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New test for genotyping Booroola mutation (*FecB*) using high resolution melt curve analysis.

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Chapter 1 - Review of Literature: The Booroola Mutation

1.1 Introduction

The Booroola mutation (*FecB*) increases the ovulation rate, and thus fecundity, in ewes and therefore has been a mutation that has been thoroughly studied. The discoverer of the mutation's effect, in the 1950s, was Dr. Helen Newton Turner (Turner, 1978). This work ultimately led to the introgression of the *FecB* gene into many different breeds and countries (Davis, 2009). Despite the discovery of the Booroola effect in Australia, the gene is also naturally found in several native breeds of sheep in China and India where it is thought to have originated (Turner, 1982). Since the description of the Booroola effect, many other fecundity genes have been identified and studied to increase the prolificacy of sheep as the polygenic trait of fecundity has a low heritability (Abdoli et al., 2016). The Booroola mutation was subsequently mapped to the bone morphogenetic protein 1B receptor (BMPR1B) and is shown to be the causative mutation affecting ovulation rates (Mulsant et al., 2001). The mutation causes many different effects on the ovary, culminating in an additive increase in ovulation rates. Consequently, ewes that possess the Booroola mutation have a higher number of multiple lambs per pregnancy and therefore a potentially desirable trait. However, homozygous carriers of the *FecB* in most breeds and production systems have excessive litter sizes and lamb mortality therefore the heterozygote carrier is the desired genotype (Walkden-Brown et al., 2009). The current genotyping method is by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) developed by (Wilson et al., 2001). High resolution (HRM) melt curve analysis is a more recent genotyping method (Wu et al., 2008), with several advantages over RFLP-PCR, and has the potential to be used for genotyping sheep for the Booroola mutation. Developing a test based on HRM would provide a simpler and lower cost method for *FecB* to differentiate non-carrier ($FecB^{++}$), homozygote ($FecB^{BB}$) and heterozygote ($FecB^{B+}$) sheep. The gene is not currently widely utilized in the Australian sheep population due to the low survival rates of lambs but is used in some commercial wool and meat production systems (Earl et al., 2017). The Booroola mutation is an effective way to rapidly increase the prolificacy in sheep and a cheaper, more accessible test for the presence of the mutation which would facilitate future research and use of *FecB* in the industry.

1.2 History and Discovery of the Booroola mutation

1.2.1 Discovery of the effects of the Booroola mutation

The discovery of the Booroola fecundity gene (*FecB*) was a significant step in influencing the lambing rates in sheep breeds. The influence of the gene was first seen in 1953 by Dr. Helen Newton Turner in a Merino flock, from a property named 'Booroola' near Cooma (NSW), owned by the Sears brothers. The Sears brothers had been selecting for high lambing rates for 10 to 15 years and by 1959 their lambing percentage were at 170 - 180% (Turner, 1978). Turner (1978) had developed an experiment to determine the influence of selection for lambing rates with 3 groups of ewes between 1954 and 1972. The flock groups were a selection of ewes born as single lambs (O), ewes born as a twin lambs (T) and the Booroola ewes (B) that were purchased from the Sears brothers in 1959 (Turner, 1978). Management methods changed over the years but O ewes were joined to single born rams, T ewes were joined with twin-born rams and the B ewes were joined to a Booroola ram throughout the entirety of the experiment (Turner, 1978). To analyze the data the management, environmental and dam age was taken into consideration (Turner, 1978). Figure 1 below shows the percentage of ewes that had at least one multiple birth over 3 lambings.

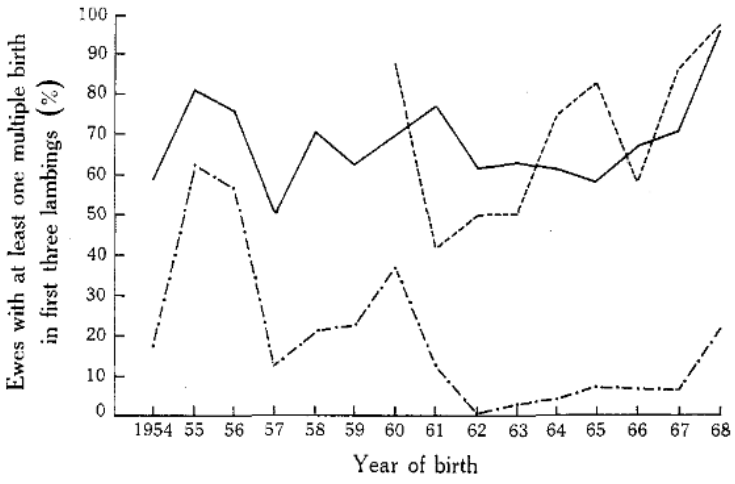


Figure 1: Percentage of ewes with at least one multiple birth in their first three lambings. Twin-born ewes in group T were given a correction of + 10.0, but no other corrections applied. -.-.-.- 0; -T. ----- B. (Turner, 1978).

This figure shows the fluctuation of the lambing rates of the different groups but it also shows the clear difference of multiple births between selecting for twins and the Booroola sheep compared to single born selection. It was hypothesized that the environmental effects on the flock influenced lambing rates and created the fluctuations (Turner, 1978). In 1963, the

selection method for ewes altered and the O ewes were culled if they had multiple lambs and the T and B ewes were culled if they didn't lamb or if they didn't produce at least one multiple birth in 3 lambings (Turner, 1978). Table 1 shows the lambing rates of ewes born in 1969 and 1970, revealing that the lambing rates for the B ewes were significantly higher than either of the other groups.

Table 1: Number of lambs born per ewe joined per year up to 1972, for 1969 and 1970 drops. (Turner, 1978)

Year of ewe birth	Number of lambings	Group	Number of Ewes	Lambs born/ ewe joined/ year
1969	2	O	13	0.96
		T	59	1.27
		B	80	1.69
1970	1	O	11	1.07
		T	40	1.15
		B	29	1.66

Based on this experience, some ewes were sold to other sheep producers who also found that the Booroola crossbreds had higher lambing rates than their own stock (Turner, 1978). Turner (1978) concluded that the Booroola flock had the highest annual lambing performance and had the potential to increase lambing rates in Merino flocks. She did also note however, that lambing survival was affected negatively by the increased lambing rates. Dr. Helen Newton Turner made an important discovery with the Booroola flock that has had a significant influence on the research into increasing lambing rates in ewes.

1.2.2 Discovery of High Ovulation Rates

After Turner's discovery, some producers purchased sheep from the Booroola flock and studies in the 1970s and 80s discovered that the Booroola ewes had higher ovulation rates than non-carriers. Piper & Bindon (1976); Kelly et al. (1980); (Davis et al. 1982) conducted studies that showed that there were higher ovulation rates in Booroola-cross flocks than the pure-bred sheep. Table 2 below are the results of a study by Piper et al. (1976) showing the ovulation rates of different strains of Merino Booroola crosses compared to other pure-bred strains of Merinos.

Table 2: Endoscopy ovulation results of Merino Booroola crosses compared to pure-bred Merinos (Piper et al., 1976).

Location of Study	Strain of Merino	Number of ovulations					
		1	2	3	4	5	Mean
New Zealand	Fine Wool	62	12	0	0	0	1.16
	Booroola - cross	33	41	19	6	2	2.04
Western Australia	Collinsville	27	3	0	0	0	1.10
	Booroola cross	16	37	11	0	0	1.92
	Peppin	9	4	0	0	0	1.31
	Booroola cross	12	12	8	0	0	1.88
Riverina, NSW	Collinsville	30	8	0	0	0	1.21
	Booroola cross	28	10	2	0	0	1.35
New England, NSW	Medium Non-Pepin	16	2	0	0	0	1.11
	Booroola cross	8	8	5	0	0	1.86

These results show that the Booroola crosses have a higher average of ovulations than the pure-bred Merinos. The study by Kelly et al. (1980) found similar results comparing Romney Booroola crosses against Romney ewes. This determined that the crosses have a significant increase in ovulations and multiple births. Kelly et al. (1980) also determined that there was a significantly negative impact on birth live weight and fleece weight. These studies led to selecting Booroola ewes based on their ovulation rates in other research papers. Using the ovulation rates to identify the Booroola ewes proved to be more efficient compared to litter size as there are high embryonic losses associated with large numbers of ovulations (Hanrahan 1974). Piper et al. (1976); Kelly et al. (1980)confirmed that the Booroola crosses had higher fecundity levels than pure-bred.

Davis et al. (1982), was also a significant contributor to the development and research of the Booroola mutation. They suggested that there was a single locus responsible for the high lambing rates of the Booroola Merinos (Davis et al., 1982). Their selection criteria denoted that ewes producing 3 or more ova possessed the Booroola mutation. This criteria also influenced other subsequent research on the Booroola mutation (Davis, 2009). The selection criteria characterizes *FecB*$^{B+}$ ewes as producing more than three ovulations and *FecB*^{BB} ewes

as producing more than five ovulations per cycle (Davis et al., 1982). Using Mendelian theory, their gene segregation experiment measured the ovulation rates of F1 non-carrier Merino cross Booroola, Booroola back-cross (¾ Booroola) and Merino back-cross (¼ Booroola) progeny (Davis et al., 1982). The selection criteria for a Booroola carrier was that ewe ovulation rate were ≥ 3 and those less than were assumed to be non-carriers (Davis et al., 1982). Table 3 below shows the proportion of the F1 progeny that ovulated 3 or more eggs, the results were expected to show that the rams were Booroola homozygous (Davis et al., 1982).

Table 3: Number of F1 progeny of each Booroola-type sire and non-carrier Merino dams, and the proportion of progeny with at least one record of ≥3 litter size/ovulation rate in 3-6 records. (Davis et al., 1982)

Sire	**Litter size**		**Ovulation proportions**		
	No. of progeny	**Proportion ≥3**	**No. of progeny**	**Proportion ≥3**	**Proportion ≥3 (adjusted) +**
A	41	0.61	37	0.62	0.63
B	44	0.30**	39	0.46	0.47
C	46	0.48	32	0.5	0.51
D	41	0.27**	39	0.31*	0.32*
E	31	0.45	36	0.56	0.57
F	40	0.35	37	0.49	0.5
G	25	0.56	25	0.88**	0.91**
H	41	0.34*	38	0.55	0.56
Mean		0.41		0.53	
Merino (control)	118	0.03	111	0.03	

* Proportion significantly different from 0.50 (P<0.05)

**Proportion significantly different from 0.50 (P<0.01)

+ Adjusted for number of records

The results were significantly different from the hypothesized results that all rams were *FecB*^{BB}. Using the Mendelian theory, the rams should have had progeny that all ovulated 3 or

more ova. From these results, the Booroola rams were assumed to be heterozygous as 50% of their progeny produced ≥3 litter size/ovulation rate (Davis et al., 1982). Therefore, the expected results were adjusted. Continuing with the Booroola backcross and Merino backcross, their results showed that the Booroola mutation fitted the Mendelian theory. The results supported the hypothesis that the Booroola effect was influenced by a major gene as there was no significant difference between the expected and the actual results (Davis et al., 1982). The results of this experiment had a significant influence on the Booroola studies as researchers looked to determine which single locus influenced the fecundity in Booroola Merinos (Davis et al., 1982). During this study, Davis labeled the suspected allele and locus *F* but after the discovery of other fecundity genes the allele was labeled as Booroola and the locus was labeled *FecB* (Davis, 2009). Furthermore, the selection criteria of this experiment proved to be an effective method to select for the Booroola mutation and therefore was used in other research.

1.2.3 Determination of Gene Responsible

Many studies were conducted to locate the *FecB* locus responsible for the high fecundity in Booroola ewes. By testing different markers suspected to influence sheep and cattle reproduction the *FecB* mutation was determined to be linked to the human markers on chromosome 4q (Montgomery et al., 1993). Montgomery et al. (1994) confirmed that the *FecB* mutation was linked to the human 4q chromosome and also determined that the gene was located on the sheep chromosome 6. Three different papers in 2001 determined that the mutation was a single nucleotide polymorphism (SNP) located on the bone morphogenetic protein receptor 1B (BMPR1B) (Mulsant et al., 2001; Souza et al., 2001; Wilson et al., 2001). Åström et al. (1999) determined that the human BMPR1B was located on human chromosome 4q21–25 which correlated with the region that the Booroola mutation was determined to be on by Montgomery et al. (1993) (Souza et al., 2001). Furthermore, Montgomery et al. (1995) found that the Booroola mutation was between genes for secreted phosphoprotein 1 (SPP1) and epidermal growth factor (EGF) on the chromosome 6 of sheep. BMPR1B also was suspected because type-β transforming growth factors (TGFβ) are highly involved in signaling during gametogenesis to granulosa cells, oocytes and primordial germ cells (Lawson et al., 1999; Shimasaki et al., 1999). In 1987, the BMPR1B receptor was identified as belonging to the TGFβ receptor family (Massagué, 1987). There are two types of receptor

families for TGFβ, type I and type II (Massagué, 1998). The BMPR1B were determined to belong to a group of 6 serine-threonine kinase receptors known as active receptor-like kinase (ALK) in the type I TGFβ receptors (Yamashita et al. 1995). BMPR1B was determined to be ALK-6 and has an influence on the bone morphogentic protiens (BMP), hence the change of name from ALK-6 to BMPR1B (Yamashita et al., 1995). The BMP have been found to influence fertility, oocyte growth and ovaries and the *FecB* mutation inhibits certain aspects of their function (Wilson et al., 2001). Bone morphogenetic proteins (BMP) contribute to the mechanisms within the ovaries (Shimasaki et al., 1999) and the BMPR1B regulates some of the BMPs therefore it was logical that the BMPR1B gene was the site of the *FecB* mutation (Souza et al., 2001). The effect of BMPR1B on the fecundity in sheep is more thoroughly discussed in section 1.4. Due to the research showing that the BMPR1B had subsequent effects on the BMP, and therefore the fecundity of sheep, it was suspected and then proven that the *FecB* mutation was located on the BMPR1B locus in the Mulsant et al. (2001); Souza et al. (2001); Wilson et al. (2001) papers.

