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Having better genetic control over fertility in New Zealand maternal Sheep breeds?

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Summary

An important trait in sheep breeding is the number of lambs born (NLB) per ewe that survive to weaning, with the amount of meat produced per ewe, to a great extent being determined by litter size. Research suggests that Single Nucleotide Polymorphisms (SNPs) in the *GDF9* gene (*Growth differentiation factor-9*) could lead to different number of lambs born per ewe, and thus *GDF9* could be used to increase ewe performance and meat production. The overall aim of this research is to investigate whether there is *GDF9* sequence variation in some common New Zealand sheep breeds, and if so, whether this variation is associated with variation in fecundity in different breeds of sheep. In the initial study, PCR-SSCP analysis revealed three *GDF9* variants (*A*, *B* and *C*) with frequencies of 79.1%, 13.8% and 7.1% respectively and three genotypes *AA*, *AB* and *AC* with the frequency of 63.5%, 27.5% and 8.8%, respectively in 134 Finn and Finn × Texel-cross ewes. The heterozygote *BB* and *CC* genotypes were not observed in the sheep studied. The average litter size for *AB* genotypes in Finn and Finn × Texel cross ewes was 2.36 and 2.50, respectively and overall the litter size of heterozygous *AB* ewes was 0.7 higher than ewes without the *B* variant. The investigation is ongoing with the hope of finding functional variation in the more common NZ maternal breeds.

Keywords: fertility, sheep, GDF9 gene, PCR-SSCP, variation

Introduction

Two important traits with high economic value to sheep production are ewe ovulation rate and litter size or fecundity(Notter, 2008). New Zealand's economy is predominantly based on agriculture with the export of sheep products including meat and wool. Accordingly, improvement in the production performance of sheep has economic impact. Lambing performance is routinely measured, both on-farm and nationally, and this enables the industry to measure its progress. Ovulation rate is different in different breeds and the range is from one egg per ovulation, as is typical for the Texel or Suffolk breeds, up to ten eggs per ovulation for prolific breeds such as the Booroola Merino or Finn sheep (Souza *et al.*, 2001). Maximising reproductive success and producing litters of intermediate size in breeding programs is deemed more desirable than simply increasing the number of lambs per ewe (SanCristobal-Gaudy *et al.*, 2001). This is especially the case if nutrition for the ewe cannot be maintained at an appropriate level across the calendar year and/or is adversely affected by season.

Sheep fertility and reproductive performance depends on many factors, but attention has in part focussed on the genes that might underpin variation in fertility and fecundity. The benefit of this approach is not only important from an animal production perspective, but also because it enables improved understanding of animal infertility and other genetic disorders that affect reproduction(Jansson, 2014). Genetic improvement in these traits is slow because they are only expressed in one gender (the ewe) and because accurate recording of the traits can only be achieved at the end of the ewe's lifetime.

Any technology that positively influences lambing performance and gives us a better understanding of fertility and fecundity genes is desirable. This explains the ongoing research emphasis on improving such things as fertility and fecundity, lamb survival, etc., and the overall efficiency of sheep farming systems from the perspective of inputs (feed costs, animal health costs, etc.) and outputs (lambs available for sale).

One gene that appears to have a large effect on sheep prolificacy is the gene for

61 growth differentiation factor 9 (*GDF9*) known as *GDF9* or *FecG* (McNatty *et al.*, 2005).
62 *GDF9* is expressed from the primary stage of follicular development (McGrath et al., 1995).
63 It is an autosomal gene (ovine chromosome 5) and a member of the mammalian growth
64 transforming family (TGF-Beta superfamily), which plays an important role in ovulation
65 development and folliculogenesis (McPherron and Lee, 1993, Sadighi *et al.*, 2002, Juengel *et al.*,
66 2004).

67 Various polymorphisms have been described in ovine *GDF9* and other genes of the
68 transforming growth factor beta superfamily that have been associated with increased litter
69 size(Souza *et al.*, 2014) For example, Embrapa and G7 are mutations in *GDF9* that appear to
70 have an additive effect on litter size, while the high fertility and Thoka mutations increase
71 prolificacy in the heterozygous state, but are associated with sterility in homozygous
72 ewes.(Davis, 2005, Pramod *et al.*, 2013)

73 The aim of this study was to investigate associations between *GDF9* variation and
74 the number of lambs born per ewe in a known high fertility flock, and as start to gaining a
75 better understanding of the gene in common NZ maternal breeds. An improved
76 understanding of potentially more benign variation in *GDF9*, may allow us to better control
77 the number of lambs born per ewe per year on individual farms, and not least to better match
78 lambing performance to feed supply.

