter size (p<0.0001) at both 1 year and 2 year. The mean and least square means (lsmeans) for number of lambs born at 1 year and 2 years per genotypes are shown in Table 3.3. The mean litter size at 2 years is bigger than that of 1 year. The lsmeans among the genotypes for the phenotypic number of lambs born at 2 year also differed from 1 year and was higher indeed. Ewes that were homozygous for the mutant GDF variant (AA) produced the largest litter size for both years, whereas animals homozygous for the wild type had the smallest litter size and heterozygous had an intermediate litter size. Ewes homozygous for c.1111A gave birth to more lambs than their contemporaries (GG and GA). The difference in genotype mean was highest between animals with homozygous wild type and homozygous

mutant allele for both traits. Also for lsmeans ewes with mutant homozygote allele had larger litter size than other genotype groups for both phenotypes. The mean of litter size per genotypes for first and second year are shown in Figure 3.1 and Figure 3.2.

Table 3.3: Mean and least-square means of LS per genotype

Phenotype	Genotypes	Means±sd	lsmeans
LS**1**	GG	1.922±0.674	1.955
	GA	2.066±0.705	2.085
	AA	2.415±0.831	2.496
	Mean	2.061±0.736	
LS**2**	GG	2.349±0.784	2.271
	GA	2.834±0.835	2.777
	AA	3.113±1.145	3.141
	Mean	2.671±0.921	

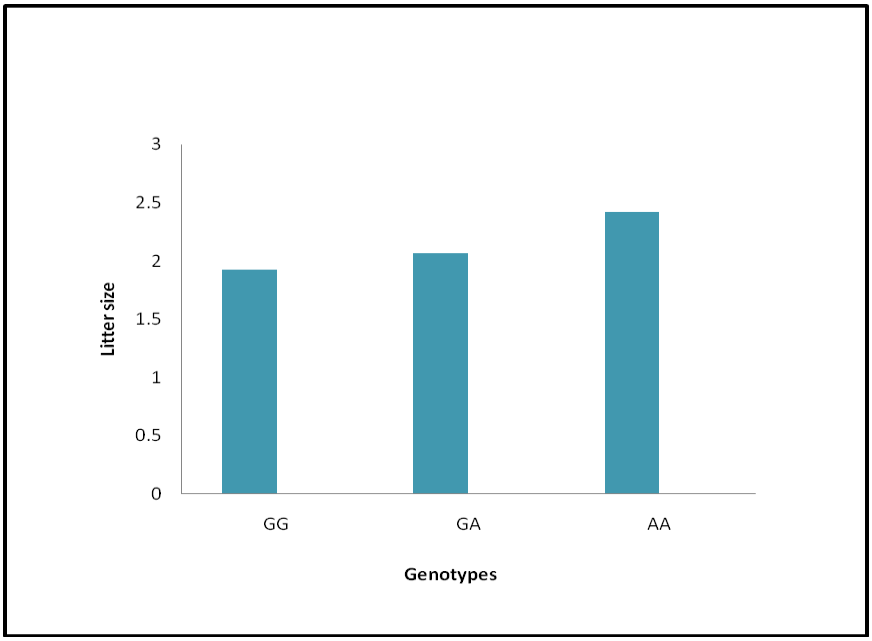

Figure 3.1: Average litter size at 1 year of age per genotype

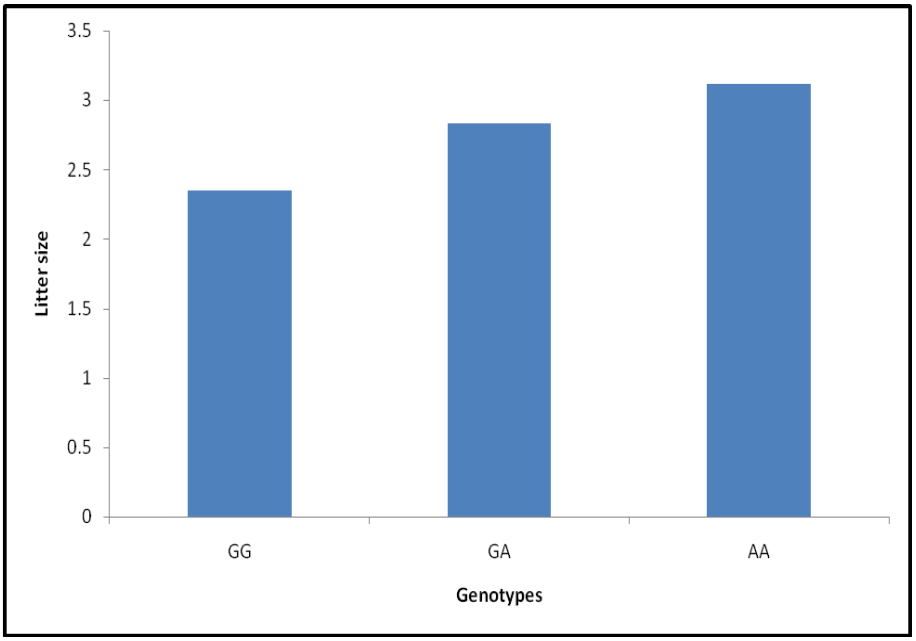

Figure 3.2: Average litter size at 2 year of age per genotype

The genotype has a significant effect on LS1 and LS2 phenotypes. The difference was largest between animals homozygous for the wild type allele and those homozygous for the mutant, both for LS1 and LS2. The effect of genotype on number of lambs born was significant (p<0.0001) for both phenotypes. Farm year only proved significance for second year (p=0.4682) but did not shown a significant effect on second year (Table 3.4).

Table 3.4: Effect of different sources on phenotype

Phenotype	Effects			
	Genotype		Farm_yr	
	F value	Pr > F	F value	Pr > F
LS1	21.21	<0.0001	2.00	0.0015
LS2	20.65	<0.0001	0.98	0.4682

Difference between least square means of three different genotypes (GG, GA and AA) is shown in Table 3.5. Ewes with the mutant allele (homozygous) showed highest difference in mean number of lamb production (0.541-0.87) compared to the wild homozygous contemporaries. For the heterozygous (GG vs GA) the difference was lowest among the three difference groups. But the GA vs AA group is different from the other two genotype groups.