1.3 Other Fecundity Genes

The Booroola mutation was the first mutation to be identified to have affected ovulation rates in sheep but since then other mutations have been discovered that influence sheep fecundity. The use of genes that increase prolificacy in sheep is widely researched as selecting for multiple births has a low heritability. The heritability of litter size has been thoroughly studied in many breeds and can range between 0.01 and 0.26 depending on the breed (Safari and Fogarty, 2003). There are many different genes that influence the ovulation rates within sheep and therefore there is polygenetic control of fertility in sheep in addition to the single gene effects (Abdoli et al., 2016). Studies have discovered causative mutations on major genes that influence the prolificacy in certain breeds which enables sheep breeds to gain greater prolificacy in a shorter amount of time than through selection for polygenic traits. These gene mutations have different allele locations and differ between sheep breeds therefore, knowledge of the genes is still being researched (Abdoli et al., 2016). Booroola, Inverdale, FecG, FecL are three prolific mutations identified on the BMPR-1B, BMP15, GDF9 and B4GALNT2 genes respectively, and genetic testing has been developed for them (Abdoli et al., 2016). Woodlands and Thoka genes have had their inheritance mode identified however the specific mutation has not been located and genetic testing is not available

(Towe, 2014). Other prolific genes have been putatively suggested in some breeds as they have been tested for the Booroola and Inverdale gene with negative results but produce high ovulation and lambing rates. These breeds include Belle-Ile and Perendale breeds and require further research to determine the cause of the increased fecundity. Table 4 is a summary some of the known fecundity mutations, their distribution and base change. The effect of the genes has been researched to determine the influence the genes have on each other. Some homozygous genes cause streak ovaries and it has been observed that having multiple fecundity genes may also cause infertility for instance. It has been determined that two copies of a BMP15 ($FecX^{I}$, $FecX^{H}$, $FecX^{B}$, $FecX^{G}$) mutation causes infertility (Towe, 2014). In other circumstances, crossbreeding a Booroola and a Inverdale mutation increase ovulation rates to an average of 4.36 (Towe, 2014). Furthermore, it was discovered that a ewe with Booroola, Ivendale and Woodlands gene produced an ovulation rate of 5 at 1.5 years old and 12 at 2.5 years (Towe, 2014). The interactions between these genes needs much more research to determine the impact. These genes may have a big impact in future lambing rates in breeds and the development of readily available genetic testing will facilitate this.

Table 4: Known Major Genes for Prolificacy in Sheep (Abdoli et al., 2016)

Gene	**Chromosome location**	**Allele**	**Nucleotide change**	**Breed**	**Distribution**	**References***
BMPR1B Booroola	*6*	*FecB*	g.746A>G	Booroola Merino	Australia	Souza et al. (2001)
				Javanese	Indonesia	Davis et al. (2006)
				Small Tailed Han	China	Chu, Liu, et al. (2007)
				Hu	China	Chu, Liu, et al. (2007)
				Garole	India	Fogarty (2009)
				Kendrapada	India	Fogarty (2009)
BMP15 Inverdale	*X*	*FecX G*	g.718C>T	Belclare/Cambridge	Ireland and England	Hanrahan et al. (2004)
		FecX B	g.1100G>T	Belclare	Ireland and England	Hanrahan et al. (2004)
		FecX I	g.896T>A	Romney	New Zealand	Davis (2005)
		FecX H	g.871C>T	Romney	New Zealand	Davis (2005)
		FecX L	g.962G>A	Lacaune	France	Bodin et al. (2007)

		FecX R	17 nt deletion (525-541)	Rasa Aragonesa	Spain	Martinez-Royo et al. (2008)
		FecX O	g.1009A>C	Olkuska	Poland	Demars et al. (2013)
		FecX Gr	g.950C>T	Givette	France	Demars et al. (2013)
GDF9 FecG	*5*	*FecG H*	g.1184C>T	Belclare/Cambridge	Ireland and England	Hanrahan et al. (2004)
		FecG T	g.1279A>C	Icelandic	Northern Europe	Nicol et al. (2009)
		FecG E	g.1034T>G	Santa Inês	Brazil	Silva et al. (2011)
B4GALNT2 FecL	*11*	*Fec L*	g.803A>G	Lacaune	France	Drouilhet et al. (2009)

*Access these sources from (Abdoli et al., 2016)

1.4 Booroola Mutation Effect

1.4.1 Effects on follicular and ova development

The Booroola mutation effects the processes of the BMPR1B gene and how this influences ovarian function, which is not completely understood, however it is known that BMPR1B regulates BMP. A study by Ruoss et al. (2009) suggested that the BMPs affect the signaling pathway to the ovaries and either decreases follicle atresia or increases follicle maturation. The mutation causes an attenuation in the BMP production and therefore inhibits the role of the BMP in the ovaries. Rouss et al. (2009), found that the rate of primordial follicle recruitment in the ovaries decreased with the presence of the Booroola mutation compared to a *FecB*$^{++}$ Merino. This suggested that BMP increases the rate at which primordial follicles and follicle recruitment occur and the BMPR1B mutation slows this process and decreases the rate of atresia of recruited follicles (Rouss et al., 2009). The Booroola mutation also increases the production of progesterone and follicle secreting hormone (FSH) as the BMP is inhibited. The increase in progesterone and FSH causes the increase in follicle maturation and ovulation rates (Ruoss et al., 2009). It has been recently discovered that the presence of the Booroola mutation decreases the production of anti-Müllerian hormone (AMH) (Estienne et al., 2015). The study by Estienne et al. (2015), found that the presence of the Booroola mutation caused the follicles to mature at a smaller size, and increase the number of antral follicles and the ovulation rates in ewes. It has been found that lower AMH levels increases follicle growth activation and follicles sensitivity to FSH and luteinising hormone (LH) therefore decreasing follicle atresia (Estienne et al., 2015). These factors are suggested to be

the reason why the Booroola ewes have higher ovulation rates, as the increase in smaller mature follicles allows more follicles to develop to dominant follicles and ovulate (Estienne et al., 2015). The Booroola mutation on the BMPR1B, has an effect on BMPs which is not completely understood however, clearly it has a role in follicle development and the increase in the ovulation rates in ewes.

1.4.2 Effect on fetal and lamb growth and survival

The Booroola mutation increases ovulation rates and litter size but it also has been recorded by many sources that lambing survival decreases. It has been suggested that the BMPR1B influences the development of the fetus and other organs which effects the lambs' growth and survival. A study conducted by Smith et al. (1993), observed the effects of the Booroola mutation on litter size, ovarian development and fetal life during pregnancies. The study suggested that the fetal weight within a ewe with 1 or more *FecB* genes, during the pregnancy, was less than a $FecB^{++}$ but this may be due to the litter size rather than a direct effect of the Booroola mutation. Smith et al. (1993) found that the Booroola carrier ewes had a slower, retarded ovarian development than the $FecB^{++}$ (Smith et al., 1993). It suggests that the development of follicles, fetal size, and fetal organs in Booroola carriers are slower than $FecB^{++}$ which impacts on the lambs' rate of growth *in utero* (Smith et al., 1993). The Booroola mutation increases ovulation rates and litter size due to its impact on follicular development furthermore has an impact on fetal development.

The Booroola mutation appears to have an additive effect on the prolificacy of sheep depending on the number of copies of the mutation are present (Fogarty, 2009). The high ovulation rates and litter sizes have a major impact on the energy stores of a ewe and it may be argued that sheep are not physiologically designed to support the high prolificacy that the Booroola mutation produces (Hinch, 2009). Studies find that there is a great loss of lambs both in utero and after parturition between ovulation recordings and marking time for ewes that possess the Booroola mutation. Walkden-Brown et al. (2009) found that the ovulation rates per ewe were 1.27, 2.48 and 3.86 for $FecB^{++}$, $FecB^{B+}$ and $FecB^{BB}$ ewes respectively, however the weaning rates were significantly lower at 0.94, 1.36 and 1.09 respectively. Farquhar et al. (2006) reported that with higher ovulation rates there is also an increase in barren ewes as they found that only 4.1% of $FecB^{++}$ ewes were barren compared to 7.6% of $FecB^{B+}$ and 16.4% of $FecB^{BB}$ ewes were barren. The loss of lambs can be at embryonic, fetal,

or post parturition stages and the ewe energy stores before, during and after pregnancy have a big impact (Hinch, 2009). The nutritional supply affects embryo survival and uterine efficiency, which is greatest with high nutrition prior to and during mating.

Kleemann et al. (1990) indicated that fertilisation failure in ewes with the Booroola mutation was not a large component to the reproductive loss. Fetal losses increase in ewes that have ovulation rates that are higher than 4 and is associated with placental insufficiency (Hinch, 2009). Fetal mortality has been recorded up to 30% between day 30 and 70 in highly prolific ewes by Fahmy et al. (1994). Hinch (2009) showed that fetuses at 146 days of pregnancy in ewes with twins have higher numbers of placentomes per fetus than triplets. The study also showed that the triplet fetuses weighed less, at 3.4kg, than the twin fetuses, at 3.9kg, indicating that the triplets have a higher risk for mortality (Hinch, 2009). By the last trimester of the pregnancy the ewe has limited capability to consume the nutritional requirements for fetal growth, especially ewes with large litter sizes (Hinch, 2009). The energy stores of the ewe therefore has an impact on the fetal growth and ewes with higher litter sizes have a limited supply of energy fat reserves and this consequently impacts on the fetal growth (Hinch, 2009).

The Booroola mutation causes high ovulation rates and thus high litter sizes which has an effect on lamb growth and mortality. Lambs born in litter sizes of 3 or more generally have a smaller birth weights with a greater risk to mortality than those born as twins or singles (Hinch, 2009). The maternal ability of the mother is a major contributing factor to lamb survival and it is found that most lamb deaths between 24 and 48 hours after birth are due to starvation (Hinch, 2009). Ewes with multiple lambs also have a higher risk of hypoglycaemia and hypoxaemia during late pregnancy which impacts on the lambs' growth and survival post-partum. Studies suggest that lambs born as multiples do not physiologically adapt well after birth and have a lower probability of survival and a higher risk to hypothermia than single and twin lambs (Hinch, 2009). Furthermore, lambs born as multiples will have lower levels of colostrum available to them and may also receive milk later than normal (Hinch, 2009). This impacts on the lambs ability to survive and its growth rate as they have less milk available to them (Hinch, 2009). The Booroola mutation increases the ovulation rates and the litter sizes therefore the risk of lamb mortality rises. Studies such as Walkden-Brown et al. (2009) on Booroola Merinos show that the loss of lambs is much greater in *FecB*^{BB} ewes and therefore

is undesirable however, the *FecB*$^{B+}$ ewe is desirable as lamb survival is greater and it produces more lambs than the *FecB*$^{++}$ and *FecB*^{BB} ewes. Gootwine (2009) also demonstrated that the lamb survival of *FecB*^{BB} in Afec-Awassi breed was lower than the *FecB*$^{B+}$ and *FecB*$^{++}$ and recommended the *FecB*$^{B+}$ as the desirable genotype. As the Booroola mutation effect each breed differently, it depends on the breed which genotype is best suited however, most studies conclude that the *FecB*$^{B+}$ is the most desirable genotype. An increase in ovulation rates and litter size increases the risk of lamb mortality however the *FecB*$^{B+}$ produces a litter size that the ewe can manage.

1.5 Detection of *FecB* carriers

The location of the *FecB* mutation was discovered simultaneously by Mulsant et al. (2001); Souza et al. (2001); Wilson et al. (2001). All three studies determined the location of the mutation using reverse-transcriptase polymerase chain reaction (RT PCR) and then sequencing the purified samples (Mulsant et al., 2001); Souza et al. (2001); Wilson et al. (2001). RT PCR produces DNA complementary (cDNA) to mRNA so the cDNA is the functional DNA without the exons and therefore only the functional *FecB* mutation will be replicated. By sequencing they were then able to determine the location of the mutation at Q249R on the ovine chromosome 6 which changed the base A to a G (Wilson et al., 2001). Another site of mutation was located at 1113 that changes C into a A however it doesn't change the amino acid coding and therefore does not affect the BMPR1B function (Souza et al., 2001). Figure 2 below shows the difference between the three genotypes and the base changes: *FecB*^{BB} GG, *FecB*$^{B+}$ A/GG and *FecB*$^{++}$ AG (Souza et al. 2001).

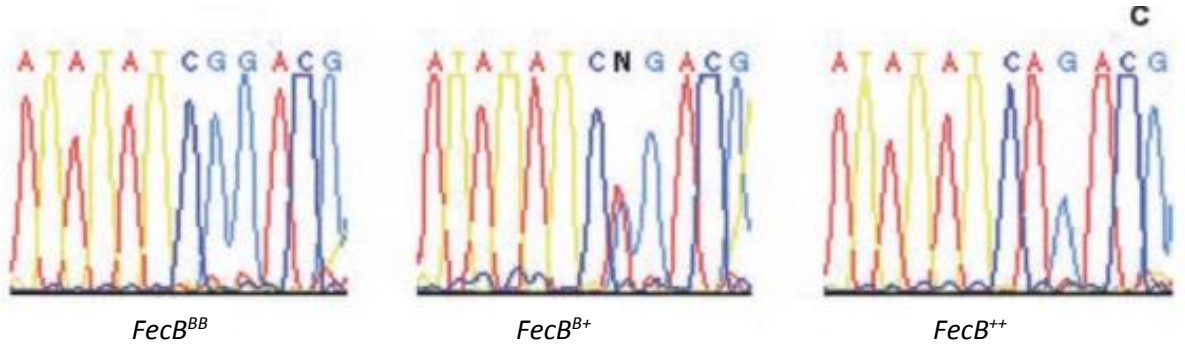


Figure 2: Sequence obtained for each of the genotypes and illustrating the presence of both homozygous in the heterozygotes. (Souza et al. 2001)

There were slight differences between the three studies but all produced the same conclusion about the location of the *FecB* mutation. Wilson et al. (2001) also developed the restriction fragment length polymorphism - polymerase chain reaction (RFLP-PCR) method to test and identify the $FecB^{BB}$ and $FecB^{B+}$. The discovery of the location of the *FecB* mutation lead to the development of the RFLP-PCR test and has further identified the role of the BMPR1B.

1.5.1 Restriction Fragment Length Polymorphism –Polymerase Chain Reaction

Restriction fragment length polymorphism – polymerase chain reaction (RFLP-PCR), is a method used to determine the Booroola genotype of sheep. The RFLP-PCR method uses the PCR to replicate the DNA sequence that contains the specific gene, which is then digested with the selected restriction endonuclease, causing the length of the base pairs to vary depending on the genotype (NCBI, 2016). Then gel electrophoresis is used to separate the fragments by size, enabling accurate genotyping. Wilson et al. (2001) developed the RFLP-PCR method for the *FecB* mutation which was then patented in 2002 by Wilson and Wu (2001). The RFLP-PCR method developed used Primers 5'-GTCGCTATGGGGAAGTTTGGATG- 3' and 5'-CAAGATGTTTTCATGCCTCATCAACACGGTC- 3' to replicate the *FecB* mutation and then the restriction endonuclease AvaII was added to fragment the DNA (Wilson et al., 2001). The AvaII fragments the site of mutation when the G base is present, therefore making a DNA strand with the *FecB* mutation shorter (Wilson et al., 2001) The DNA was then run through 2.5% agarose gel electrophoresis to determine the Booroola genotype. After the fragmentation, the $FecB^{BB}$ DNA strands were 110 base pairs, the $FecB^{++}$ would be 140 base pairs and the $FecB^{B+}$ had fragments of both lengths. Figure 3 shows the electrophoresis result of the Wilson et al. (2001) paper.

