Molecular genetics of flystrike susceptibility in New Zealand sheep

A thesis
submitted in fulfilment
of the requirements for the Degree of
Doctor of Philosophy

at
Lincoln University
By
Lucynda Emma Ruth Burrows

Lincoln University
2018

Abstract

Aspects of the molecular genetics of flystrike susceptibility in New Zealand sheep

by

Lucynda Emma Ruth Burrows

Flystrike is a problem for most sheep industries worldwide as it affects animal welfare and production. The cost of flystrike is multifactorial, and includes production losses resulting from reductions in wool and body growth, morbidity, and treatment and control costs. Flystrike incidence is environmentally dependent and its prevalence is variable. However, several factors predispose individual sheep to flystrike, such as variation in the fleece and skin of the sheep, and variation in their immune response. Identifying genes that are involved with these factors and the predisposition to parasitic disease, will potentially allow for flocks to be genetically improved for flystrike resilience, thus reducing its impact in the NZ sheep industry.

This study aimed to identify variation in five candidate genes associated with phenotypic factors that do, or may, predispose sheep to flystrike, and determine whether the genetic variation (if any) was associated with susceptibility to disease. The genes chosen had either previously been implicated in resistance to fleecerot (*FBLN1* and *FABP4*), or potentially affected the wax content of the fleece (*ABCC11*), or were involved in immune response (*TLR4* and *RASGRP1*).

In the sheep studied, variation in ovine *FABP4, FBLN1* and *RASGRP1* was associated with susceptibility to flystrike. Sheep with the A_1 variant of *FABP4* were found to be less likely (P = 0.014, with an odds ratio of 0.689) to have flystrike than those without A_1. The likelihood of flystrike occurrence continued to decrease as copy number of A_1 increased from one copy to two (P= 0.002, with an odds ratio of 0.674). The *FBLN1* B_1 variant was found to be associated with reduced flystrike occurrence (P = 0.029, odds ratio = 0.286), while the *FBLN1* C_1 variant was associated with increased occurrence (P = 0.002, odds ratio = 1.687). The *RASGRP1 A* (P = 0.015, odds ratio = 1.557) and *C* (P = 0.047, odds ratio = 0.733) variants had a significant association with flystrike occurrence.

This study showed identified *FABP4, FBLN1* and *RASGRP1* as good candidate's genes for flystrike resilience in sheep. Further research is required to verify these genes as markers for flystrike resilience.

Keywords: Flystrike, sheep, resistant, susceptible, *FABP4, FBLN1, TLR4, RASGRP1, ABCC11*.

Publications arising from this study

Burrows, L. E. R., Zhou, H., Frampton, C. M. A., Forrest, R. H. J., Hickford, J. G. H. (2018). Variation in ovine RAS Guanyl Releasing Protein 1 (RASGRP1) gene and its association with flystrike in New Zealand sheep. *Proceedings of the World Congress on Genetics Applied to Livestock Production*, 11. 641

Nucleotide Sequences Submitted to the NCBI GenBank

Sheep –*FBLN1* A_1 – D_1: MG704148 –MG704151

Sheep –*FBLN1* A_2 – D_2*:* MG704152 –MG704155

Sheep –*FBLN1 A3 – D3*: MG792344 –MG792347

Sheep –*RASGRP1 A – C*: MF925341 –MF925343

Acknowledgements

"Whatever you do in life will be insignificant but it is very important you do it, because nobody else will" – Mahatma Gandhi.

First of all, I would like to express my sincere gratitude to my supervisor Professor Jon Hickford for the opportunity to do a PhD through the Lincoln University Gene-Marker Lab. Your vast knowledge and support was of immense help during my PhD.

Thank you to Rachel Forrest, for reading my thesis with a fine tooth comb. Your encouragement, quick feedback and relaxed personality has been greatly appreciated. I could not have done it without your support.

I would like to thank members of the Gene Marker Laboratory, Huitong Zhou, Freeman Fang and Seung-Ok (Mimi) for all the technical support during my research. A special thanks to Mimi for teaching me everything I needed to know in the lab. Your support and encouragement had been much appreciated during my PhD. Jane Ekegbu for your positive attitude and Yuihai, Wenqiong Cai and Qingming An, *qui qui* for helping me learn lab skills at the beginning of my PhD. And other staff and postgraduates of the Faculty of Agricultural and Life Science who have helped and supported me along the way.