Table 3.5: Least means for LS contrasts between GDF9 c.1111 genotypes

Phenotype	GG vs GA	GA vs AA	GG vs AA
LS1	0.13	0.411	0.541
LS2	0.506	0.364	0.87

All three genotypes for Least-square means differed from each other. For phenotype LS1, the homozygous mutant ewes had significant difference (p<0.0001) from both the wild type homozygote ewes and from the heterozygote and these two genotype groups also differed from each other (p=0.0234l). Least-square means for LS2, homozygous ewes (AA) also differed significantly from wild type ewes (p<0.0001) and heterozygous ewes (p<0.05). Wild type ewes had difference (p<0.0001) in litter size than the other two genotype groups, while heterozygote individuals showed difference from mutant homozygote (p<0.05) and wild type individuals (p<0.0001) (Table 3.6).

Table 3.6: Adjusted *p*- values for pair wise LS-means difference of effect genotype for LSs

LS1	GG	GA	AA
GG	-	0.0234	<0.0001
GA	0.0234	-	<0.0001
AA	<0.0001	<0.0001	-
LS2			
GG	-	<0.0001	<0.0001
GA	<0.0001	-	0.0175
AA	<0.0001	0.0175	-

The data on litter size at 1 year and 2 years for three different genotypes are shown in Table 3.7. Total number of lambs produced by homozygote mutant ewes was smallest among the three genotype groups for both phenotypes. More lambing data was available for 1 year than for the 2 year phenotype. Number of lambs born per genotype differed greatly from each other in this study. Wild type homozygote ewes had the highest number of lambs born at 1 year and heterozygote ewes had highest for 2 year than their contemporaries in this study. Ewes homozygote for c.1111A had the lowest number of observations for 1, 2 and 3 lambs for both

phenotypes and both year than the other two genotype groups. Homozygote (mutant) ewes at first year (10) and heterozygote ewes at second year (31) produced the highest incidence for 4 lambs among the three genotype groups. No observation found on 5 lambs at first year but all three genotype had incidence of 5 lambs at second year lambing. The only incidence (1) of 6 lambs at 1 year and 2 year of lambing was observed in wild homozygote and mutant homozygote, respectively while two other genotypes did not have any incidence of 6 lambs in any of the two year of ewe lambing.

Table 3.7: Number of lambs born per genotype with different observations

Genotypes	**No. of lambs at 1yr**						**Total**	**No. of lambs at 2yr**						**Total**
	1	**2**	**3**	**4**	**5**	**6**		**1**	**2**	**3**	**4**	**5**	**6**	
GG	70	188	35	4	0	1	298	21	59	61	4	1	0	146
GA	65	187	73	7	0	0	332	7	51	77	31	3	0	169
AA	15	46	46	10	0	0	113	5	5	19	11	3	1	44
	150	421	150	21	0	1	743	33	157	157	46	7	1	359

4. Discussion

The number of live lambs born per breeding ewe is an important trait in commercial sheep breeding, since the litter size largely determines the amount of meat produced per ewe. In this study we have investigated the phenotypic effect of a mutation in the ovine *GDF9* that recently has been reported to segregate in the Norwegian White Sheep population (Våge et al., 2013). A material of 853 ewes has been genotyped for the GDF9 c.111G>A mutation. All three possible genotypes (GG, GA and AA) were well represented in the genotyped material, making it possible to perform reliable comparisons between the different genotype groups.

We found that the phenotypic effect of the mutation was smaller for ewes at 1 year of age compared to ewes at 2 years of age. Since the average number of lambs born also is lower for ewes at 1 year of age compared to those at 2 years of age, this is not very surprising. However, since this material is limited to ages of 1 and 2 year, we cannot conclude whether the effect of the mutation can be even bigger for ewes older than two years. The phenotypic effect of being homozygous for the mutant allele at age 1 year was 0.54, while the effect of being homozygous for the mutant allele at age 2 year was 0.87. This is a higher phenotypic effect than reported by Våge et al., 2013, supporting the assumption of these authors that the phenotypic effect based on the EBVs of the AI rams is slightly underestimated.

The frequency for c.1111A allele was found to be 0.38 in this NWS experimental ewe population. This is a relatively high frequency compared to the corresponding frequency among the NWS AI rams (Våge et al., 2013). However, one must keep in mind that our experimental ewe population is heavily selected for large litter size. Given the strong association between this allele and litter size, there is reason to believe that this selection has influenced the frequency among these ewes. It is therefore likely that the frequency in the general ewe population is lower than this. However, by using this highly selected ewes in the present study we succeeded in obtaining a reasonable high number of animals in all the three genotypic classes (337 GG, 388 GA, 128 AA), making it possible to get a reliable estimate of the effect of the c.1111A allele.

The reason for the increased frequency of c.1111A allele in Norwegian White Sheep AI ram population and also among the ewes (as shown in this study), may be due to the introduction of Finnish Landrace sheep in Norway for genetic improvement of NWS in 1960s and 1970s, also supported by Vage et al., (2013); Donald and Read (1967) and Ercanbrack and Knight (1985). Finnish Landrace or Finnsheep is a prolific (multiple births are very common) sheep breed and its crosses with other sheep breeds have been recognized for their higher fertility.

Increased meat production in Norway can be obtained increasing litter size by selection, both traditional phenotypes based breeding and/or by using the c.1111A allele for genotype assisted selection. However, NWS is a small size sheep and it might have some impact with really big LS

in ewes. No adverse effect on ewe has yet been reported in NWS ewes, but higher number of lambs born can cause lower birth weight and increased post-natal mortality. So, an optimum number of lambs born should be desired for Norwegian White ewes. The Norwegian Sheep and Goat Breeder's association will take the decision how the ram with c.1111A allele will be used in Norwegian White sheep breeding program.

The genotyping success rate in this study was more than 90% and it can be said that the quality of the extracted DNA was generally good. The "no calls" obtained during genotyping might be due to technical error, human error or poor DNA in some samples.