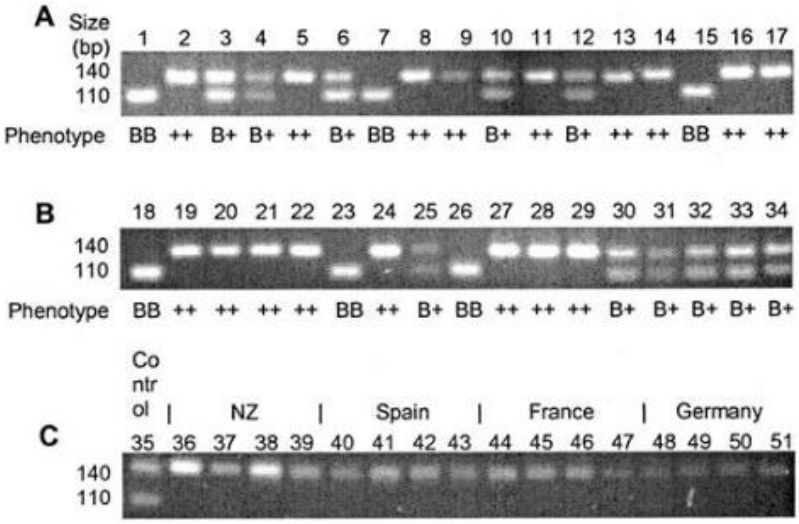


Figure 3: RFLP analysis showing the A G transition, resulting in an *Ava*II site being generated for animals that contain a 'G'. Panels **A** and **B** show representative animals from the backcross flock with the phenotype assigned shown underneath. Panel **C** represents non-Booroola merinos from the countries shown with lane 35 containing a B+ control (Wilson et al., 2001).

These results showed that the RFLP-PCR method is a successful method to identify the Booroola genotypes of sheep. This process however is time consuming and costly as it is a multistep process with a risk for potential sample contamination. The method is currently offered by 'GenomNZ®' which is a DNA testing lab in New Zealand and due to the patent, commercial Booroola DNA testing in Australasia is limited to that lab. To test for the Booroola mutation in a flock, blood samples on a $1.50 blood card are sent to the GenomNZ® lab and the results should be received within 4 weeks. Depending on the number of samples the cost of testing can range between $20 to $35 per sample (Muriel Dufour *pers. comm.)*.

1.5.2 SNP Microarray

Genotyping the *FecB* has been done via the RFLP-PCR method since its development by Wilson et al. (2001) however, since the development SNP microarray technologies genotyping methods are evolving. SNP microarrays can genotype thousands of genetic polymorphisms simultaneously by using annotated markers for each SNP (Kawęcka et al., 2016). BeadArray is the technology most commonly used practice for SNP genotyping which uses 3 micron silica beads with probes, designed for specific SNP markers, attached in microwells on the microarray surface (Kawęcka et al., 2016). Amplified and fragmented DNA is applied to the microarray and the DNA hybridizes with the probes to highlight nucleotides with fluorescence which enables the identification of the sheeps' genotype (Kawęcka et al., 2016). This method

has low error rates and is easier to calibrate in laboratories compared to RFLP-PCR method for genotyping (Kawęcka et al., 2016). Genotyping the *FecB* gene is now becoming available via SNP chip genotyping as well; for example, it forms part of the Sheep CRC 15k SNP test for parentage which will differentiate between homozygous and heterozygous carriers (Julius Van der Werf and Klint Gore *pers. comm.*). The newly released, April 2017, Sheep CRC Parentage SNP test includes the *FecB* SNP and the test currently costs $22 + sampling costs (Julius Van der Werf and Klint Gore *pers. comm.*). The SNP microarray genotyping method supplies the genotypes of many different genes and is increasingly utilized in sheep breeding strategies.

1.5.3 Polymerase Chain Reaction (PCR) – High Resolution Melting (HRM) Curve Analysis

Polymerase chain reaction – high resolution melting (PCR-HRM) is potentially an alternative method to identify the *FecB* genotype of sheep. Like the RFLP-PCR, the primers and PCR are used to amplify a specific DNA sequence around the causative polymorphism. However, instead of using gel electrophoresis on the final product, an intercalating dye is incorporated into the amplicons, up to a 304bp length, and a HRM cycle in certain real-time PCR machines is used to record the temperature as the DNA denatures and differentiate the genotypes on this basis Gundry et al. (2003). The development of HRM curve analysis with real-time PCR was first introduced by Lay and Wittwer (1997); Ririe et al. (1997) and was further developed by Gundry et al. (2003) to genotype. The HRM analysis relies on the presence of a fluorescent dye within the sample and as the temperature gradually heats up from 50°C to around 95°C the DNA strands denature and separate, inactivating the dye so the fluorescence fades (Tong and Giffard, 2012). The fluorescence is measured throughout this process and 2 graphs are produced which are then used for genotyping. The HRM curve results can change because of single base (A,G,T,C) change and therefore this has been used for SNP genotyping, DNA mapping, mutation scanning, species identification, zygosity testing, DNA methylation analysis, and DNA fingerprinting. The Booroola mutation changes an A to a G therefore, in theory HRM curve analysis should be able to detect the change. Wu et al. (2008) conducted a study which is an example of SNPs genotyping for almonds as an easy and low-cost method of genotyping. Leaves from 25 almond cultivars were used to extract DNA which were then run through the HRM-PCR process. Both the PCR and HRM processes were conducted in the RotoGene3600 machine that replicates the DNA strands and analyses the fluorescence

presence. Figure 4 below provides an example of the HRM analysis discriminating SNPs in one amplicon.

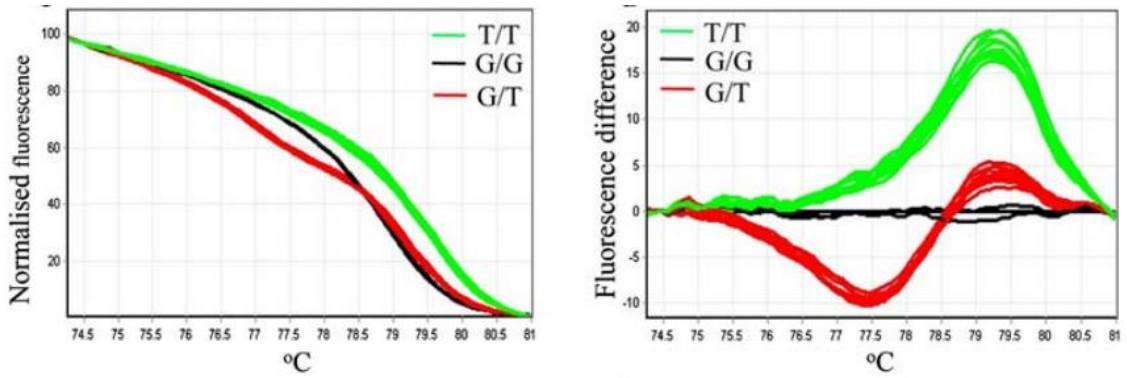


Figure 4: High resolution melting curve analysis for the Xneas109a mutation in almonds. First graph is the normalized high resolution melt curve, and the second is the melt curve. TT represents non-carrier, GG is homozygous and G/T is heterozygous (Wu et al., 2008).

It is clear, that there is a difference between the curves of the three genotypes. As the HRM process is basically a two-step process, DNA extraction and then placing the samples in the Rotorgene3600, the process of SNP genotyping is time efficient and low-cost (Wu et al., 2008). As the *FecB* has been identified as a mutation at Q249R on chromosome 6 from a base A to G, theoretically the SNPs genotyping with HRM-PCR should be a suitable method of identification.

1.5.4 DNA Sample Collection Methods

Genotyping requires a submission of a sample that contains DNA and for sheep this is generally via dried blood card samples, tissue samples, or fresh or frozen blood tubes. Each sample requires a different method to extract the DNA for genotyping. The current method for collecting, transporting and extracting DNA for Booroola genotyping is via FTA blood cards sent to GenomNZ®. The blood cards are a suitable method for transporting DNA samples as they able to be stored at room temperature (GenomNZ®, 2017). There are two types of blood cards, standard FTA card and FTA Elute cards. In standard FTA cards, the DNA binds to the matrix of the card from which it is released after washing away contaminants (GEHealtcare, 2010). Compared to the FTA Elute cards which binds contaminants in the matrix and elutes DNA directly in the elution buffer (GEHealtcare, 2010). Standard FTA card DNA extraction methods involve the use of kits with specific buffers to remove the DNA from the card. DNA extractions methods and kits include: organic extraction, Chelex 100 resin, QIAamp™ DNA

Investigator Kit, illustra™ tissue and cells genomicPrep Mini Spin Kit, and DNA IQ™ Kit (GEHealtcare, 2010). These methods extract the DNA from the matrix of the cards and each method removes a different amount of DNA from the cards (GEHealtcare, 2010). Figure 5 below shows the different amount each extraction method releases.

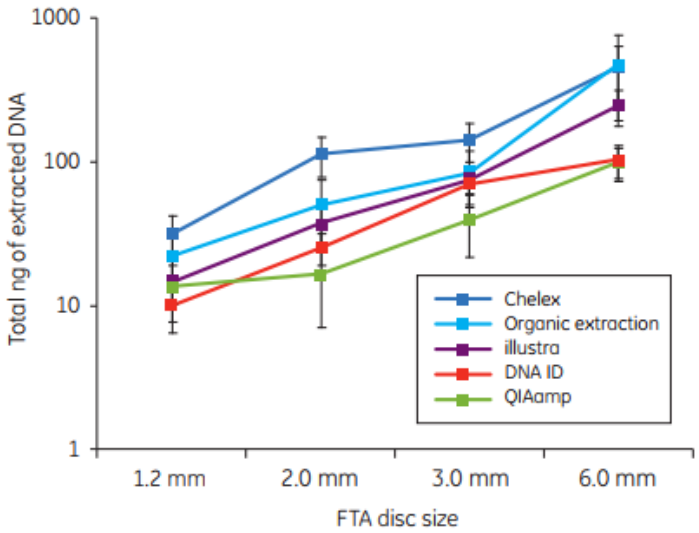


Figure 5: Amount of DNA extracted from blood FTA cards using 5 extraction methods (GEHealtcare, 2010).

The FTA elute cards produce better quality DNA that is suitable for qPCR and SNP genotyping (GEHealtcare, 2011). Song et al. (2013) tested four different methods of DNA extraction from such cards using accesible laboratory solutions and determined that they were all viable furthermore, the extracted DNA was suitable for PCR and RFLP genotyping. The use of easily available solutions provides a more affordable method of extraction compared to the methods and kits mentioned previously. The solutions were Tris–HCl (10 mM, pH7.4), Tris-EDTA (TE) buffer (10 mM, pH7.6), sterilized water and DN131 (Molecular Research Center, InC) (Song et al., 2013). Their study determined that each method extracted a sufficient amount of DNA although Tris-HCl had a greater PCR and genotyping success rate than the other solutions (Song et al., 2013). See Table 5.

Table 5: Success rates of amplifying caspase-12 C125T SNP (rs497116) by PCR and a Taqman genotyping assay using four different reagents (Song et al., 2013)

Method	**PCR success rate (PCR mix 1) %**	**PCR success rate (PCR mix 2) %**	**Taqman genotyping success rate %**

Tris-HCl	89.5	96.1	81.9
TE	73.3	88.5	70.5
H2O	72.4	78.1	79.1
DN131	87.6	71.4	70.5

DNA extractions from blood cards with relatively common solutions for PCR and genotyping provides a simple and affordable method compared to the use of kits and other methods. The use of blood cards provides a convenient method for transportation and storage of DNA samples and provide quality DNA for PCR and genotyping.

Tissue collection in sheep for genotyping is usually collected from the ear by a tissue sampling unit (TSU). Transporting tissue samples to laboratories are costly but they provide high quality and quantity of DNA. The DNA extraction from tissue is done via kits and the targeted outcome will determine the kit that is used. Table 6 provides some examples of DNA extraction kits that are used for tissue samples.

Table 6: Examples of DNA Extraction Kits from tissue and their applications (ThermoFisher, 2017)

Tissue DNA extraction Kit	**Applications of DNA Extraction Kit**	**Tissue starting material**	**Yield**	**Isolation method**	**Compatible applications**
DNAzol™ Reagent	Process the largest amount of tissue	Up to 50 mg	Up to 250 µg	Organic extraction	Cloning, qPCR, sequencing
PureLink™ Genomic DNA Mini Kit	Fast isolation of gDNA from a variety of samples	Up to 25 mg	5-10 µg	Silica membrane	Cloning, qPCR, sequencing, genotyping
PureLink™ Pro 96 Genomic DNA Purification Kit	High -yield, high-purity gDNA in a plate format	Up to 25 mg	5-10 µg	Filter plate	Cloning, qPCR, sequencing, genotyping
MagMAX™-96 DNA Multi-Sample Kit	Rapid and automated extraction of DNA	Up to 25-50 mg	10-80 µg	Magnetic beads	Cloning, qPCR, sequencing, genotyping

There are also organic extraction protocols which disaggregates the tissue sample and then adds detergent to the lyse cell membranes, as well as a proteinase for protein digestion. To separate the DNA from the lipids and proteins, phenol is added and then chloroform removes the phenol. The DNA is purified via precipitation in salt and ethanol and the final product is resolubilized in Tris-EDTA buffer (Fan and Gulley, 2001). Tissue samples are easily obtained, especially with TSU equipment and there is a low risk for contamination. Blood sampling requires either a veterinarian or an experienced personnel who can draw blood. It is also the most time-consuming procedure (Demeny et al., 1997). Fresh blood samples also require an anticoagulant such as citrate, EDTA or heparin and shipping can be expensive. DNA extraction methods from blood also use DNE extraction kits to isolate the DNA. These include an extraction buffer, a phenol chloroform extraction, an alcohol precipitation and a resuspension buffer such as Tris EDTA (Samadi Shams et al., 2011). Blood samples provide high levels of DNA and provide a large sample for multiple DNA extractions. There is a low risk of contamination but the collection of samples is time consuming. DNA extraction is vital for genotyping and the samples provided will impact on the extraction method, cost and the quality and quantity of the DNA.