Thank you to John Bates for taking me around sheep farmers at the beginning of my PhD.

Chris Fampton, Simon Hodges and Marian Hodges. Thank you for all the statistical support during my PhD, the struggle was real. Thank you for the time you all took to make sure I knew what to do.

Graham Burrell for the reference letters that helped me receive my scholarships and for helping with my writing, your quick feedback has been very helpful.

Thank you for funders of my research, it is greatly appreciate, Vernon Willy Memorial Fellowship, JW and C McLean Scholarship and Miss Clarice Bell Memorial Scholarship. Thank you to Jane Edwards and Sue Smart for helping with scholarships.

The New Zealand Corriedale Association for your on going support throughout my study, not only the financial support, and awarding me the 2016 Corriedale Young Ambassador but also for your members providing blood samples for my research. I really appreciate your support and contribution towards my study.

All the farmers involved in my research. Tom Burrows, John Burrows, John Wiggly, John Jebson, Malcolm Menzie, Greg Lassen, John McLachlan, Rob James, Kerry Prenter, Mike Smith, James Thompson, Charlie Croft, John Olerenshaw, Richard Murchison, Shane Bertaceo and Richard Watson,

Alistair Lawerance, Angus Johnson, Rowan Nesbit, Christen Beaton, Eric Ross, Fliss Gardnier, Doc Sidey, Penni Loffigan, Digger McCulloch, Helen Heddell, Reuben Carter, Paul Gardiner, Delia Jones, Bob Masfield, Dave Clark, Chris Hampton, Neville Moorhead, Roscoe Taggart and Ike Williams. A special thank you to Helen Heddell during my PhD, who would always look out for flystrike sheep for me.

A big thank you to my family Mum, Dad, John, Robb, Kayla and Jaime for all the support encouragement and food given to me during the last few years. Especially to my Dad for reading my thesis and helping me collect blood samples from fly blown sheep during my PhD.

Thank you to all my friends and flatmates for all the support, encouragement and distractions. Special mention to my office mates Michele, Ivy and Yuihai. You were awesome office mates, your dedication to your work kept me motivated to keep writing.

Rhiannon, Kirsty, Grace and Lisa and all the post-grads in the Animal Science department for having someone to talk about the stress of PhD life.

Table of Contents

List of Tables

List of Figures

Abbreviations

$	New Zealand Dollars
%	Percentage
ºC	celcius
µL	microlitre
µM	micromolar
aa	amino acid
A	adenine
ABCC11	ATP-binding cassette transporter sub family C member 11
AFLP	Amplified Fragment Length Polymorphism
bp	base pair
CFW	clean fleece weight
C	cytosine
cm	centimetre
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
FABP4	fatty acid binding protein 4
FADS1	fatty acid desaturase 1
FEC	faecal egg count
FBLN1	fibulin
G	guanine
GI	gastrointestinal
GFW	greasy fleece weight
GWAS	Genome Wide Association Study
h	hour
Ha	Hectare
IGR	Insect growth regulators
kbp	kilobasepair
kg	kilogram
km	kilometre
LRR	Leucine Rich Repeats
MHC	Major Histocomplability Complex
mg	milligram
mL	millilitre
mM	millimolar
mm	millimetre
NCBI	National Centre for Biology Information
ng/mL	nanograms per millilitre
NZ	New Zealand
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
OAR	*Ovis aries* chromosome
Pten	Phosphatase and tension homology
RAPD	Random Amplified Polymorphic DNA
RASGRP1	Ras guanyl releasing protein 1
RFLP	Restriction Fragment Length Polymorphism
SSCP	Single Strand Conformation Polymorphism
SNP	Single Nucleotide Polymorphism
T	thymine
Taq	*Thermus aquaticus*
TBE	tris-borate-EDTA
TCR	T-Cell Receptor
TE	tris-EDTA
ThO	T helper cell type O
TLR4	Toll-like Receptor 4
TM	Transmembrane

Tris	tris (hydroxylethyl) aminomethane	**U**	unit

Amino Acid	Three-letter Abbreviation	One-letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chapter 1. Introduction