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6. Appendix

Commands used in SAS 9.2 Editor for ananlysis

```
data lamb;
infile 'lamb_data.txt' delimiter='09'x;
input Sample ID Genotype Flock_yr Flock_no Birth_yr lamb1yr lamb2yr;
proc sort; by Sample ;
run;
proc freq;
table Flock_yr;
run;
proc freq;
table Genotype;
run;
proc freq;
table Flock_no;
run;
proc freq;
table Birth_yr;
run;
proc freq;
table lamb1yr;
run;
proc freq;
table lamb2yr;
run;
proc freq;
table Flock_yr*Genotype;
run;
proc freq;
table Birth_yr*Genotype;
run;
proc freq;
table Flock_no*Genotype;
run;
proc freq;
table lamb1yr*Genotype;
run;
proc freq;
table lamb2yr*Genotype;
run;
proc means data=lamb;
var Genotype Flock_yr Flock_no Birth_yr lamb1yr lamb2yr;
run;
proc glm data=Lamb;
  class Genotype Flock_yr;
  model lamb1yr lamb2yr=Genotype Flock_yr/solution;
  means Genotype;
  lsmeans Genotype/pdiff;
run;
```

Commands used for the model for bivariate analysis

```
proc glm data=Lamb;
  class Genotype Flock_yr;
  model lamb1yr lamb2yr=Genotype Flock_yr/solution;
  means Genotype;
  lsmeans Genotype/pdiff;
run;
```

Measurement of frequency for Flock_yr (Flock year)

The SAS System 11:42 Monday, March

The FREQ Procedure

Flock_yr	Frequency	Percent	Cumulative Frequency	Cumulative Percent
12972010	23	2.70	23	2.70
12972011	22	2.58	45	5.28
24972010	22	2.58	67	7.85
24972011	38	4.45	105	12.31
29712010	60	7.03	165	19.34
29712011	104	12.19	269	31.54
33902010	24	2.81	293	34.35
33902011	31	3.63	324	37.98
38032010	15	1.76	339	39.74
38032011	27	3.17	366	42.91
43262010	11	1.29	377	44.20
43262011	15	1.76	392	45.96
43482010	14	1.64	406	47.60
43482011	13	1.52	419	49.12
45622010	53	6.21	472	55.33
45622011	70	8.21	542	63.54
53762010	25	2.93	567	66.47
53762011	28	3.28	595	69.75
67462010	24	2.81	619	72.57
67462011	23	2.70	642	75.26
78842010	23	2.70	665	77.96
78842011	32	3.75	697	81.71
84702010	16	1.88	713	83.59
84702011	23	2.70	736	86.28
85742010	16	1.88	752	88.16
85742011	17	1.99	769	90.15
86292010	19	2.23	788	92.38
86292011	12	1.41	800	93.79
95072010	19	2.23	819	96.01
95072011	34	3.99	853	100.00

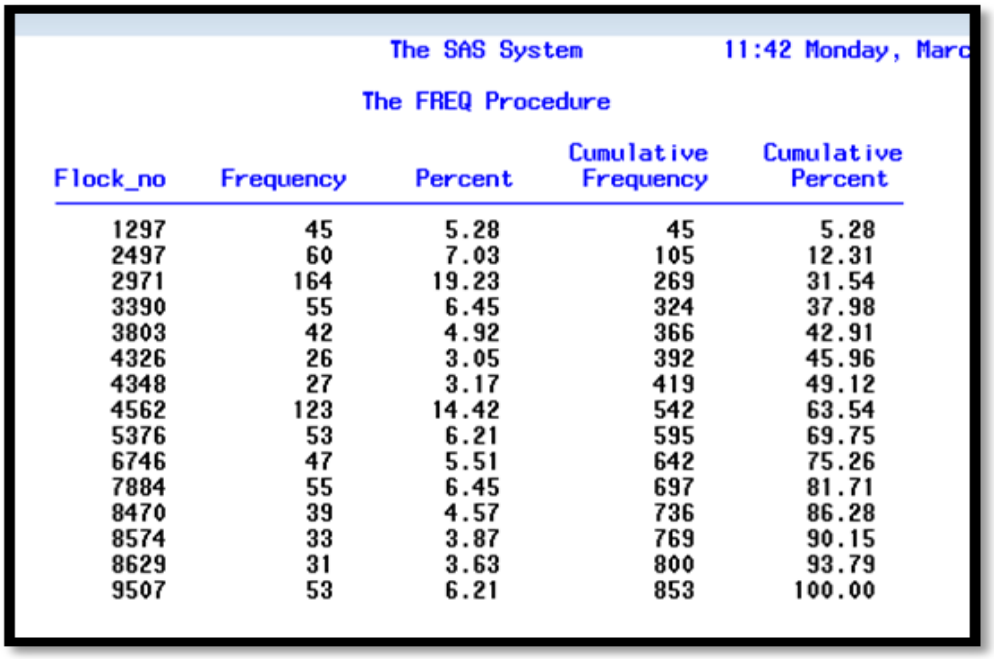
Measurement of frequency for different Genotypes (GG=1, GA=2, AA=3)

The SAS System 11:42 Monday, Ma

The FREQ Procedure

Genotype	Frequency	Percent	Cumulative Frequency	Cumulative Percent
1	337	39.51	337	39.51
2	388	45.49	725	84.99
3	128	15.01	853	100.00

Measurement of frequency of Flock_no (Flock number)

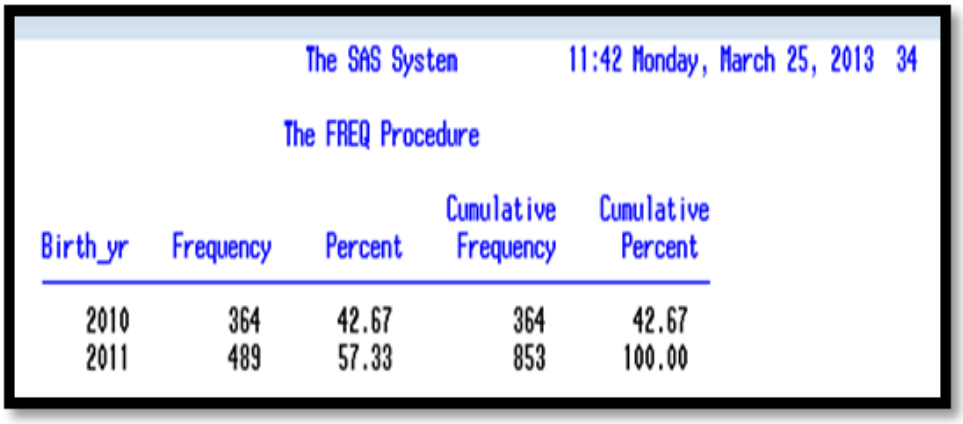


The SAS System 11:42 Monday, Marc

The FREQ Procedure

Flock_no	Frequency	Percent	Cumulative Frequency	Cumulative Percent
1297	45	5.28	45	5.28
2497	60	7.03	105	12.31
2971	164	19.23	269	31.54
3390	55	6.45	324	37.98
3803	42	4.92	366	42.91
4326	26	3.05	392	45.96
4348	27	3.17	419	49.12
4562	123	14.42	542	63.54
5376	53	6.21	595	69.75
6746	47	5.51	642	75.26
7884	55	6.45	697	81.71
8470	39	4.57	736	86.28
8574	33	3.87	769	90.15
8629	31	3.63	800	93.79
9507	53	6.21	853	100.00