1.6 Use of the Booroola mutation

1.6.1 Determining the origins of the Booroola mutation.

After the discovery of the of the high fecundity of the Booroola sheep, Dr. Helen Newton Turner began to investigate the origins of the Booroola sheep. Turner (1982) traced the Booroola Merinos from the Sears Brothers to an Egelabra strain of Merinos that originated from a shipments of Bengal sheep to Australia in 1792 and 1793. The Australian Stud Merino Flock register recorded the origins, as determined by Turner (1982), and based on an initial reports the origin was the highly fecund Garole breed in Bengal (Ghalsasi and Nimbkar, 1993). Piper and Bindon (1996) suspected that the modern relative of the Bengal sheep brought over to Australia was this ‘Garole’ sheep. Garole sheep are from West Bengal and have fleece and body characteristics that are similar to those recorded as being brought to Australia in the 1790s (Piper and Bindon, 1996). This theory was then substantiated by Davis et al. (2002) when they conducted DNA testing for the Booroola and Inverdale mutations on seven different breeds including the Garole breed. The seven breeds tested included: Javanese, Thoka, Woodlands, Olkuska, Lacaune, Belclare, Cambridge, and Garole sheep (Davis et al.,

2002). The ewes from each breed were selected for records of high ovulation or lambing rate and therefore providing ewes that could potentially have a fecundity gene (Davis et al., 2002). The study found that Javanese and Garole breeds possessed the Booroola mutation but not the Inverdale gene and the five remaining breeds contained neither genes (Davis et al., 2002). These researchers also suggested that the Booroola mutation was fixed within the Garole population and that *FecB*^{BB} was the original genotype for the breed. The results of the DNA test support Piper and Bindon's theory that the Booroola mutation originated from the Garole breed from West Bengal and was crossed with Merinos to produce the Booroola Merinos (Davis, 2009). Another 21 breeds were tested for the Booroola and the Inverdale gene by Davis et al. (2006) due their prolific reputation. The breeds studied were Romanov (2 strains), Finn (2 strains), East Friesian, Teeswater, Blueface Leicester, Hu, Han, D'Man, Chios, Mountain Sheep (three breeds), German Whiteheaded Mutton, Lleyn, Loa, Galician, Barbados Blackbelly (pure and crossbred) and St. Croix (Davis et al., 2006). This study conclude that only the Han and Hu breed had the Booroola mutation but not the Inverdale gene and remaining breeds did not possess either gene (Davis et al., 2006). This study also concluded that, like the Garole breed, the Hu sheep have a fixed *FecB*^{BB} genotype (Davis et al., 2006). Due to the significant phenotypic differences between the Garole sheep and the Hu sheep it is unclear how they are related but due to the fixed presence of the Booroola mutation it is suspected that they have a common ancestor (Davis, 2009). It is also been found that the Kendrapada (Kumar et al., 2008), Nilagiri (Sudhakar et al., 2013) and Shahabadi (Debnath and Singh, 2014) sheep of India, as well as the Zel (Jafari-Joozani et al., 2012) and Lori sheep (Nanekarani et al., 2016) in Iran have the polymorphic presence of the Booroola mutation detected. The use of the RFLP-PCR method by Wilson et al. (2001) enabled researchers to determine the suspected origins of the Booroola mutation and dismiss the prolific breeds that did not contain the gene as potential sources.

1.6.2 Introgression of the Booroola mutation into Other Breeds

Since the discovery of the high fecundity Booroola sheep in the 1950s many countries have imported Booroola sheep to cross with other breeds in hope to increase their fecundity. Table 7 is a compilation of the distribution of the Booroola mutation into other breeds by Davis (2009).

Table 7: Distribution of *FecB* from the Booroola Merino into other Sheep breeds in alphabetical order (Davis, 2009).

Breed	**Country**	**Reference(s)***
Assaf	Israel	Gootwine et al. (2008)
Awassi	Israel	Gootwine et al. (2008)
Border Leicester- Merino	Australia	Piper et al. (1988)
Border Leicester	Australia, New Zealand	Davis and Meyer (1983); Piper et al. (1988)
Borderdale	Australia	Piper et al. (1988)
Cheviot	UK	Haley (1991)
Columbia	USA, Canada	Fahmy and Castonguay (1991); Young (1991)
Coopworth	New Zealand, Canada	Davis and Meyer (1983); Fahmy and Castonguay (1991)
Corriedale	New Zealand, Poland Uruguay	Davis and Meyer (1983); Fernandez-Abella (1991); Nowak and Charon (2001)
Chzechish Merino	Czechoslovakai	Veress et al. (1988)
Dorset-Leicester-Suffolk	Canada	Fahmy and Castonguay (1991)
Dohne Merino	South Africa	Davis et al. (1991)
Dorset Horn	Australia, Canada, UK	Piper et al. (1988); Haley (1991); Fahmy (1996)
Finn	Canada, USA	Fahmy and Castonguay (1991); Young (1991)
German Blackhead Mutton	Germany	Kaulfuss et al. (2004)
German Mountain	Germany	Wassmuth et al. (1991)
German Mutton Merino	Germany, Belgium	Decuypere et al. (2004); Kaulfuss et al. (2004)
Hungarian Mutton	Hungary	Veress (1996)
Hyfer (Dorset-Merino)	Australia	Piper et al. (1988)
Ile de France	UK	Haley (1991)
Merino	Australia, New Zealand, Chile, Uruguay	Piper and Bidon (1982); Hinch et al. (1985); Fernandez-Abella (1991); Cristian (1994)
Merinoland	Germany	Wassmuth et al. (1991)
Merinos d'Arles	France	Fernandez-Abella et al. (2005)
Olkuska	Poland	Klewiec et al. (2004)
Perendale	New Zealand	Davis and Meyer (1983)
Polish Merino	Poland	Klewiec et al. (2001)
Poll Dorset	NewZealand	Davis (1991)
Polwarth	Uruguay	Fernandez-Abella (1991)

Polypay	Canada	Fahmy and Castonguay (1991)
Rambouillet	USA, Canada	Fahmy and Castonguay (1991); Southey et al. (2002)
Romanov	France, Canada	Driancourt et al. (1986); Fahmy and Castonguay (1991)
Romney	New Zealand, UK, Brazil	Hinch et al. (1985); Haley (1991); Souza et al. (1995)
Scottish Blackface	UK	Boulton et al. (1995)
South Australian Merino	Australia	Kleemann et al. (1991)
Suffolk	UK, USA, Chile, Canada	Haley (1991); Fahmy and Castonguay (1991); Cristian (1994); Bunge et al. (1995)
Targhee	USA	Bunge et al. (1995)
Texel	Netherlands, Belgium, UK	Anderson et al. (1997); Visscher et al. (2000); Decuypere et al. (2004)
Welsh Mountain	UK	Haley (1991)
Western Whiteface	USA	Young (1991)

*Access these sources from (Davis, 2009).

The introgression of the Booroola Merino into other breeds generally increases the lambing rates however lamb mortality is also higher and lamb body weights tend to be lower than in the original breed (Davis, 2009). The results of the case study by Gootwine (2009) of the Awassi and Assaf sheep in Israel support this. The study found that one copy of the *FecB* gene increased the lambing rates of the Awassi breed by 0.62 and the Assaf breed by 0.72 lambs (Gootwine, 2009). Consequently, the presence of the Booroola mutation negatively affected the ewes body weight, milk production and lambs birth weight of the two breeds (Gootwine, 2009). Although these negative effects of the gene are present, the increase in lambing numbers by integrating Booroola mutation still provides overall economic benefits (Gootwine, 2009). However, the economic impact of the gene integrating into another breed is also dependent on the economic environment such as lamb, mutton and feed prices. The impacts of integrating the Booroola mutation is different for each breed and situation but the consensus is that the gene increases the prolificacy of the breed but has a negative impact on the lamb survival and weight averages. The Booroola Merino is not the only breed being used to integrate the gene into another breed. Since the development of the RFLP-PCR DNA test, breeds such as the Garole, Hu, Romney, Romanov and other breeds have been used to distribute the gene. The gene has been spread to 48 different breed (Adkinson and Adkinson,

2013) and continues to do so. Fecundity genes will continue to be utilized to increase the prolificacy of other breeds.

1.6.3 Adoption of Booroola sheep in Australia

The Booroola mutation has been a known source of increased prolificacy in the Merino population since the 1970s however the uptake of the gene has not been widespread (Walkden-Brown et al., 2009). The identification of the Booroola mutation and the development of the RFLP-PCR genotyping test has not increased the uptake of the gene (Walkden-Brown et al., 2009), most likely due to the known negative effects observed in the *FecB*^{BB}. The Multimeat composite breed overcomes the problems of the *FecB*^{BB} by using *FecB*^{BB} rams of a composite maternal breed to produce *FecB*$^{B+}$ first-cross Merino prime lamb dams (Walkden-Brown et al., 2009). The Multimeat composite breed with the Booroola mutation was developed in South Australia at Straun Agriculture Research Station near Naracoorte (Walkden-Brown et al., 2009) and is based on the White Suffolk breed (Earl et al., 2017). The heterozygous first-cross Merino prime lamb dams express desirable reproductive traits and are crossed with a terminal sire for lamb production (Earl et al., 2017). Because there is no further breeding amongst the progeny (both males and females sold for meat) no *FecB*^{BB} are produced, except in the stud producing the homozygous rams, and the benefits of the Booroola heterozygote can be realized (Walkden-Brown et al., 2009). The use of the Multimeat composite increases the lambing rates of first cross Merino ewes to near 200% and the weaning rates by 25-30% (Earl et al., 2017). Table 8 shows an example of the scanning rates using the Multimeat composite compared to a Border Leicester.

Table 8: Litter size distribution determined by ultrasound scanning of 1.5 year old crossbred ewes bred from merino ewes by Border Leicester or Multimeat rams and mated to terminal sires (Earl et al., 2017).

		Litter Size				
		0	**1**	**2**	**3**	**Lamb %**
Property 1	Border Leicester	10	50	40	0	129
	Multimeat cross	5	17	46	31	203
Property 2	Border Leicester	8	73	19	0	110
	Multimeat cross	4	17	61	16	190

Property 3	Border Leicester	-	8	32	55	5	153
	Multimeat		4	14	34	47	226

One consequence of the Booroola mutation is that the ewes require greater nutritional management to ensure increased production and limit lamb mortality. Maiden 1.5 year old ewes have the highest lamb mortalities but that decreases as the ewes get older and more experienced (Earl et al., 2017). The weaning rates are expected to be 100% for ewe lambs, 140% for 1.5 year old ewes and 165% for mature ewes (Earl et al., 2017). Earl et al. (2017) recommends 4 key management practices for the ewe lambs when utilizing the Multimeat composite breed. At the start of their joining period, the ewes need to be over 40 kilograms and gaining at least 100 grams per day throughout that the joining period (Earl et al., 2017). Ewes begin joining at 8 months of age and the rams should be joined for at least 8 weeks (Earl et al., 2017). As the ewes get older their management practices change but the principles are the same, ensuring that the ewes are at a body condition score 3 before joining and ensure that they continue to gain weight as their pregnancy progresses (Earl et al., 2017). Ewes with more multiples at scanning will have a greater nutritional requirement and as the Booroola mutation ensures 80% of the flock will have multiples, the flock in general will have a higher feed requirement (Earl et al., 2017). At lambing, single bearing ewes should be 60 kilograms while multiple bearing ewes should be between 65-75 kilograms depending on the number of multiples (Earl et al., 2017). Post lambing survival are dependent on feed availability, weather conditions and predators and due to increased multiples there is an increase in lamb mortality but the weaning rates should be higher, with the correct management, than pure-bred Merinos (Earl et al., 2017). The use of the Booroola composite breed increases the weaning rate of the flock and by only producing the $FecB^{B+}$ ewes the issues of lamb survival with the $FecB^{BB}$ is not an issue. With further developments on an accessible genotyping test and further research, the introgression of the Booroola mutation may increase.

1.7 Summary

The Booroola mutation increases the ovulation rates in sheep in an additive way and provides an alternative method to increase litter size instead of relying on low heritable reproductive traits. Dr. Helen Newton Turner paved the way with the discovery of the Booroola mutations effects which led to the many studies on other fecundity genes. The mutations effect on the BMPR1B causes slower maturation of follicles and fetuses which increases the ovulation rates and decreases the growth of the fetus. Studies have found that $FecB^{BB}$ ewes have higher lamb mortality rates due to the Booroola mutations effects but the $FecB^{B+}$ produces desirable lambing outcomes. Therefore, genotyping sheep for the Booroola mutation is not only used for research but in a commercial setting as well. Advancing technologies in the genotyping industry, such as SNP microarrays and HRM curve analysis, enables viable and cheaper options for researchers and sheep producers. Currently, there is limited utilisation of the Booroola mutation in Australia due to the high lamb mortalities of the $FecB^{BB}$ however, with cheaper options for genotyping the introgression of the Booroola mutation into commercial flocks may improve. The Booroola mutation was an important discovery and more research is needed to further understand the implications of the *FecB* gene.

Chapter 2

New test for genotyping Booroola mutation (*FecB*) using high resolution melt curve analysis.

2.1 Abstract

The Booroola SNP mutation of the BMPR1B gene (*FecB*) increases ovulation rates in a Mendelian fashion with additive effect. However, the high lamb mortality of the homozygous $FecB^{BB}$ genotype makes fixing the trait in a sheep population generally undesirable. Genotyping is thus required to ensure that the desirable heterozygous $FecB^{B+}$ is being utilised effectively. Current methods of genotyping are complicated and expensive so the aim of this project was to develop and validate a one-step high resolution melt curve analysis (HRM) test to differentiate between the non-carrier ($FecB^{++}$), homozygous ($FecB^{BB}$) and heterozygous ($FecB^{B+}$) Booroola genotypes. DNA was collected by utilising a simple method adapted from Song et al. (2013) to extract DNA from dried blood cards which was shown to be suitable for HRM genotyping. Two sets of primers were designed to amplify a 65bp amplicon and a 110bp amplicon containing the *FecB* SNP. Both primers proved to be suitable for genotyping *FecB* via HRM however the 65bp primer provided an easier interpretation. SYTO9 dye was used as the fluorescence to produce the HRM data and premium ingredients for the PCR master mix were used. The results of the HRM curve analysis demonstrated the differentiation between the three different Booroola genotypes. HRM was validated against 46 known genotypes and produced 100% accuracy. 137 other sheep, which had suspected genotypes from breeding strategies, were also genotyped. Furthermore, the effects of the *FecB* genotypes on litter size was analysed determining that there was a significant difference ($P<0.008$) between the genotypes. The use of HRM method to genotype the Booroola mutation will be more cost effective and have a greater time efficiency than the genotyping method used currently.