Flystrike (also known as cutaneous myiasis Figure 1.1) is a problem for most sheep industries worldwide because it affects both animal welfare and production. In New Zealand (NZ) there are four species of flies that cause flystrike: *Lucilia cuprina, L. sericata, Calliphora stygia* and *Chrymosmya rufifacies*. Historically flystrike has had an estimated prevalence of 3 % to 5 % in the NZ flock (Heath and Bishop, 1995), but more recent work has reported a range in prevalence from 3.4 % to 24 % in Romney and Composite type sheep (Scobie and O'Connell, 2010), and with an average prevalence across NZ of 2.15 % from 2009 to 2011 in Romney sheep (Pickering, 2013). Flystrike is estimated to cost the NZ sheep industry around $60.2 million annually (Eleanor Linscott, personal communication, August 30, 2016; Ludemann *et al*. 2010).

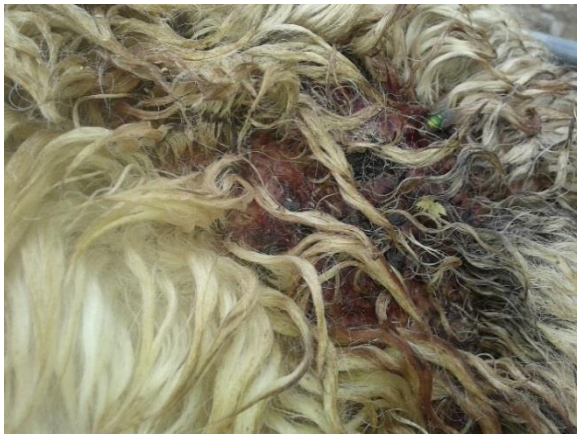


Figure 1.1. Breech strike. Note the cluster of freshly laid eggs and the fly.
(Photo taken by L. Burrows 2015).

The cost of flystrike is multifactorial and includes production losses from reduction in wool and body growth, morbidity, and the cost of treatment and control (Pickering *et al*. 2012). The latter includes the use of insecticides, with dipping costs estimated to be $34 million annually, along with the on-farm cost of prophylactic lamb shearing and ewe crutching (Linscott. E, personal communication, August 30, 2016; Ludemann *et al*. 2010). The long-term use of organophosphorus insecticides as a method of control has led to the development of resistance to this class of insecticide, and this necessitating other methods of control (Levot *et al*. 1999; Horton *et al*. 1997). There is also a demand for less chemical residues in wool (Levot and Sales, 1997). The practice of mulesing, which is the removal of wool-bearing skin from around the breech to prevent flystrike, has come under scrutiny from animal welfare organisations (Lee and Fisher, 2007). Finally, flystrike is unpleasant to treat (Lucas and Horton, 2013), and all these things taken together make reducing its occurrence a very attractive proposition.

The occurrence of flystrike is environmentally dependent and its incidence varies temporally and spatially. It is therefore difficult to make genetic gains by the selective breeding of sheep for resilience to flystrike. Although there is a large environmental influence on flystrike occurrence, other intrinsic

factors predispose sheep to flystrike, such as variation in the fleece and skin of the sheep, and variation in their immune response (Otranto, 2001; Smith *et al*. 2010). Flystrike resistance (strictly speaking this is resilience as the effect is not absolute at the flock level) has been estimated to have a greater than 90% genetic correlation with fleecerot in Merino sheep (Raadsma, 1991), and thus indirect selection for traits such as fleecerot resilience, might lead to genetic improvement in flystrike resilience (Greeff and Karlsson, 2009; Smith *et al*. 2009).

To date, most research into flystrike resilience has been done on Merino sheep in Australia, and in NZ there has been less research in this area (Bishop and Morris, 2007; Pickering, 2013). Identifying genes that are associated with the reduced occurrence of flystrike could allow for flocks to be genetically improved for resilience, and thus reduce the impact of flystrike in the NZ sheep industry.

The research described in this thesis (Figure 1.2) aims to identify variation in several candidate genes that may be associated with phenotypic factors that predispose sheep to flystrike, and to ascertain whether variation in those genes is associated with flystrike susceptibility. If so, it is anticipated that the genetic variation could be exploited as a gene-marker, and thus used in breeding for flystrike resilience, or more specifically breeding against flystrike susceptibility. The candidate genes chosen for analysis have been previously implicated in resistance to fleecerot (*FBLN1* and *FABP4*, Smith *et al*. 2010), may affect the wax content of the fleece (*ABCC11*, Smith *et al*. 2010), or are involved in immune responses (*TLR4*, Zhou *et al*. 2007; *RASGRP1*, Pickering, 2013). Polymerase chain reaction - single-strand conformation polymorphism (PCR-SSCP) analysis will be used to detect variation in the genes and DNA sequencing will be used to characterise the genetic variation detected. Associations between the flystrike incidence and nucleotide variation within the genes studied will be tested statistically.