Measurement of frequency of Birth_yr (Birth year)



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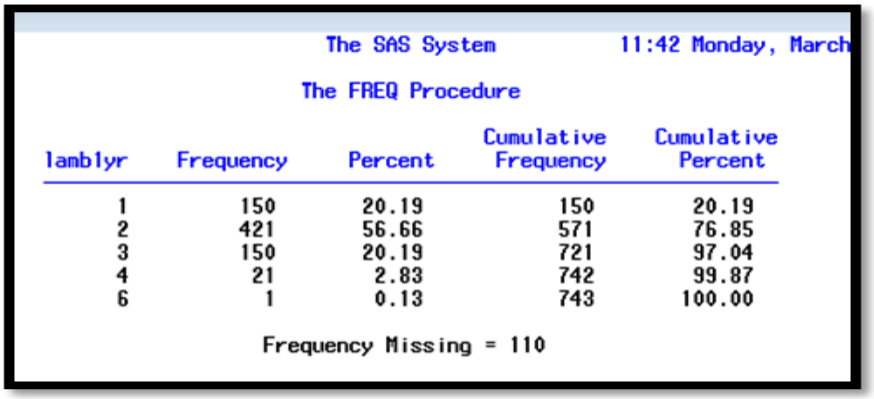
The FREQ Procedure

Birth_yr	Frequency	Percent	Cumulative Frequency	Cumulative Percent
2010	364	42.67	364	42.67
2011	489	57.33	853	100.00

Measurement of frequency of lamb1yr (No. of lambs at 1 year)



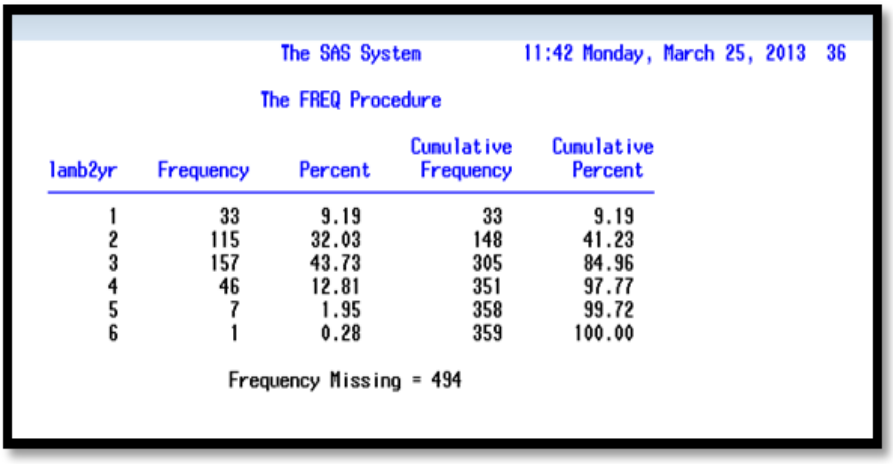
The SAS System 11:42 Monday, March

The FREQ Procedure

lamb1yr	Frequency	Percent	Cumulative Frequency	Cumulative Percent
1	150	20.19	150	20.19
2	421	56.66	571	76.85
3	150	20.19	721	97.04
4	21	2.83	742	99.87
6	1	0.13	743	100.00

Frequency Missing = 110

Measurement of frequency of lamb2yr (No. of lambs at 2 year)



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The FREQ Procedure

lamb2yr	Frequency	Percent	Cumulative Frequency	Cumulative Percent
1	33	9.19	33	9.19
2	115	32.03	148	41.23
3	157	43.73	305	84.96
4	46	12.81	351	97.77
5	7	1.95	358	99.72
6	1	0.28	359	100.00

Frequency Missing = 494

Frequency table of Flock_yr by Genotype (3 tables)

Table of Flock_yr by Genotype

Flock_yr / Frequency / Percent / Row Pct / Col Pct	Genotype 1	2	3	Total
12972010	8 0.94 34.78 2.37	13 1.52 56.52 3.35	2 0.23 8.70 1.56	23 2.70
12972011	9 1.06 40.91 2.67	11 1.29 50.00 2.84	2 0.23 9.09 1.56	22 2.58
24972010	12 1.41 54.55 3.56	7 0.82 31.82 1.80	3 0.35 13.64 2.34	22 2.58
24972011	17 1.99 44.74 5.04	15 1.76 39.47 3.87	6 0.70 15.79 4.69	38 4.45
29712010	33 3.87 55.00 9.79	24 2.81 40.00 6.19	3 0.35 5.00 2.34	60 7.03
29712011	51 5.98 49.04 15.13	41 4.81 39.42 10.57	12 1.41 11.54 9.38	104 12.19
33902010	6 0.70 25.00 1.78	15 1.76 62.50 3.87	3 0.35 12.50 2.34	24 2.81
33902011	8 0.94 25.81 2.37	16 1.88 51.61 4.12	7 0.82 22.58 5.47	31 3.63
38032010	6 0.70 40.00 1.78	6 0.70 40.00 1.55	3 0.35 20.00 2.34	15 1.76
38032011	12 1.41 44.44 3.56	14 1.64 51.85 3.61	1 0.12 3.70 0.78	27 3.17
Total	337	388	128	853

Table of Flock_yr by Genotype

Flock_yr	Genotype 1	2	3	Total
43262010	Frequency 4 Percent 0.47 Row Pct 36.36 Col Pct 1.19	4 0.47 36.36 1.03	3 0.35 27.27 2.34	11 1.29
43262011	5 0.59 33.33 1.48	3 0.35 20.00 0.77	7 0.82 46.67 5.47	15 1.76
43482010	6 0.70 42.86 1.78	7 0.82 50.00 1.80	1 0.12 7.14 0.78	14 1.64
43482011	4 0.47 30.77 1.19	4 0.47 30.77 1.03	5 0.59 38.46 3.91	13 1.52
45622010	19 2.23 35.85 5.64	27 3.17 50.94 6.96	7 0.82 13.21 5.47	53 6.21
45622011	25 2.93 35.71 7.42	30 3.52 42.86 7.73	15 1.76 21.43 11.72	70 8.21
53762010	12 1.41 48.00 3.56	13 1.52 52.00 3.35	0 0.00 0.00 0.00	25 2.93
53762011	7 0.82 25.00 2.08	19 2.23 67.86 4.90	2 0.23 7.14 1.56	28 3.28
67462010	11 1.29 45.83 3.26	12 1.41 50.00 3.09	1 0.12 4.17 0.78	24 2.81
67462011	10 1.17 43.48 2.97	12 1.41 52.17 3.09	1 0.12 4.35 0.78	23 2.70
Total	337	388	128	853