2.2 Introduction

The aim of this experiment is to SNP genotype non-carrier ($FecB^{++}$), homozygous ($FecB^{BB}$) and heterozygous ($FecB^{B+}$) genotypes sheep using the high resolution melt curve analysis (HRM). The Booroola mutation in the bone morphogenetic protein 1B receptor (BMPR1B) gene on chromosome 6 that is a causative mutation affecting ovulation rates in sheep (Mulsant et al., 2001). The mutation is a single nucleotide polymorphism (SNP) that changes a base A to a G

at Q249R (Souza et al., 2001) and impacts the ovulation rates in additive way (Walkden-Brown et al., 2009). With each copy of the mutation, the amount of ovulations increases and this has been recorded in many different breeds and countries (Davis, 2009). Davis et al. (1982) created a criterion for selecting *FecB* genotypes based on the ewes' ovulation rates where *FecB*$^{B+}$ had ovulation rates greater than three and *FecB*^{BB} had ovulation results great than five. This selection criteria was used for many research studies until the development of the genotyping test. The impact on ovulation from the mutation differs between each breed but it is suggested that the effect is multiplicative (Davis, 2009). Studies have found that the effect of having two copies of the mutation is not desirable due to the high lamb mortality in the uterus and after parturition (Walkden-Brown et al., 2009). Often the lamb numbers at weaning time suggest that the *FecB*^{BB} ewes produce no greater number of lambs than the *FecB*$^{++}$ ewes due to the high lamb mortality. However, a *FecB*$^{B+}$ genotype is more desirable as the weaning results tend to be much higher than the *FecB*$^{++}$. Figure 6 below provides an example of the effect of the Booroola mutation in Merino ewes.

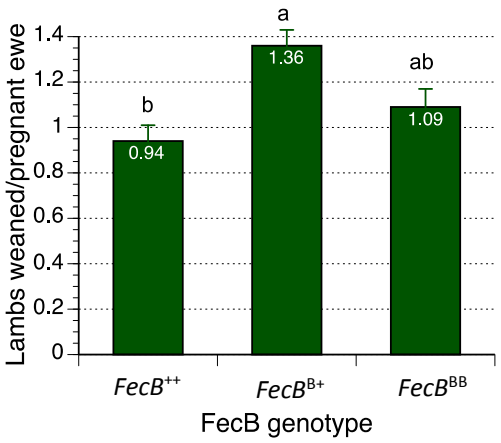


Figure 6: Weaning rates per Merino ewe of non-carrier (WW), Booroola heterozygous (BW) and Booroola homozygous (BB) (Walkden Brown et al. 2009).

The *FecB*$^{B+}$ produces a greater number of lambs even though the *FecB*^{BB} has a higher ovulation rate (Walkden-Brown et al., 2009). Therefore, because of the difference of the number of lambs produced between *FecB*^{BB} and heterozygous it is beneficial to genotype sheep and manage those genotypes.

The mutations effect was first observed by Turner (1978) and then traced back to the Garole sheep that arrived in Australia in the 1790s (Turner, 1982). The mutations location was determined by Mulsant et al. (2001); Souza et al. (2001); Wilson et al. (2001), and a

genotyping method was developed by Wilson et al. (2001) using restriction fragment length polymorphism (RFLP). This method is a multistep process that requires polymerase chain reaction (PCR), restriction enzymes and electrophoresis to determine the genotype (NCBI, 2016). It is time consuming, costly and produces many opportunities for contamination. GenomNZ® is the current supplier for the genotyping the Booroola mutation through RFLP-PCR and they receive dried blood cards for DNA extraction and testing. The two types of blood cards available are standard and elute. Standard blood cards require Kits for DNA extraction to remove the DNA from the matrix and elute cards only require a buffer solution to release the DNA from the card. Other methods of genotyping are being further developed. SNP microarray technology has recently (April, 2017) becoming available to genotype the *FecB* gene and is a part of the Sheep CRC 15k SNP test (Julius Van der Werf and Klint Gore *per. comm.*). SNP microarray genotypes thousands of SNPs simultaneously with probes in microwells that highlight specific nucleotides with fluorescence for genotyping (Kawęcka et al., 2016). This method of genotyping provides supplies the genotypes of many different genes and is being increasingly utilised in stud breeding strategies but in a commercial property it is not viable. High resolution melt curve analysis (HRM) is a method used that can be used to genotype SNP and therefore could potentially be used to genotype the Booroola mutation (Tong and Giffard, 2012). Utilising the HRM analysis for the Booroola mutation would be a simpler and cheaper option than the RFLP-PCR and SNP microarray method for the Booroola mutation specifically. HRM is based on real time PCR and the use of a florescent dye to determine the difference between genotypes. HRM increases the temperature of the DNA strands gradually from 50°C to 95°C and the Rotorgene6000 machine measures the florescence fading as the strands denature. The SNP changes the temperature that the DNA bases (A,G,T,C) denature thus producing different results between genotypes (Tong and Giffard, 2012). Figure 7 provides an example of the graphs produce by HRM curve analysis.

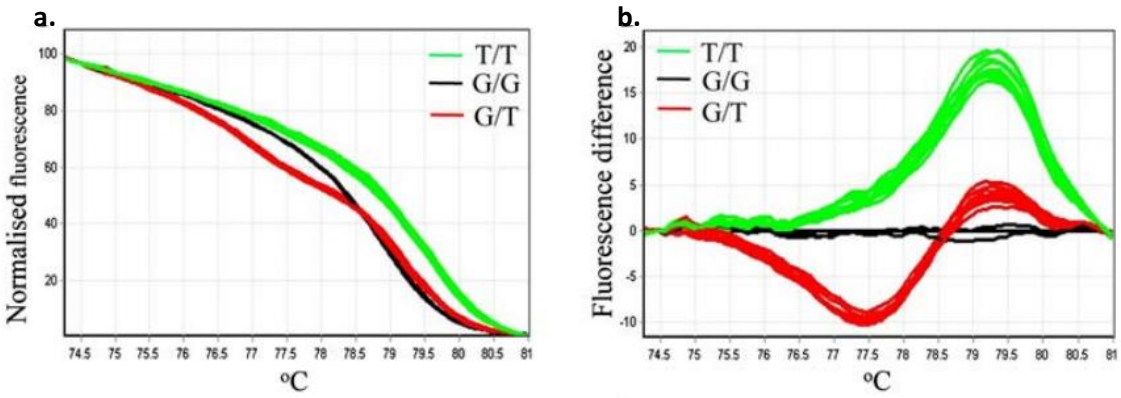


Figure 7: High resolution melting curve analysis for the Xneas109a mutation in almonds. **a.** Normalized melting plot, **b.** The difference plot of **a**. TT represents non-carrier, GG is homozygous and G/T is heterozygous (Wu et al., 2008).

Each genotype forms a distinct curved which can be analysed to determine the genotype. This method is less time consuming, costly and difficult than the current method by Wilson et al. (2001) that is used (Wu et al., 2008). The hypothesis of this experiment was that the HRM method can be used to genotype the Booroola mutation because of the SNP mutation.

2.3 Experimental Approach

The experimental approach aimed to design a process to validate that HRM could be utilised to genotype for the *FecB* gene. This was completed first by designing primers that could produce an amplicon containing the *FecB* gene. Various samples of sheep with different Booroola genotypes for DNA extraction were supplied, majority of the samples were in dried blood card form. A DNA extraction method adapted from Song et al. (2013) was validated with 6 samples first to ensure the extracted DNA was suitable for the HRM process. The extraction method was then used to extract DNA from the supplied Booroola samples to be used for HRM genotyping. Using the HRM method for genotyping for the Booroola mutation was required to be authenticated. 46 sheep that samples had been received from had known genotypes from GenomNZ® were used to confirm the HRM method can be used to genotype. A further 137unknown samples were genotyped using the new HRM method. Samples were also sent to AGRF (Sydney) for Sanger sequencing to confirm that the targeted region of DNA was amplified. Finally, to demonstrate the effects of the Booroola genotype on litter size, 171 ultrasound scanning records from genotyped ewes were analysed to confirm the biological effect of the FecB mutation on litter size. The objectives of this experiment was to determine that the utilisation of the HRM method can be used to genotype the Booroola mutation.

2.4 Method and Materials

2.4.1 Primer Design

The primers were designed to amplify the region of the Booroola mutation site. Two primers were designed to replicate the results. One produced a 65bp long amplicon (p65) and the other a 110bp long amplicon (p110) as shown below.

65 base pair amplicon

5'**AGCTGGTTCCGAGAGACAGA**AATATATC**A/G**GACGGTGTTGATGAG**GCATGAAAACATCTTGGG TGA** 3'

110 base pair amplicon

5'**GGCGAAAAGGTAGCTGTGAA**AGTGTTCTTCACTACAGAGGAGGCCAGCTGGTTCCGAGAGACAG AAATATATC**A/G**GACGGTGTTGATGAG**GCATGAAAACATCTTGGGTGA** 3'

The bold are the forward and reverse primers and the red is the location of the mutation.

2.4.2 Sample Collecting and DNA Source

Merino sheep DNA was sourced from 4 different suppliers: Sheep CRC UNE, Ross Baldwin, David Wolfenden and UNE Kirby farm. 20 samples of stored sheep DNA which were suspected to be the progeny of a *FecB*^{BB} ram were obtained from Sheep CRC UNE. Blood cards were supplied from Sheep CRC and sent to property owners with Booroola Merinos. 169 blood cards in total were obtained by clipping the ear of the sheep and bleeding onto a blood card and then dried, with University of New England Animal Ethics Committee approval (UNE AEC 17-029). 15 blood cards were received from Ross Balwin with records of their suspected Booroola genotype. David Wolfenden provided 147 blood cards; 46 samples had genotypes confirmed by GenomNZ and 102 samples were labelled with their suspected genotypes from breeding selection. 134 samples were personally collected with David Wolfenden over the span of one week. 6 blood cards were also collected from UNE farm, Kirby, sheep for DNA extraction method testing which were assumed to be non-carrier sheep. As described in Table 9.

Table 9: Number of samples collected from each source.

Source	Material	Known Genotype*	Suspected	Unknown	Total
Sheep CRC – Stored samples	Extracted DNA	-	20	-	20
David Wolfenden – Rand, southern NSW	Blood Cards	46	102	-	148
Ross Balwin – Young, central NSW	Blood Cards	-	-	15	15
Kirby Sheep	Blood Cards	-	-	6	6
Total		46	122	21	189

2.4.3 Validating Tris-HCl method for DNA extraction

The DNA extraction method was adapted from Song et al. (2013). Six blood cards were used to determine the suitability of Song et al. (2013) extraction method for extracting DNA from blood cards. Two different solutions were tested, Tris-HCl and TE, which were compared to positive samples of already extracted DNA. For four samples, only half the blood card was used and 2 samples used the whole blood cards. These samples were assumed to not possess the Booroola mutation. The extracted DNA was tested by the nanodrop spectrophotometer for quality and quantity and went through the PCR and HRM process to determine if the amplification and melt curve was suitable.

2.4.4 DNA extraction from dried blood cards

Each dried blood card was completely cut up it sterile conditions and placed in 1.5ml Eppendorf tube and then the blood card was submerged in 10 mM Tris-HCl (pH 7.4) or TE (Tris EDTA 10 mM, pH7.6) buffer solution. The tubes were rotated overnight in -4°C. The next day each tube was placed in a 95°C heat block for 10 minutes and then centrifuged at 10 000g for 10 minutes. A supernatant was produced that was pipetted into a new tube which was the

final product of the DNA sample. Each sample was measured for DNA quality and quantity by the nanodrop spectrophotometer.

2.4.5 Polymerase Chain Reaction (PCR) master mix

The polymerase chain reaction (PCR) master mix was made up of 10 x PCR buffer, 50mM $MgSO_4$, 2.5mM dNTP, 5µM of both forward and reverse primer, 50µM Syto® 9 green fluorescent dye and 5 U/µL Platinum Taq (Invitrogen). The total reaction volume was 10µL with 8µL of PCR master mix and 2µL of diluted DNA. Each sample was duplicated and 2 different primers were used as described above. The samples were placed in the RotorgeneQ 6000 realtime PCR Thermocycler (Corbett Research, Sydney, Australia) for the PCR and HRM cycle.

2.4.6 PCR and HRM cycle

The PCR cycle was performed with a touchdown PCR protocol. The first denaturation at 95°C for 2 minutes, then 50 cycles at 95°C for 5 seconds, annealing and extension for 10 seconds at 62°C for the first cycle and the and then decreasing from there by 0.5°C for 10 cycles. The final extension at 72°C for 2 minutes. A random duplex strand formation proceeded with 5 seconds of 95°C and then 50°C for 30 seconds. The HRM cycle started with 70°C for 90 seconds and then began the melt curve by starting at 20°C and increasing every 2 seconds by 0.1°C. It finally held at 25°C when that cycle was complete.

2.4.7 Analysis of the HRM curve

To confirm that the HRM curve analysis method was a suitable to genotype the Booroola mutation, the samples that had known genotypes were processed first through the PCR and HRM cycles. To confirm that the HRM was successful, three different curves had to be present for each different genotype and were expected to look similar to the results of studies such Han et al. (2012). 6 samples were tested first, two of each genotype. These samples were then used as positive controls to determine the genotypes of the suspected samples. Each sample was analysed separately to determine if the sheep was *FecB*$^{++}$, *FecB*$^{B+}$ or *FecB*^{BB}. Once the known samples were processed the suspected genotyped samples were tested using the known samples as positive controls. Analysis was conducted by comparing the samples against the positive controls to determine their genotype. A valid result was defined as a singular curve at a set temperature, samples that produced an invalid result were retested.

2.4.8 Sequencing for primer conformation

8 samples of purified PCR product were sent to AGRF (Sydney) for Sanger sequencing to confirm the Booroola mutation was present in the amplicon and to confirm each genotype. 2 samples of *FecB*$^{++}$, 4 samples of *FecB*$^{B+}$ and 2 samples of *FecB*^{BB} genotype as according to the HRM curve analysis results. A different primer was used for sequencing but targeted the same region as shown below.