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80 ## Materials and methods

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82 All research involving animals was conducted under license from the Animal
83 Welfare Act 1999 (New Zealand Government). The Lincoln University Animal Ethics
84 Committee approved the collection of blood from animals used in this study.

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86 **Blood samples and DNA purification**

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88 Two-hundred and two ewes were studied. They were of the Finn breed, or a Finn ×
89 Texel cross. Records were available for these sheep including litter size (i.e. number of lambs

born per ewe) taken over one, or more seasons. Blood was collected on FTA cards, from a small incision in the ear of the sheep. DNA was extracted from the blood samples by punching a 1.2 mm disc from the FTA card, followed by genomic DNA purification using a two-step procedure described by(Zhou *et al.*, 2006). In brief, the FTA card punch was placed in tubes containing 200 µL of 20 mM NaOH, left for 20 to 30 min at 62 °C, or until the disk became white. All the liquid was then removed and the disk equilibrated in 200 µL of 1× TE^{-1} buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). After this washing and equilibration, the liquid was again removed and the disks were left to air dry in the tubes overnight.

PCR amplification and SSCP analysis

The Polymerase Chain Reaction (PCR) primers used in this study as follows: *GDF9* exon 2 (The forward primer used was *5′- ATAAGCGATTGAGCCATCAGG* and the reverse primer was 5′- GCTGAGGGTGTAAGATCGTC). The primers were designed based on GenBank sequence AF07854.2 to amplify a fragment that encompassed SNPs reported in the literature that had an association with litter size. Amplification was undertaken as follows: denaturation at 94 ºC for 5 min, then 35 cycles at 94 ºC for 30 s followed by 59 ºC for 30 s and 40 ºC for 40 s and final extension at 72 ºC for 5 min. Genotyping was undertaken using a PCR-SSCP approach. 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), and after denaturation at 95 ºC for 5 min, the samples were rapidly cooled on wet ice and immediately loaded on to 16 cm × 18 cm, 12% acrylamide:bisacrylamide (37.5:1; Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad), at 350 V for 18 h at 7 ºC in 0.5 × TBE buffer. DNA fragments were visualized using a silver nitrate staining method (Byun *et al.*, 2009). In brief, the gels were bathed in a solution of 10% ethanol, 0.5% acetic acid and 0.2 silver nitrate for 10 min. Next the gels were rinsed with distilled water then developed with a solution of 3% NaOH and 0.1 % HCOH till dark staining bands appear on the yellow back ground of the SSCP gel.

Sequencing of variants and sequence analyses

PCR amplicons representing different banding patterns from sheep that appeared to be

121 homozygous were sequenced in both directions at the Lincoln University DNA Sequencing
122 Facility, NZ to confirm that variants detected represented unique sequences. Variants that
123 were only found in heterozygous sheep were sequenced using an approach described by
124 (Gong *et al.*, 2011). Briefly, a band corresponding to the variant was excised as a gel slice
125 from the polyacrylamide gel, macerated, and then used as a template for re-amplification
126 with the original primers. This second amplicon was then sequenced. Sequence alignments,
127 translations and phylogenetic analysis were carried out using DNAMAN (version 5.2.10,
128 Lynnon BioSoft, Vaudreuil, Canada).

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130 **Statistical analysis**

131 Analysis of fertility data is carried out in the R environment for statistical computing.
132 A least-squares mean analysis was used to examine the number of lambs born per ewe with
133 different *GDF9* genotypes, including an analysis of variant and genotype differences between
134 the pure Finn and Finn × Texel cross ewes. Analysis performed to assess the presence or
135 absence of each of three *GDF9* variants separately on six parities. The calculation of variant
136 and genotype frequencies and Hardy-Weinberg equilibrium were performed using
137 POPGENE V1.32 software.

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139 Results and Discussion

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141 The SSCP analysis of *GDF9* detected three banding patterns (*A*, *B* and *C*), and three
142 combinations of these banding patterns (*AA*, *AB* and *AC*) were found in two different sheep
143 breeds. The sequencing of heterozygous genotypes confirm that the three detected variants
144 were unique sequences. Our results revealed no homozygous *BB* or *CC* sheep.

145 In total, 134 ewes were genotyped using the PCR-SSCP approach. Variant and
146 genotype frequencies are summarised in Table 1.