Frequency table of Birth_yr by Genotype

The SAS System 11:42 Monday,

The FREQ Procedure

Table of Birth_yr by Genotype

Birth_yr	Genotype 1	2	3	Total
2010	Frequency 149 Percent 17.47 Row Pct 40.93 Col Pct 44.21	169 19.81 46.43 43.56	46 5.39 12.64 35.94	364 42.67
2011	188 22.04 38.45 55.79	219 25.67 44.79 56.44	82 9.61 16.77 64.06	489 57.33
Total	337 39.51	388 45.49	128 15.01	853 100.00

Frequency table of Flock_no by genotype (2 tables)

The FREQ Procedure

Table of Flock_no by Genotype

Flock_no / Genotype Frequency Percent Row Pct Col Pct	1	2	3	Total
1297	17 1.99 37.78 5.04	24 2.81 53.33 6.19	4 0.47 8.89 3.13	45 5.28
2497	29 3.40 48.33 8.61	22 2.58 36.67 5.67	9 1.06 15.00 7.03	60 7.03
2971	84 9.85 51.22 24.93	65 7.62 39.63 16.75	15 1.76 9.15 11.72	164 19.23
3390	14 1.64 25.45 4.15	31 3.63 56.36 7.99	10 1.17 18.18 7.81	55 6.45
3803	18 2.11 42.86 5.34	20 2.34 47.62 5.15	4 0.47 9.52 3.13	42 4.92
4326	9 1.06 34.62 2.67	7 0.82 26.92 1.80	10 1.17 38.46 7.81	26 3.05
4348	10 1.17 37.04 2.97	11 1.29 40.74 2.84	6 0.70 22.22 4.69	27 3.17
4562	44 5.16 35.77 13.06	57 6.68 46.34 14.69	22 2.58 17.89 17.19	123 14.42
Total	337 39.51	388 45.49	128 15.01	853 100.00

(Continued)

The SAS System 12:45 Friday,

The FREQ Procedure

Table of Flock_no by Genotype

Flock_no	Genotype			
Frequency Percent Row Pct Col Pct	1	2	3	Total
5376	19 2.23 35.85 5.64	32 3.75 60.38 8.25	2 0.23 3.77 1.56	53 6.21
6746	21 2.46 44.68 6.23	24 2.81 51.06 6.19	2 0.23 4.26 1.56	47 5.51
7884	33 3.87 60.00 9.79	20 2.34 36.36 5.15	2 0.23 3.64 1.56	55 6.45
8470	13 1.52 33.33 3.86	25 2.93 64.10 6.44	1 0.12 2.56 0.78	39 4.57
8574	10 1.17 30.30 2.97	16 1.88 48.48 4.12	7 0.82 21.21 5.47	33 3.87
8629	8 0.94 25.81 2.37	17 1.99 54.84 4.38	6 0.70 19.35 4.69	31 3.63
9507	8 0.94 15.09 2.37	17 1.99 32.08 4.38	28 3.28 52.83 21.88	53 6.21
Total	337 39.51	388 45.49	128 15.01	853 100.00

Ferquency table of lamb1yr by Genotype

The SAS System 11:42 Monday, Marc

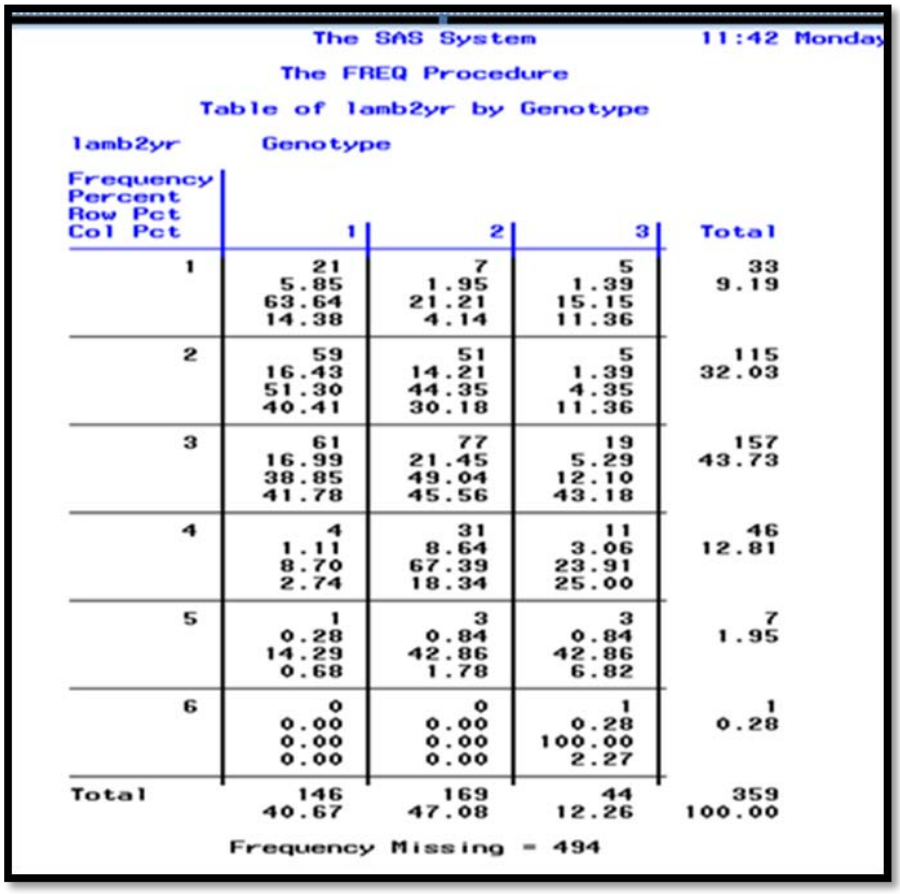
The FREQ Procedure

Table of lamb1yr by Genotype

lamb1yr	Genotype			
Frequency Percent Row Pct Col Pct	1	2	3	Total
1	70 9.42 46.67 23.49	65 8.75 43.33 19.58	15 2.02 10.00 13.27	150 20.19
2	188 25.30 44.66 63.09	187 25.17 44.42 56.33	46 6.19 10.93 40.71	421 56.66
3	35 4.71 23.33 11.74	73 9.83 48.67 21.99	42 5.65 28.00 37.17	150 20.19
4	4 0.54 19.05 1.34	7 0.94 33.33 2.11	10 1.35 47.62 8.85	21 2.83
6	1 0.13 100.00 0.34	0 0.00 0.00 0.00	0 0.00 0.00 0.00	1 0.13
Total	298 40.11	332 44.68	113 15.21	743 100.00