613 base pair amplicon

5'**GATCGAACCCGAGTCTCTTG**TGTCTGCTGTATTGGCACACACATTCTTTACCACTAGCGCCACCTC
GGAAACCCATATAAAGAAAAACTACTGGCTAAATATATTTTACATGCAGTTGTTTTCTTCTCTGAAGG
AAAAAAGAAAACATTAAACAATCTGTAGTGCCGTGAACGCACTAACAGTGTGTTGGGGGATTTAAC
AGGTCCAGAGGACGATAGCAAAGCAAATTCAGATGGTGAAACAGATTGGAAAAGGTCGCTATGGG
GAAGTTTGGATGGGAAAGTGGCGTGGCGAAAAGGTAGCTGTGAAAGTGTTCTTCACTACAGAGGA
GGCCAGCTGGTTCCGAGAGACAGAAATATATC**A/G**GACGGTGTTGATGAGGCATGAAAACATCTTG
GGTGAGTATAAGTCTGTATTAGCGATGTTCAGGGTTTCCTAGCTTTCCTCTCTTTTTTTGTTAACATCT
GCATATTAACTCATCTGTCTAGATCCGCTATGAAATATATAGACCTTTTTTTTTTTTTTTGCCATGAGCC
CAAGAATGTTATTTGCTTCTAATGGGAGACCTCACAGGAAGAATGACTAAGGTACTTCTA**TGAATGG**
ATGGTGGACTTGA 3'

The bold are the forward and reverse primers and the red is the location of the mutation. The samples were placed in the RotorgeneQ 6000 for the PCR and HRM cycle.

2.4.9 *FecB* genotypes effects on litter size

Over the period at David Wolfendens', the records of the ultrasound scannings of a Booroola flock were collected. A statistical analysis of ultra sound pregnancy scanning records of 171 sheep from a Booroola flock were also obtained from David Wolfendens'. Records were collected from 2014 to 2017 for each genotype: *FecB*$^{++}$, *FecB*^{BB} and *FecB*$^{B+}$. The ultrasound scanning results were compiled and scanning data was analysed using a mixed model fitting animal as a random factor and the *FecB* genotype, year and their interaction as fixed factors in JMP12 (SAS institute, Cary NC, USA).

2.5 Results

2.5.1 Optimising DNA extraction from blood cards

The result of the adapted Song et al. (2013) DNA extraction method produced crude DNA. The results in Figure 8 below show the mean nanodrop spectrophotometer for 6 blood card DNA extractions with Tris-HCl or TE and the results of the different means of using half a blood card and the full blood card for DNA extraction. These results show that the concentration of DNA was much lower from only using half the blood card, it also shows that the TE extraction buffer produces a higher quality of DNA than the Tris-HCl. The standard error of each graph is very small. Figure 9 shows the PCR amplification efficiency mean results for both primer and comparing the amplification effieciency of the different buffer solutions used for the DNA extraction.

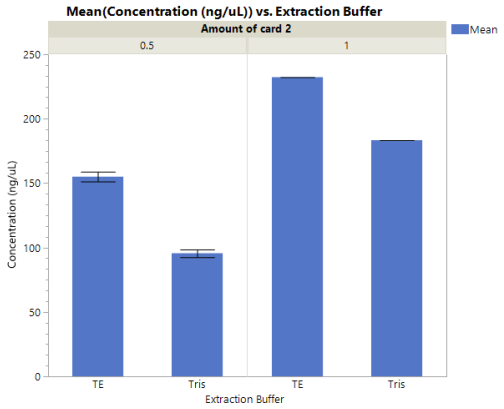


Figure 8: Nanodrop spectrophotometer mean results of DNA using the two different extraction buffers and the amount of blood card used in the extraction method.

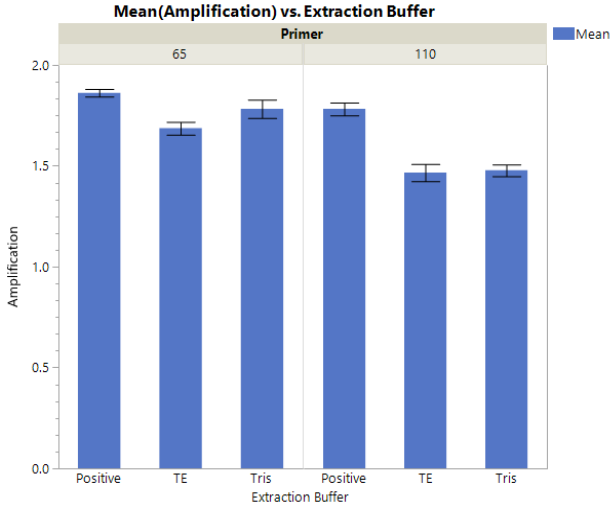


Figure 9: Mean results of PCR amplification effieciency and the comparison between the p65 and p110 comparing the two extraction buffers used in DNA extraction and the postive DNA

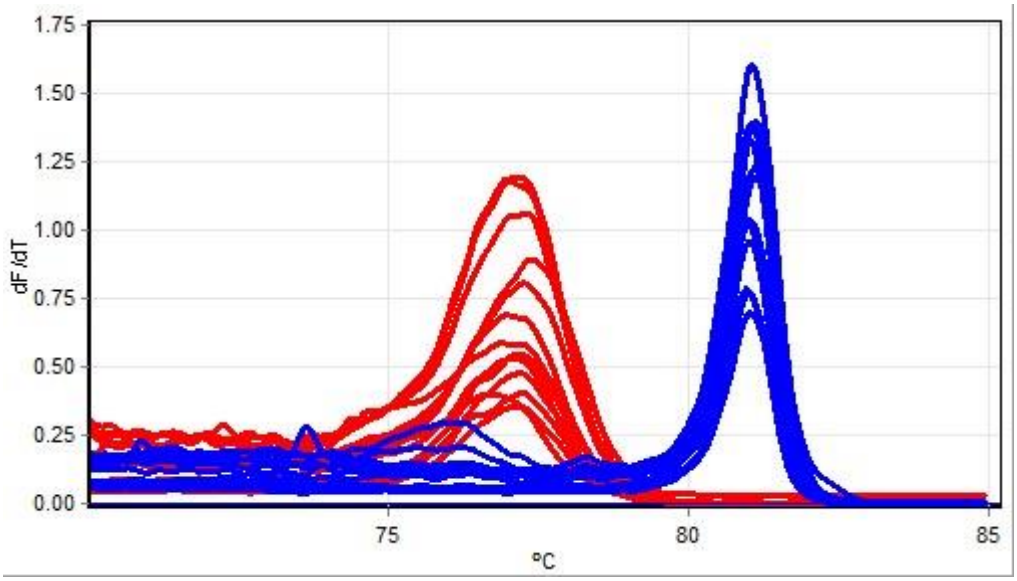


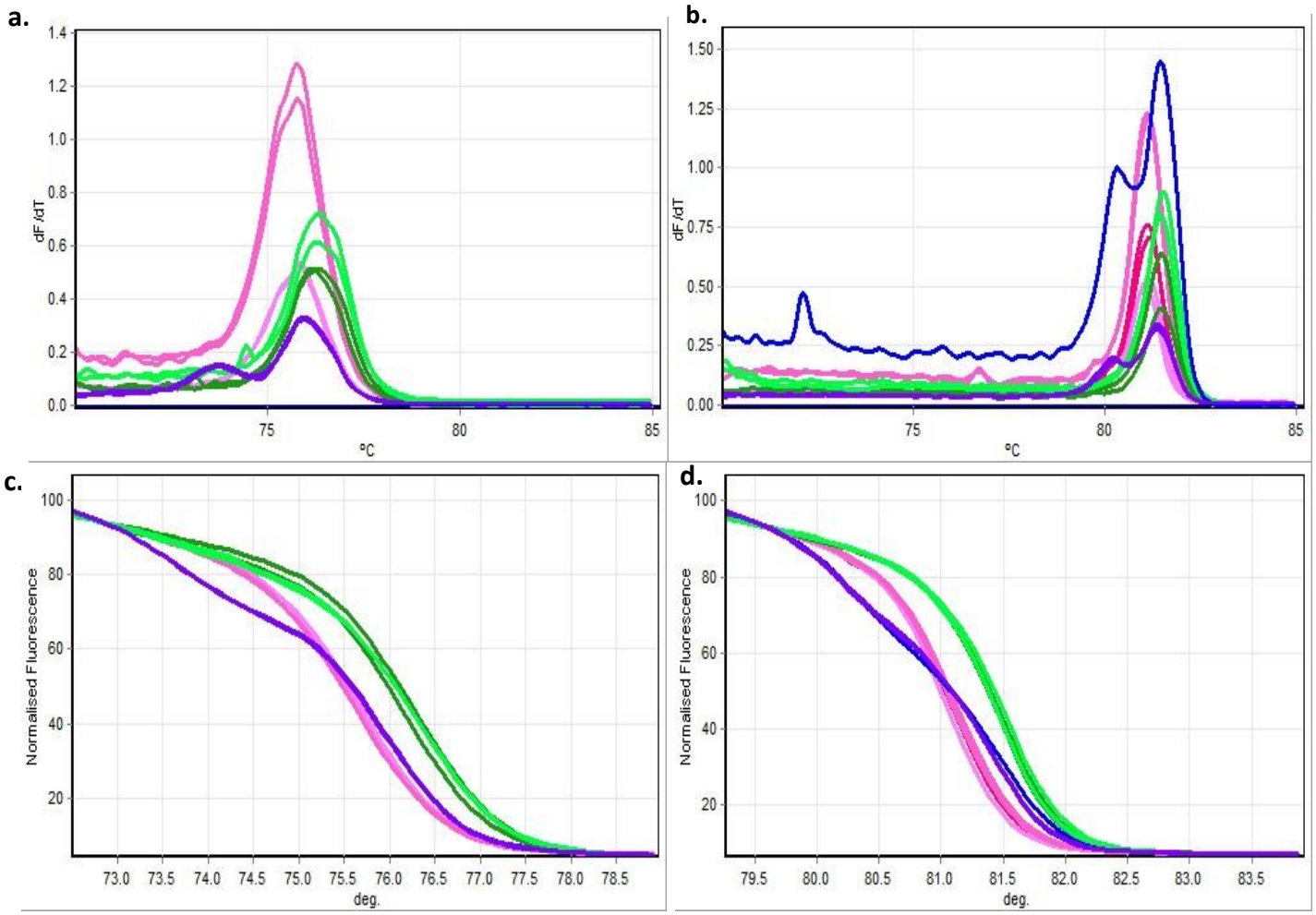
Figure 10: Melt curve results of DNA extraction validation, illustrating the temperatures that the highest amount of DNA denatures for primer 65 (red) and primer 110 (blue).

Figure 1 above shows the melt curve results of the DNA extraction validation results and the difference between the primers. The two separate peaks represent the temperature at with the DNA denatures. Primer 65 produces an amplicon that denatures between 72°C and 80°C and primer 110 produces an amplicon that denatures between 79°C and 83°C.

30 samples were received on Whatman blood cards. Using the DNA extraction method on Whatman blood cards produced a turbid red supernatant instead of a translucent supernatant and the nanodrop spectrophotometer results revealed that there was no DNA in the sample. These samples did not contribute to the results of the experiment as no DNA was obtained.

2.5.2 The Utilisation of HRM to Genotype for *FecB*

The results determined the success of the HRM curve analysis for genotyping the sheep for the Booroola mutation. The resulting graphs of the HRM cycle were analyzed and determined with curve represented each genotype. Primer 65 peaks occur between 73°C and 80°C and the primer 110 peaks were between 83°C and 88°C. 6 samples of known genotypes were tested first with HRM and the HRM results are shown in Figures 11 below.

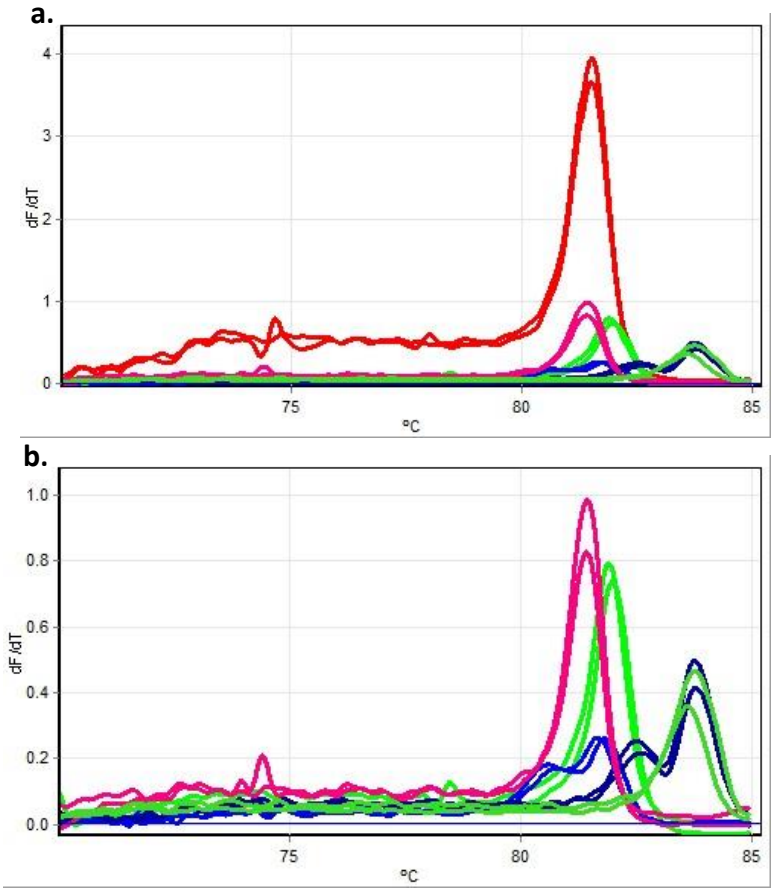


Colour	Sample Number	Known Genotype	HRM genotype
	1	*FecB*$^{++}$	*FecB*$^{++}$
	2	*FecB*$^{++}$	*FecB*$^{++}$
	3	*FecB*^{BB}	*FecB*^{BB}
	4	*FecB*^{BB}	*FecB*^{BB}
	5	*FecB*$^{B+}$	*FecB*$^{B+}$
	6	*FecB*$^{B+}$	*FecB*$^{B+}$

Figure 11: HRM results for know samples that were genotyped by GenomeNZ®. **a.** and **c.** show the results for p65. Figures **b.** and **d.** show the results for p110. **a.** and **b.** represent the melt curve. **c.** and **d.** represent the normalised HRM curse. The table below explains the color representation and the genotypes.

Samples were obtained from David Wolfenden at two separate occasions and it was determined that the HRM analysis was different between each group of samples. The results

found that the HRM for Group 2 of samples had a different temperature at which the florescence faded compared to Group 1. Figure 12 also shows that the *FecB*$^{++}$ peaks of both Groups were at the same temperature however the *FecB*^{BB} and *FecB*$^{B+}$ peak at different temperatures between Groups. The *FecB*$^{B+}$ and *FecB*^{BB} of Group 1 had melt curve peaks were between 80°C and 83°C and Group 2 peaks were between 81°C and 85°C. *FecB*$^{++}$ of both Groups have have peaks between 80°C and 83°C. Both primers 65 and 110 show the same results of between Groups with samples containing the Booroola mutation denaturing at different temperature. Samples shown in Figure 12 were all genotyped by GenomNZ® prior to this experiment.