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152 *Table 1. Variant and genotype frequencies, observed, expected and heterozygosity of exon 1*
153 *for GDF9 in the sheep investigated.*

Gene	Variant frequencies (%)		Observed Genotype frequencies (%)		Expected Genotype frequencies (%)		X^2
	A	79.1	AA	58.2	AA	62.5	
	B	13.8	AB	27.6	AB	21.8	
GDF9	C	7.1	AC	14.1	AC	11.2	9.35**
			BB	0.00	BB	1.9	
			BC	0.00	BC	1.9	
			CC	0.00	CC	0.5	

154 ** Significantly deviated from Hardy-Weinberg equilibrium (P <0.01)

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156 The genotype frequencies differed from what was predicted from Hardy-Weinberg
157 analysis. This suggests that one or more of the Hardy-Weinberg conditions are being
158 violated, although it does not tell us which ones.

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160 *Table 2. The frequency of different genotypes in the sheep studied.*

	Genotype		
Breed	*AA*	*AB*	*AC*
Finn	45	35	10
Finn × Texel	33	2	9

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162 All three variants of *GDF9* were found in both the Finn and Finn × Texel sheep.

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169 *Table 3. The distribution and average of NLB in different genotypes in two sheep breeds*

	Finn			Finn × Texel		
NLB	*AA*	*AB*	*AC*	*AA*	*AB*	*AC*
0	*2.88%*	*1.23%*	*0.82%*	*3.66%*	*0.00%*	*0.00%*
1	*9.47%*	*5.76%*	*2.47%*	*23.17%*	*0.00%*	*6.10%*
2	*17.28%*	*25.51%*	*4.12%*	*47.56%*	*2.44%*	*7.32%*
3	*6.17%*	*18.11%*	*0.41%*	*4.88%*	*2.44%*	*2.44%*
4	*0.82%*	*4.12%*	*0.41%*	*0.00%*	*0.00%*	*0.00%*
6	*0.00%*	*0.41%*	*0.00%*	*0.00%*	*0.00%*	*0.00%*
Average NLB	*1.80*^{b}	*2.36*^{a}	*1.65*^{b}	*1.68*^{b}	*2.50*^{a}	*1.77*^{b}

170 * Levels not connected by the same letter are significantly different (p<0.05).
171 ** Duncan's multiple range test was applied to determine differences between averages within each breed
172 separately.

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174 Finding major genes influencing fertility is important to improve genetic gain in New
175 Zealand sheep breeds. Generally, genetic gain is very slow for fertility traits because firstly, it
176 cannot be measured before maturity and secondly these traits expressed in only one sex.
177 Moreover, the accurate measurement of the fertility traits can be difficult and expensive at
178 farm level. On the other hand, the high cost of SNP genotyping limits commercial use and a
179 large training population is often required to calculate the SNP effects. Accordingly, the use
180 of genomic selection with high-density SNP markers is currently somewhat limited in sheep
181 breeding programmes. In contrast, the identification and use of single gene markers for key
182 traits can be an appropriate and suitable method to improve production performance.

183 Table 3 shows that in Finn × Texel crossbred ewes, the frequency of the variant
184 associated with increased litter size (i.e. variant *B*) was low. Whereas in Finn ewes, the

185 frequency of the *B* variant was relatively high (0.27) and the average litter size was
186 significantly higher than the Finn × Texel sheep ($p < 0.05$). The results of this investigation
187 suggests the *GDF9* variation increases litter size by about 0.7 lambs per ewe lambing The
188 average litter size for *AA* vs *AB* genotypes in Finn and Finn × Texel ewes was 1.80 vs 2.36
189 and 1.68 vs 2.50, respectively.The novel variant *C* described here did not appear to have an
190 effect on litter size, but more sheep will need to be studied to confirm this observation.
191 Variant C contains the amino acid substitution (V371M) described by Hanrahan et al. in
192 Cambridge and Belclare sheep, but the frequency of this variant was very low in our study
193 and we could not find any association between allele C and litter size ($p<0.05$). (Mullen and
194 Hanrahan, 2014) reported that in Finnish Landrace ewes there was no statistically significant
195 effect of a single copy of V371M on litter size. (Kaczor, 2017)found that in Olkuska ewes
196 with one copy of the V371M substitution showed an increase in litter size of +0.55 lamb,
197 while those with the V332I mutation showed a decrease of 0.18 lamb/litter.

198 The finding of 3 variants using the PCR-SSCP approach suggests a justification for
199 looking at more sheep using this approach, and not just Texel and Finn sheep. This lays a
200 strong theoretical foundation to further this type of analysis with more common NZ breeds,
201 not least the main maternal breeds, the Romney, Perendale and Coopworth.

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