Frequency Missing = 110

Ferquency table of lqmb2yr by Genotype



The SAS System 11:42 Monday

The FREQ Procedure

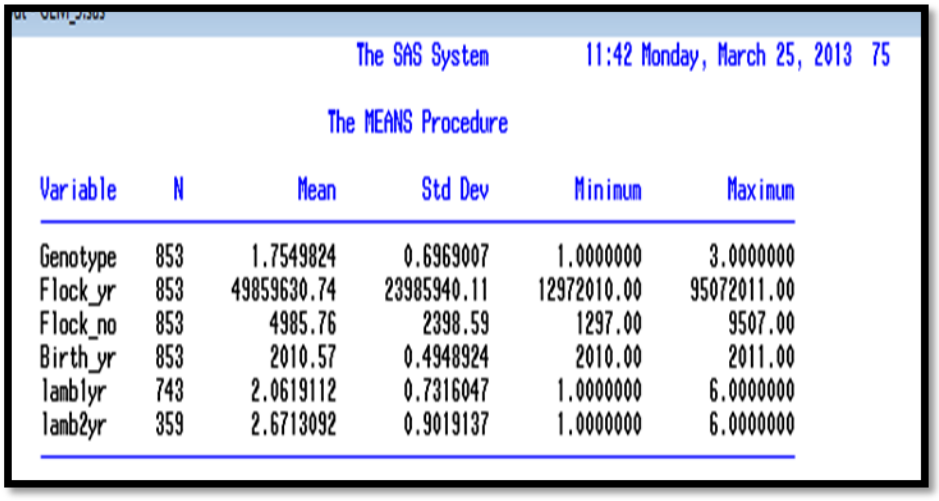
Table of lamb2yr by Genotype

lamb2yr Genotype

Frequency / Percent / Row Pct / Col Pct	1	2	3	Total
1	21 5.85 63.64 14.38	7 1.95 21.21 4.14	5 1.39 15.15 11.36	33 9.19
2	59 16.43 51.30 40.41	51 14.21 44.35 30.18	5 1.39 4.35 11.36	115 32.03
3	61 16.99 38.85 41.78	77 21.45 49.04 45.56	19 5.29 12.10 43.18	157 43.73
4	4 1.11 8.70 2.74	31 8.64 67.39 18.34	11 3.06 23.91 25.00	46 12.81
5	1 0.28 14.29 0.68	3 0.84 42.86 1.78	3 0.84 42.86 6.82	7 1.95
6	0 0.00 0.00 0.00	0 0.00 0.00 0.00	1 0.28 100.00 2.27	1 0.28
Total	146 40.67	169 47.08	44 12.26	359 100.00

Frequency Missing = 494

Means of different variables used for analysis in this study



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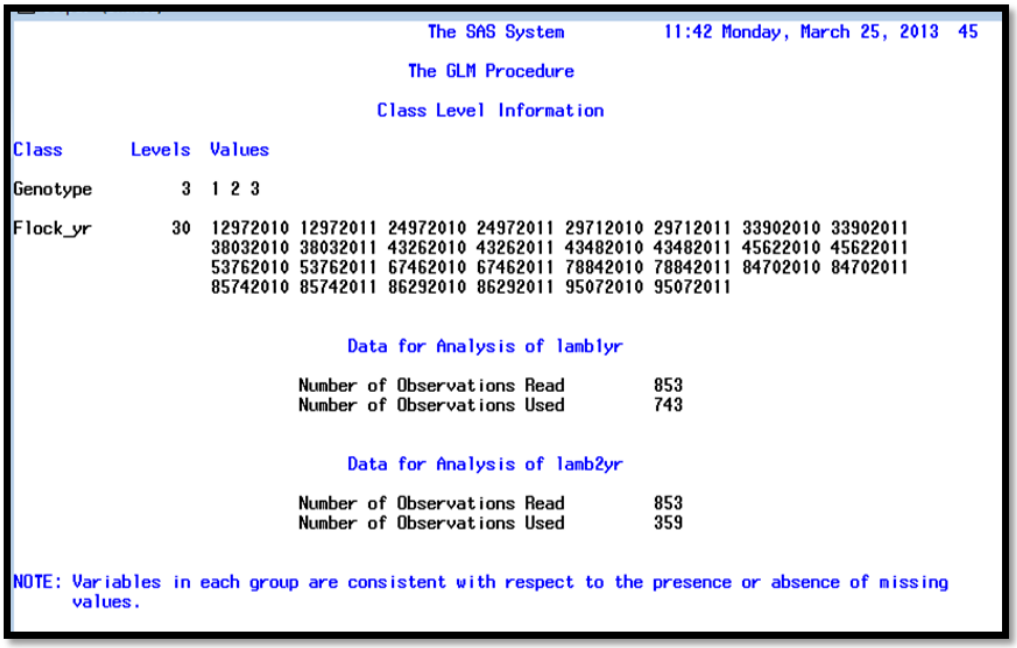
The MEANS Procedure

Variable	N	Mean	Std Dev	Minimum	Maximum
Genotype	853	1.7549824	0.6969007	1.0000000	3.0000000
Flock_yr	853	49859630.74	23985940.11	12972010.00	95072011.00
Flock_no	853	4985.76	2398.59	1297.00	9507.00
Birth_yr	853	2010.57	0.4948924	2010.00	2011.00
lamb1yr	743	2.0619112	0.7316047	1.0000000	6.0000000
lamb2yr	359	2.6713092	0.9019137	1.0000000	6.0000000

Data level in the model



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The GLM Procedure

Class Level Information

Class	Levels	Values
Genotype	3	1 2 3
Flock_yr	30	12972010 12972011 24972010 24972011 29712010 29712011 33902010 33902011 38032010 38032011 43262010 43262011 43482010 43482011 45622010 45622011 53762010 53762011 67462010 67462011 78842010 78842011 84702010 84702011 85742010 85742011 86292010 86292011 95072010 95072011