Sample	Colour	Genotype	Sample group
2		*FecB*$^{++}$	Group 1
6		*FecB*$^{B+}$	Group 1
4		*FecB*^{BB}	Group 1
27		*FecB*$^{++}$	Group 2
103		*FecB*$^{B+}$	Group 2
165		*FecB*^{BB}	Group 2

Figure 12: a. and **b.** show the HRM melt curve results comparing sample Groups 1 and 2. **a.** is constructed with **all** samples shown whereas **b.** shows all but sample 2 to magnify the peaks and demonstrate the difference between Group genotypes.

2.5.3 Validation of HRM *FecB* Genotyping

The results of the genotypes for samples collected.

Table 10: Known genotype samples and the HRM curve analysis results. Genotypes were determined by GenomNZ® prior to this experiment.

Known Genotype	**No of samples**	**HRM agreement**	**HRM disagreement**	**Invalid (re-test)**
FecB^{++}	13	12	0	1
FecB^{B+}	19	19	0	
FecB^{BB}	14	14	0	
Total	46	45	0	1 (2.2%)

The results in Table # show that there was 100% accuracy with genotyping the Booroola mutation when there was a valid result.

Table 11: Suspected genotype, from breeding selection, samples and the HRM curve analysis results.

HRM Genotype	**Wolfenden**	**Balwin**	**Sheep CRC**	**Total**
Total samples	102	15	20	137
***FecB*$^{++}$**	32	10	18	60
***FecB*$^{B+}$**	12	2	1	15
***FecB*^{BB}**	48	0	1	49
Invalid (re-test)	10 (9.8%)	3 (20%)	0	13 (9%)

The results of the validation test determined that 93% of samples were genotyped in the first HRM cycle. The samples that produced invalid results were retested and their genotypes were determined.

2.5.4 Sanger sequencing for primer conformation

The results of the sequencing are shown in Figure 13 which demonstrates the base change between each genotype. The SNP for *FecB*$^{++}$ is an A, *FecB*^{BB} is a G and the *FecB*$^{B+}$ is represented by an R which means both A and G was present.

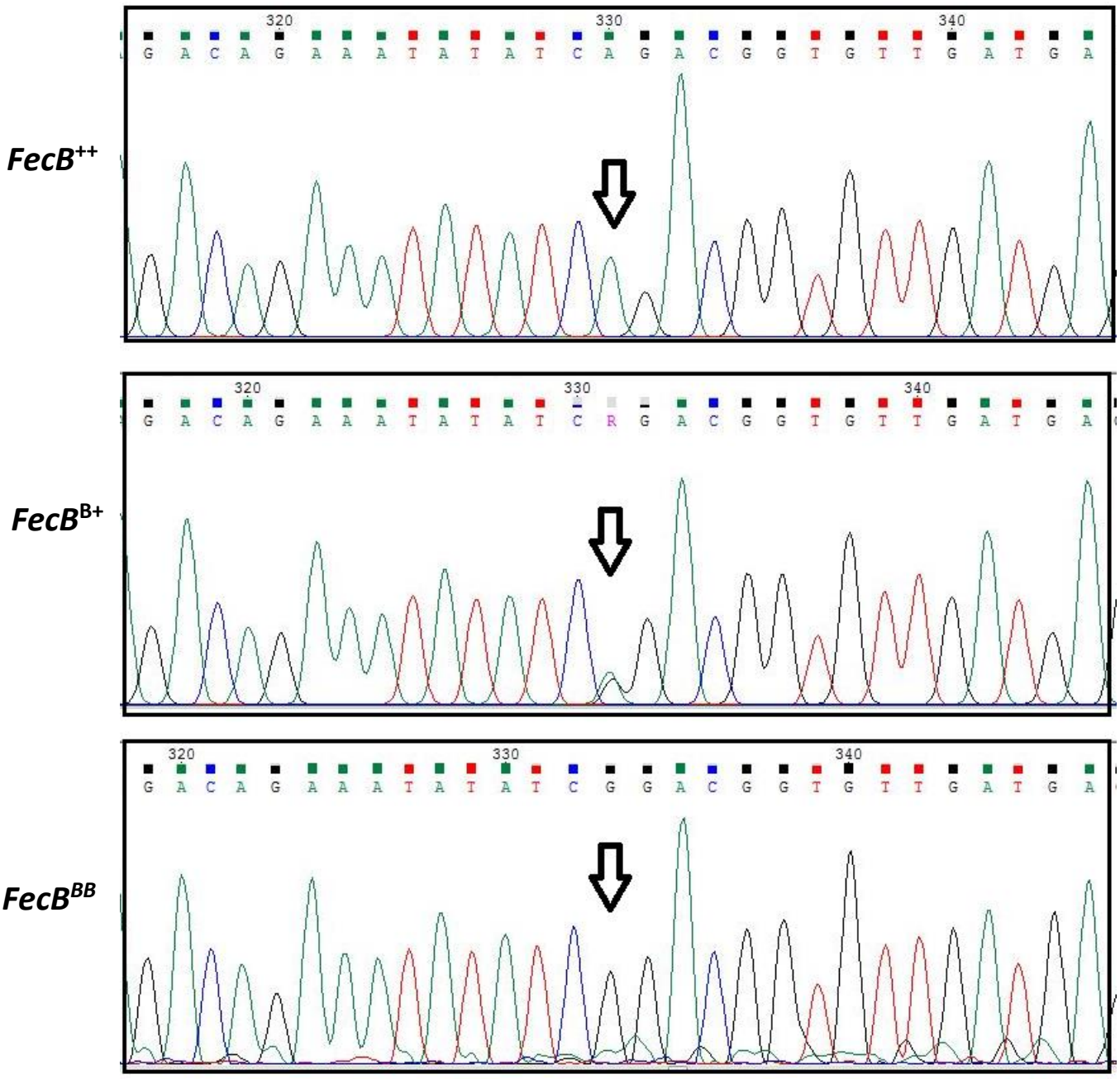


Figure 13: Results from Sanger sequencing showing the *FecB* gene within the amplicon. The arrows indicated the sight of the mutation for each genotype.

2.5.5 Effect of FecB genotype on litter size

The results of the ANOVA of the ultrasound scanning for litter size records from David Wolfendens property show that there was a significant difference (P = 0.032) in litter size between genotypes being significantly lower in *FecB*$^{++}$ than *FecB*$^{B+}$ with *FecB*^{BB} intermediate (Figure 14). The least squares means of were 1.29, 1.66 and 1.44 for *FecB*$^{++}$, *FecB*$^{B+}$ and *FecB*^{BB} ewes respectively. There was also a significant difference between years (P = 0.007) with no significant interaction between the effects of genotype and year (P=0.733).

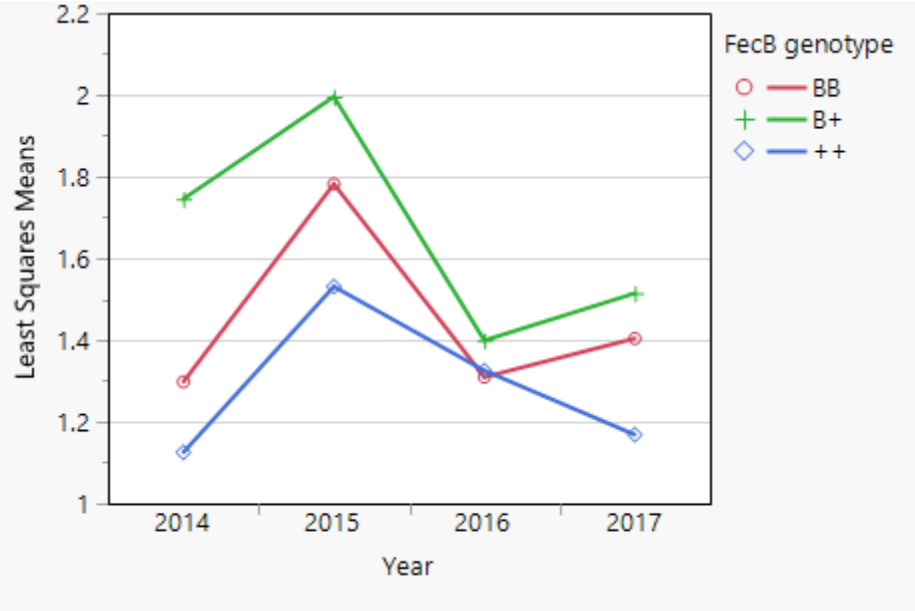


Figure 14: Least Squares Means ultra sound scanning result each year for each genotype

2.6 Discussion

The results of this experiment determined that HRM curve analysis using the selected primers was an accurate and efficient way to determine the FecB genotype of sheep (*FecB*$^{++}$, *FecB*$^{B+}$ and *FecB*^{BB}).

The Tris-HCl extraction method adapted from Song et al. (2013), proved to be a successful method to extract DNA from FTA elute cards for the use of HRM SNP genotyping. The extraction method was confirmed to be a successful method to use for HRM curve analysis genotyping by comparing the extracted DNA using the method against DNA that had been extracted by a kit. Kits are often costly and are a long, multistep process that are time consuming. The Song et al. (2013) method proved to be a cheaper and faster way to extract DNA and was therefore ideal to use. The crude DNA developed in this experiment still

produced HRM curves to genotype the Booroola mutation with. The quantities of DNA obtained in this experiment of 190 ng/µL when using the whole blood card, were similar to those obtained by Song et al. (2013). However, this study found that the TE produced a higher quantity of DNA. The results showed that although the Tris-HCl and TE extracted DNA did not amplify as well as the kit extracted DNA, it was a suitable method to use for HRM. The PCR amplification was deemed suitable for HRM if it was greater than 1.6. Primer 65 accomplished that following extraction using both extraction buffers. Primer 110 amplification results were lower than 1.6 however the DNA using the Tris-HCl and TE extraction method still produced a viable HRM curve result. The length of the amplicon affected the melting temperature to denature the DNA strands and therefore it changed where the peaks were in the melt curve data (Tong and Giffard, 2012). The results of this experiment produced 2 different peaks for each primer. This indicated that the Tris-HCl DNA extraction method can be and was used for the rest of the experiment as the Song et al. (2013) paper determined that the Tris-HCl solution produced better HRM curves. Despite this, the results of this experiment showed little difference between the Tris HCl and TE HRM curve analysis results. It was also determined that using the whole blood card compared to half a blood card produced the highest quantity of DNA. These results indicated that DNA concentrations greater that 100ng/µL were required for the HRM method to be successful. Therefore, the whole blood card was used from each sample for the remainder of the experiment. Using the whole blood card however, meant if the DNA extraction method failed then the whole sample was lost. DNA extraction kits such as Chelex 100 resin, QIAamp™ DNA Investigator Kit, illustra™ tissue and cells genomicPrep Mini Spin Kit, and DNA IQ™ Kit (GEHealtcare, 2010), often require less blood card and produces high quality and quantity DNA. This study demonstrates that this DNA extraction method which produces crude DNA can be utilised for HRM genotyping. The results of this experiment indicate that crude DNA can be used successfully to HRM genotype and enables sheep producers to send samples as blood cards and laboratories the option to genotype without the use of kits. However, this study also determined that the DNA extraction method was only successful for the Sheep CRC supplied cards that were assumed to be elute cards. It has been determined that there are two different types of blood cards: standard and elute. Standard blood cards bind the DNA in the matrix in the card and requires a specific method to extract the DNA (GEHealtcare, 2010). The elute blood cards which bind the DNA to chemicals on the card which then can be released by different solutions. Due to

the results of using Tric-HCl method on both the Sheep CRC cards and the Whatman cards it can be theorised that the Sheep CRC cards are elute and that the Whatman cards are standard blood cards. This was not confirmed by Sheep CRC however the Whatman blood cards were standard FTA cards. The Tris-HCl DNA extraction method was used for Sheep CRC blood cards for the remainder of the experiment.

The results of the HRM curve analysis determined that the HRM can be used to genotype for the Booroola mutation. As there are no other studies using HRM to genotype for the Booroola mutation, it was difficult to compare these results to other studies. However, studies such as Wu et al. (2008) and Han et al. (2012) provide a good interpretation of what the HRM curves should appear as. The HRM results of the known samples of the 3 genotypes showed clear, reproducible differences in the curves with 100% accuracy between the GenomNZ® and the HRM results, therefore the test can be used to determine the *FecB* genotype of a sheep. The results from Sanger sequencing show that both primers used contained the target region with the Booroola mutation. The melt curves show the temperature at which the highest concentration of florescence fades and therefore produces peaks at different temperatures for each genotype. In the case of the Booroola mutation, the $FecB^{++}$ genotype fades at a lower temperature than the $FecB^{BB}$ furthermore the $FecB^{B+}$ has two different peaks representing the different SNPs, which was expected due to other studies such as Han et al. (2012). The size of the peaks were irrelevant as it correlates to the PCR amplification process and the amount that was amplified. The normalised HRM curves also have three different curves due to the SNP change which were used to genotype each sample. Less than 7% of the samples tested did not produce a valid HRM curve. This could be due to insufficient DNA in the sample and/or failed PRC amplification. Insufficient DNA was caused in some cases by the minimal amount of blood on the blood card and therefore a lower amount of DNA was extracted. Human error may also be a reason for an invalid result due to contamination or insufficient PCR mix in the sample. The samples that had invalid results were retested which resulted in successfully genotyping them. The results of the HRM test with known genotypes show that the Booroola mutation can be genotyped using HRM curve analysis. These results indicate that the HRM method can be used as an alternative method to genotype for the Booroola mutation. The HRM method is a cheaper and simpler than than RFLP-PCR method and is more specific compared to the SNP microarray. Utilizing the HRM method for genotyping will

provide researchers and commercial sheep producers access to a low cost, easier method to genotype for the Booroola mutation.

Interestingly, the sample retrieved from David Wolfenden on two separate occasions, Group 1 and Group 2, produced HRM curves at different temperatures. The HRM analysis of Group 2 determined that although the peaks were produced at different temperatures than Group 1, it was still possible to genotype the samples. The melt curve shows that the *FecB*$^{B+}$ has two different peaks as expected and the HRM analysis of several other known genotyped *FecB*^{BB} results repeated the same peak thus confirming the genotype. The morphology of the peaks indicates the genotype and therefore the different temperatures between groups were irrelevant. The temperature difference may be caused by the crude DNA or a slight difference between the cards resulting in different curve. However, because the morphology of the curves indicated the genotype, the temperature was not a great concern. The applications of the HRM curve analysis for genotyping will stimulate further research and the use of the gene in commercial sheep production. Future projects may include determining the frequency of the Booroola mutation within the Australian sheep populations to analyse the distribution of the gene.