Data for Analysis of lamblyr

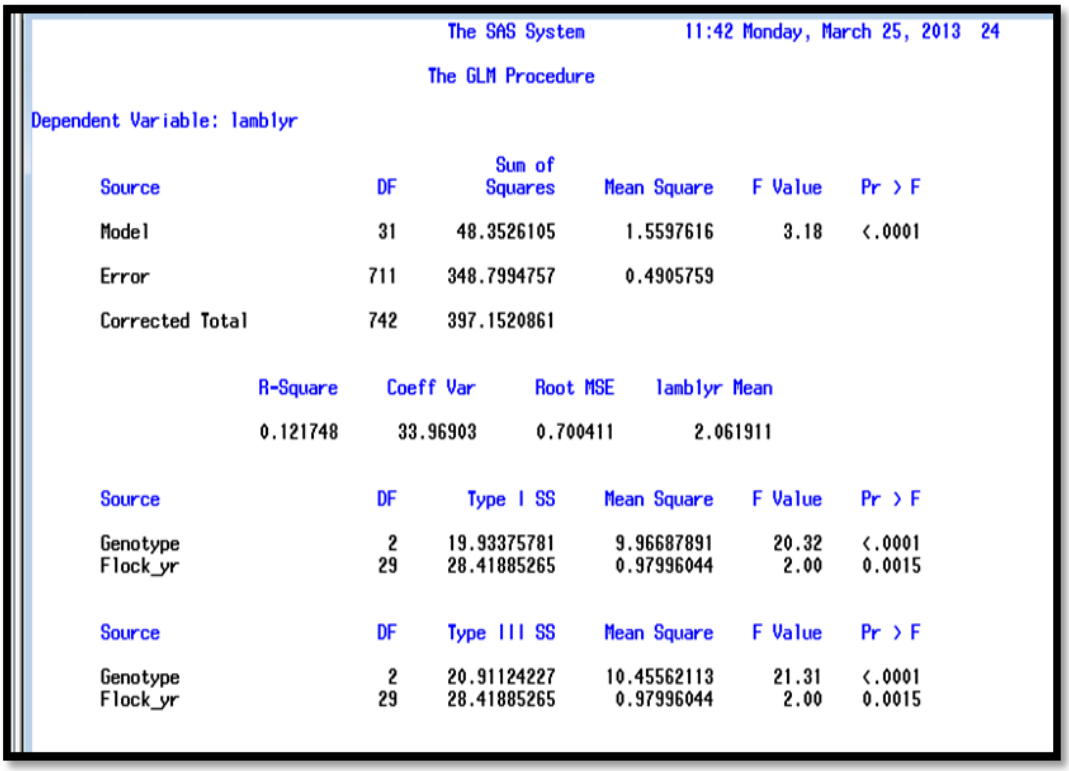
Number of Observations Read	853
Number of Observations Used	743

Data for Analysis of lamb2yr

Number of Observations Read	853
Number of Observations Used	359

NOTE: Variables in each group are consistent with respect to the presence or absence of missing values.

ANOVA Table for litter size at 1 year



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The GLM Procedure

Dependent Variable: lamblyr

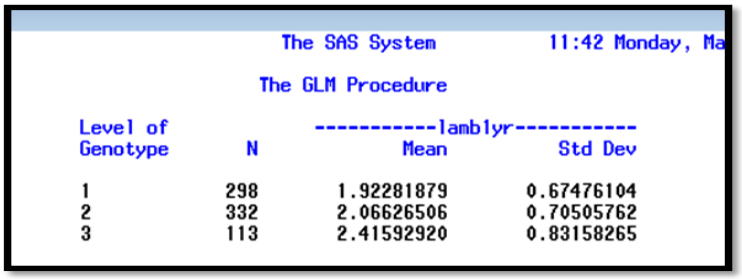
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	31	48.3526105	1.5597616	3.18	<.0001
Error	711	348.7994757	0.4905759		
Corrected Total	742	397.1520861			

R-Square	Coeff Var	Root MSE	lamblyr Mean
0.121748	33.96903	0.700411	2.061911

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Genotype	2	19.93375781	9.96687891	20.32	<.0001
Flock_yr	29	28.41885265	0.97996044	2.00	0.0015

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Genotype	2	20.91124227	10.45562113	21.31	<.0001
Flock_yr	29	28.41885265	0.97996044	2.00	0.0015

Means of litter size at 1 year per Genotypes



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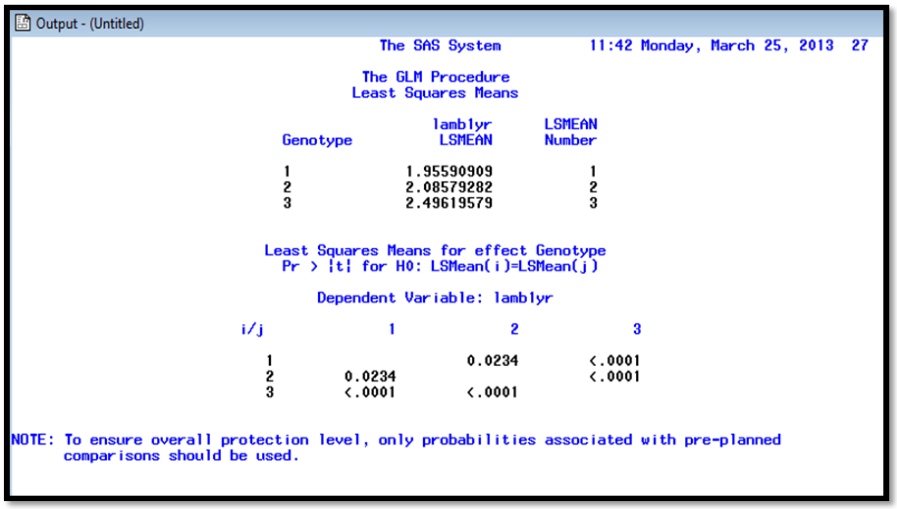
The GLM Procedure

Level of Genotype	N	lamb1yr Mean	lamb1yr Std Dev
1	298	1.92281879	0.67476104
2	332	2.06626506	0.70505762
3	113	2.41592920	0.83158265

Least-square means and difference matrix for lamb1yr



Output - (Untitled)

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The GLM Procedure
Least Squares Means

Genotype	lamb1yr LSMEAN	LSMEAN Number
1	1.95590909	1
2	2.08579282	2
3	2.49619579	3

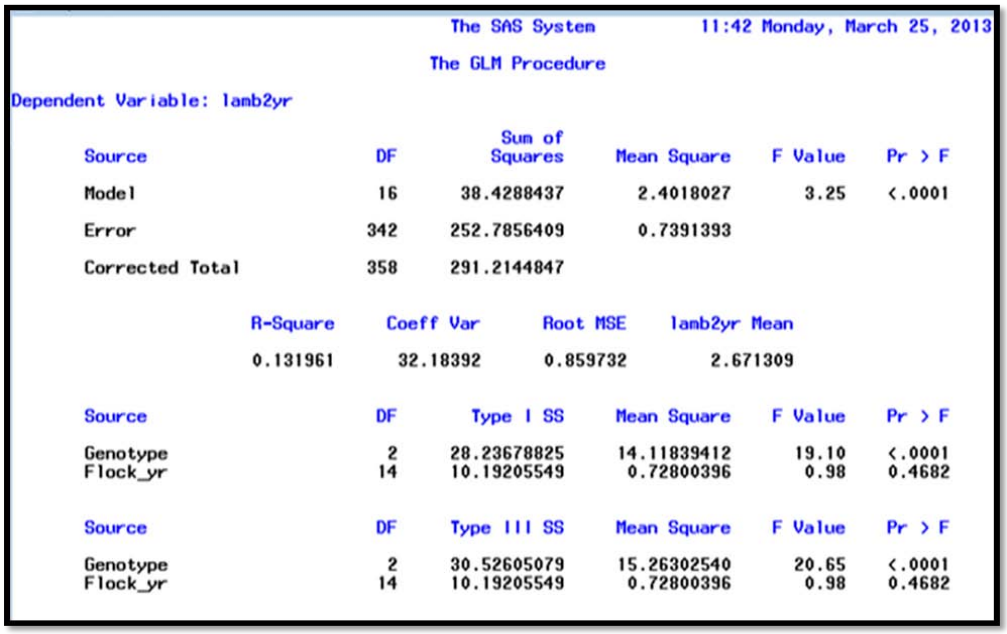
Least Squares Means for effect Genotype
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: lamb1yr

i/j	1	2	3
1		0.0234	<.0001
2	0.0234		<.0001
3	<.0001	<.0001	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

ANOVA Table for lamb2yr



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The GLM Procedure

Dependent Variable: lamb2yr

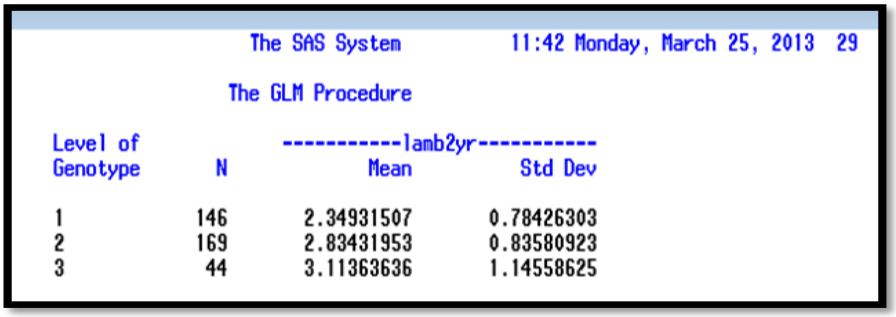
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	16	38.4288437	2.4018027	3.25	<.0001
Error	342	252.7856409	0.7391393		
Corrected Total	358	291.2144847			

R-Square	Coeff Var	Root MSE	lamb2yr Mean
0.131961	32.18392	0.859732	2.671309

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Genotype	2	28.23678825	14.11839412	19.10	<.0001
Flock_yr	14	10.19205549	0.72800396	0.98	0.4682

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Genotype	2	30.52605079	15.26302540	20.65	<.0001
Flock_yr	14	10.19205549	0.72800396	0.98	0.4682

Means of lamb2yr per Genotype



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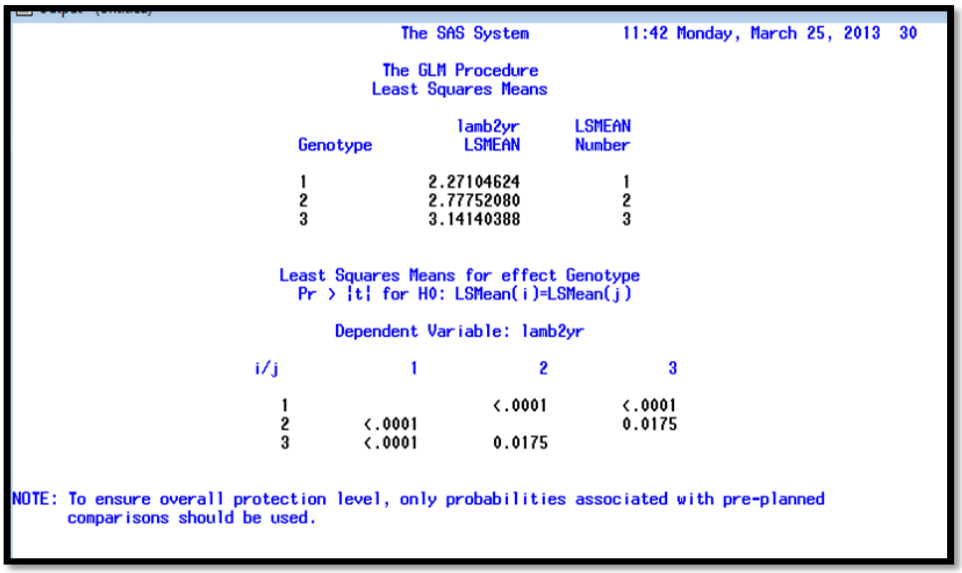
The GLM Procedure

Level of Genotype	N	lamb2yr Mean	lamb2yr Std Dev
1	146	2.34931507	0.78426303
2	169	2.83431953	0.83580923
3	44	3.11363636	1.14558625

lsmeans and difference matrix of lamb2yr for Genotypes



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The GLM Procedure
Least Squares Means

Genotype	lamb2yr LSMEAN	LSMEAN Number
1	2.27104624	1
2	2.77752080	2
3	3.14140388	3

Least Squares Means for effect Genotype
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: lamb2yr

i/j	1	2	3
1		<.0001	<.0001
2	<.0001		0.0175
3	<.0001	0.0175	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.