The results of the statistical analysis of the ultrasound scanned litter size were as expected. There are limited studies on the litter size at ultrasound scanning on the different *FecB* genotypes, but it is known there is a great reproductive wastage between ovulation and weaning both in the uterus and after birth (Farquhar et al., 2006). Walkden-Brown et al. (2009) measured the ultrasound scanned litter size of a Booroola Merino flock and found that *FecB*$^{B+}$ and *FecB*^{BB} had significantly higher litter sizes than the *FecB*$^{++}$ but were not significantly different from each other. The litter sizes were 1.22, 2.19 and 2.06 for *FecB*$^{++}$, *FecB*$^{B+}$ and *FecB*^{BB} respectively. The results of the present experiment from 2014 to 2017 show a lower average litter size than Walkden-Brown et al. (2009) but follow a similar trend. The results indicate that the *FecB*$^{B+}$ ewes have a significantly higher ultrasound scanned litter size than the *FecB*$^{++}$ ewes. There was a significant difference of litter size between years and this was most likely due to environmental impacts as well as record keeping. In 2016, a limited number of ewes had lambing records compared to the years' prior, therefore the results were restricted. These results demonstrate the litter size difference between genotype and indicate that the *FecB*$^{B+}$ has higher conception rates than *FecB*^{BB} and *FecB*$^{++}$. Studies such as

Walkden-Brown et al. (2009) show that ovulation rates are higher in *FecB*^{BB} than the other genotype, however the results of their ultrasound scannings show that the *FecB*$^{B+}$ has a greater litter size. It is reasonable to assume that the ovulation rate of Wolfendens *FecB*^{BB} ewes were higher that the *FecB*$^{B+}$ and that the results of this study demonstrate the loss of ova and embryo due to the Booroola mutation. The results of this study supports the results of other studies and provides further insight into the effect of the Booroola mutation on litter size.

In conclusion, the HRM curve analysis was an effective method that can be utilised to genotype the Booroola mutation. This study demonstrated the differentiation between *FecB*$^{++}$, *FecB*$^{B+}$ and *FecB*^{BB} and the success of the Tris-HCl DNA extraction method for HRM curve analysis. This method is cheaper, simpler and has greater time efficiency than the other genotyping methods available. The results of the litter size recorded, provided an insight into the effect of the Booroola mutation and supported other similar studies. Overall, this project confirmed the hypothesis that the HRM curve anaylsis can be used to differentiate between *FecB*$^{++}$, *FecB*$^{B+}$ and *FecB*^{BB}.

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4 Referencing

Abdoli, R., P. Zamani, S. Z. Mirhoseini, N. Ghavi Hossein-Zadeh, and S. Nadri. 2016. A review on prolificacy genes in sheep. Reproduction in Domestic Animals 51: 631-637.

Adkinson, A., and R. Adkinson. 2013. The FecB (Booroola) gene and implications for the Turkish sheep industry. Turkish Journal of Veterinary and Animal Sciences 37: 621-624.

Åström, A.-K. et al. 1999. Chromosomal localization of three human genes encoding bone morphogenetic protein receptors. Mammalian Genome 10: 299-302.

Davis, G. H. 2009. The Booroola Gene: Origin, Distribution, Use and Management of the FecB Mutation. Australian Centre for International Agricultural Research (ACIAR), Canberra, Australia.

Davis, G. H. et al. 2006. Investigation of the Booroola (FecB) and Inverdale (FecXI) mutations in 21 prolific breeds and strains of sheep sampled in 13 countries. Animal Reproduction Science 92: 87-96.

Davis, G. H. et al. 2002. DNA tests in prolific sheep from eight countries provide new evidence on origin of the Booroola (*FecB*) mutation. Biology of Reproduction 66: 1869-1874.

Davis, G. H., G. W. Montgomery, A. J. Allison, R. W. Kelly, and A. R. Bray. 1982. Segregation of a major gene influencing fecundity in progeny of Booroola sheep. New Zealand Journal of Agricultural Research 25: 525-529.

Debnath, J., and R. V. Singh. 2014. Genetic polymorphism of Boorola FecB gene and its association with litter size in Balangir, Shahabadi and Bonpala Sheep Breeds. Indian Journal of Animal Research 48: 307-314.

Demeny, D., Y. M Parsons, I. Franklin, and W. Cooper. 1997. Low Cost Sampling Method for DNA Based Testing. Animal Breeding and Genetics 12: 265-269.

Earl, C., P. Clothier, and D. McLachlan. 2017. Multimeat Composites. http://www.multimeat.com.au/information.html Accessed 31 August 2017.

Estienne, A. et al. 2015. Anti-Müllerian hormone regulation by the bone morphogenetic proteins in the sheep ovary: deciphering a direct regulatory pathway. Endocrinology 156: 301-313.

Fahmy, M. H., F. Castonguay, and J. P. Laforest. 1994. Uterine morphology and reproductive phenomena in relation to number of embryos at different stages of gestation in prolific sheep. Small Ruminant Research 13: 159-168.

Fan, H., and M. L. Gulley. 2001. DNA extraction from fresh or frozen tissues. Methods in molecular medicine 49: 5-10.

Farquhar, P. A., K. G. Dodds, and G. H. Davis. 2006. Introgression of the Booroola mutation (FecB) leads to hyper-prolificacy in a Romney sheep flock. In: 8th World Congress on Genetics Applied to Livestock Production, Belo Horizonte, MG, Brazil

Fogarty, N. 2009. A review of the effects of the Booroola gene (FecB) on sheep production. Small Ruminant Research 85: 75-84.

GEHealtcare. 2010. Reliable Extraction of DNA from Whatman FTA cards. https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1392818611307/litdoc28982222_20161015135424.pdf Accessed 24 August 2017.

GEHealtcare. 2011. Whatman FTA Elute. https://au.vwr.com/assetsvc/asset/en_AU/id/17887357/contents Accessed 25 August 2017.

GenomNZ®. 2017. Genomnz Booroola Testing. https://www.genomnz.co.nz/our-services/booroola/ Accessed 25 August 2017.

Ghalsasi, P. M., and B. V. Nimbkar. 1993. The 'Garole' – microsheep of Bengal, India. Animal Genetic Resources Information, FAO 12: 73-79.

Gootwine, E. 2009. Biological and economic consequences of introgressing the B allele of the FecB (Booroola) gene into Awassi and Assaf sheep. In: S. W. Walkden-Brown, J. Van der Werf, C. Nimbkar and V. Gupta (eds.) Use of the FecB (Booroola) gene in sheep-breeding programs. Proceedings of the Helen Newton Turner Memorial International Workshop. ACIAR

Proceedings No. 133. No. 133. p 119-127. Australian Centre for International Agricultural Research, Pune, India. 10-12 November 2008.
Gundry, C. N. et al. 2003. Amplicon Melting Analysis with Labeled Primers: A Closed-Tube Method for Differentiating Homozygotes and Heterozygotes. Clinical Chemistry 49: 396.
Han, Y., D.-M. Khu, and M. J. Monteros. 2012. High-resolution melting analysis for SNP genotyping and mapping in tetraploid alfalfa (Medicago sativa L.). Molecular Breeding 29: 489-501.
Hinch, G. N. 2009. Effects of Multiple Ovulations and Litter Size on Maternal and Foetal Physiology: prenatal and postnatal cansequences. Australian Center for International Agriculture Research, Pune, India. 10-12 November 2008.
Jafari-Joozani, R., R. Asadpour, S. Alijani, and H. Mahmoudi. 2012. Detection of polymorphism in Booroola gene (fecb) and its association with litter size in Zel sheep breed in Iran. Reproduction in Domestic Animals 47: 501.
Kawęcka, A., A. Gurgul, and A. Miksza-Cybulska. 2016. The Use of SNP Microarrays for Biodiversity Studies of Sheep – A Review Annals of Animal Science No. 16. p 975.
Kelly, R. W., G. H. Davis, and A. J. Allison. 1980. Productive Changes in Longwool Breeds in New Zealand Following Crossbreeding with Booroola-Type Rams. Proceedings of the Australian Society of Animal Production 13 413-416.
Kleemann, D. O. et al. 1990. Factors influencing lamb survival in a high fecundity Booroola Merino ◊ South Australian Merino flock. Theriogenology 33: 965-976.
Kumar, S., A. Mishra, A. Kolte, S. Dash, and S. Karim. 2008. Screening for Booroola (FecB) and Galway (FecX^G) mutations in Indian sheep. Small Ruminant Research 80: 57-61.
Lawson, K. A. et al. 1999. Bmp4 is required for the generation of primordial germ cells in the mouse embryo. Genes & Development 13: 424-436.
Lay, M. J., and C. T. Wittwer. 1997. Real-time fluorescence genotyping of factor V Leiden during rapid-cycle PCR. Clinical Chemistry 43: 2262.
Massagué, J. 1987. [17] Identification of receptor for type-β transforming growth factor. Methods in Enzymology 146: 174-195.
Massagué, J. 1998. TGF-β Signal Transduction. Annual Review of Biochemistry 67: 753-791.
Montgomery, G. W. et al. 1993. The ovine Booroola fecundity gene (FecB) is linked to markers from a region of human chromosome 4q. Nat Genet 4: 410-414.
Montgomery, G. W. et al. 1994. The Booroola Fecundity (FecB) Gene Maps to Sheep Chromosome 6. Genomics 22: 148-153.
Montgomery, G. W., J. M. Penty, E. A. Lord, and M. F. Broom. 1995. The search for the Booroola (FecB) mutation. J Reprod Fertil Suppl 49: 113-121.
Mulsant, P. et al. 2001. Mutation in bone morphogenetic protein receptor-IB is associated with increased ovulation rate in Booroola Merino ewes. Proceedings of the National Academy of Sciences of the United States of America 98: 5104-5109.
Nanekarani, S., M. Goodarzi, S. Khederzadeh, S. Torabi, and N. Landy. 2016. Detection of polymorphism in booroola gene and growth differentiation factor 9 in Lori sheep breed. Tropical Journal of Pharmaceurical Research 15: 1605-1605.
NCBI. 2016. Restriction Fragment Length Polymorphism (RFLP). https://www.ncbi.nlm.nih.gov/probe/docs/techrflp/ Accessed 17 August 2017.
Piper, L. R. et al. 1976. Ovulation rate in high fecundity Merino crosses. Theriogenology 6: 622.
Piper, L. R., and B. M. Bindon. 1996. The Booroola merino. In: M. H. Fahmy (ed.) Prolific Sheep. p 152-160. CAB International, Oxon, UK.
Ririe, K. M., R. P. Rasmussen, and C. T. Wittwer. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Analytical biochemistry 245: 154-160.
Robinson, J. J. 1990. Nutrition in the reproduction of farm animals. Nutrition Research Reverve 3: 253-276.

Ruoss, C., A. Tadros, T. O'Shea, J. McFarlane, and G. Almahbobi. 2009. Ovarian follicle development in Booroola sheep exhibiting impaired bone morphogenetic protein signalling pathway. Reproduction 138: 689-696.

Safari, A., and N. M. Fogarty. 2003. Genetic Parammeters for Sheep Production Traits: Estimates from the Literature. Technical Bulletin 49: 26.

Samadi Shams, S. et al. 2011. Highly Effective DNA Extraction Method from Fresh, Frozen, Dried and Clotted Blood Samples. BioImpacts : BI 1: 183-187.

Shimasaki, S. et al. 1999. A functional bone morphogenetic protein system in the ovary. Proc Natl Acad Sci U S A 96: 7282-7287.

Smith, P. et al. 1993. Effects of the Booroola Gene (Fecb) on Body-Weight, Ovarian Development and Hormone Concentrations During Fetal Life. Journal of Reproduction and Fertility 98: 41-54.

Song, Y. et al. 2013. A reliable and effective method of DNA isolation from old human blood paper cards. SpringerPlus 2: 616.

Souza, C. J. H., C. MacDougall, B. K. Campbell, A. S. McNeilly, and D. T. Baird. 2001. The Booroola (FecB) phenotype is associated with a mutation in the bone morphogenetic receptor type 1 B (BMPR1B) gene. Journal of Endocrinology 169: R1–R6.

Sudhakar, A., R. Rajendran, and P. Rahumathulla. 2013. Detection of Booroola *(FecB)* mutation in Indian sheep—Nilagiri. Small Ruminant Research 113: 55-57.

ThermoFisher. 2017. DNA Extraction from Tissue. https://www.thermofisher.com/au/en/home/life-science/dna-rna-purification-analysis/dna-extraction/genomic-dna-extraction/tissue-dna-extraction.html Accessed 27 Oct 2017.

Tong, S. Y. C., and P. M. Giffard. 2012. Microbiological Applications of High-Resolution Melting Analysis. J Clin Microbiol 50: 3418-3421.

Towe, J. 2014. Genes Involved in Ovulation Rate and Litter Size in Sheep. Department of Animal Breeding and Genetics 433.

Turner, H. 1978. Selection for reproduction rate in Australian Merino sheep: direct responses. Australian Journal of Agricultural Research 29: 327-350.

Turner, H. N. 1982. Origins of the CSIRO Booroola. In: The Booroola Merino. Proceedings of a workshop held at Armidale, NSW. p 1-7.

Walkden-Brown, S. W., D. H. Wolfenden, and L. R. Piper. 2009. Biological and economic consequences of introgression of the FecB mutation into Merino sheep in Australia. In: S. W. Walkden-Brown, J. Van der Werf, C. Nimbkar and V. Gupta (eds.) Use of the FecB (Booroola) gene in sheep-breeding programs. Proceedings of the Helen Newton Turner Memorial International Workshop. ACIAR Proceedings No. 133. No. 133. p 100-108. Australian Centre for International Agricultural Research, Pune, India. 10-12 November 2008.

Wilson, T. et al. 2001. Highly Prolific Booroola Sheep Have a Mutation in the Intracellular Kinase Domain of Bone Morphogenetic Protein IB Receptor (ALK-6) That Is Expressed in Both Oocytes and Granulosa Cells1. Biology of Reproduction 64: 1225-1235.

Wilson, T. M., and X. Y. Wu. 2001. Mutated bmp1b receptor as regulator of ovulation rate. Google Patents.

Wu, S.-B., M. G. Wirthensohn, P. Hunt, J. P. Gibson, and M. Sedgley. 2008. High resolution melting analysis of almond SNPs derived from ESTs. Theoretical and Applied Genetics 118: 1-14.

Yamashita, H. et al. 1995. Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. The Journal of cell biology 130: 217-226.