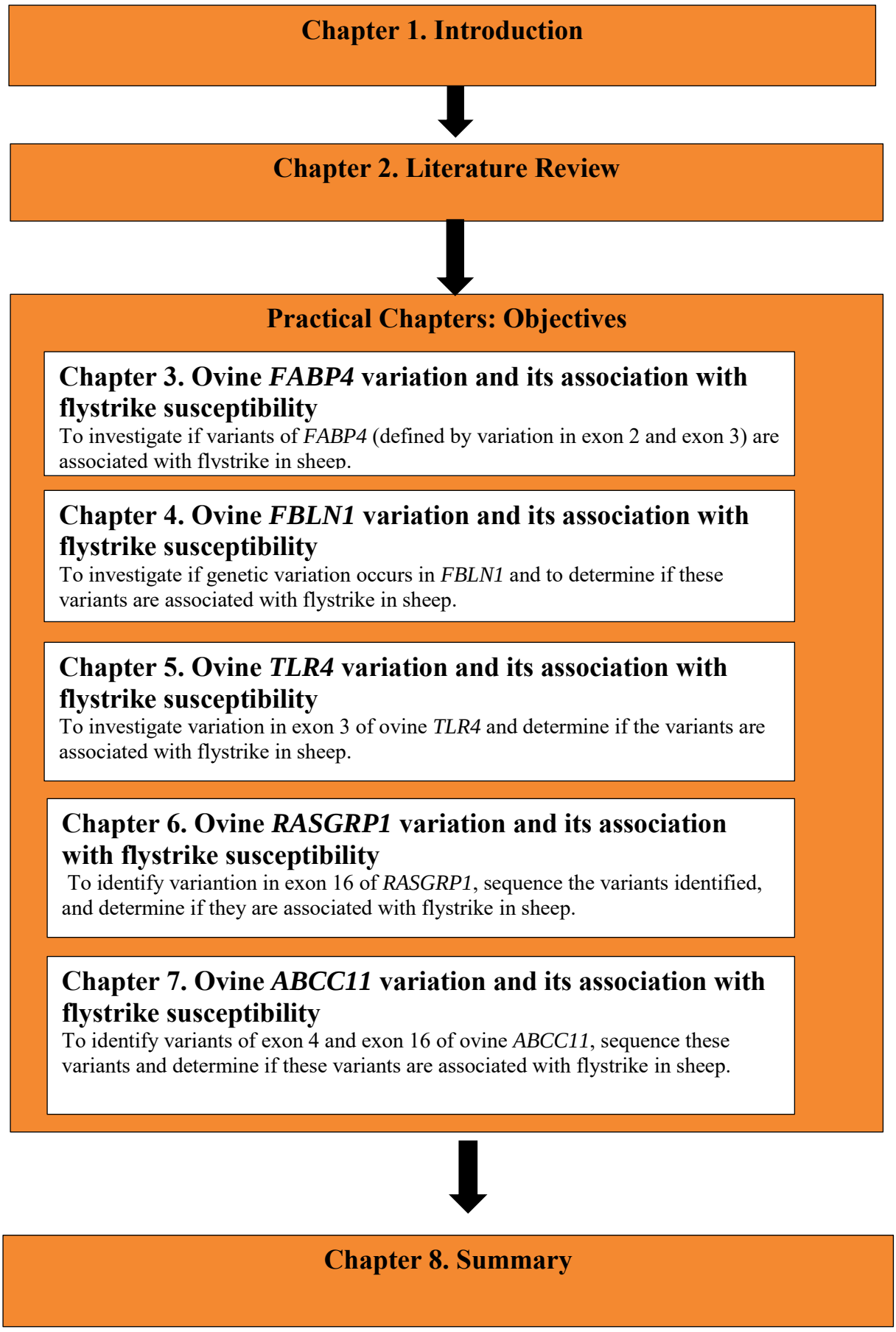


Figure 1.2 Outline of the thesis chapters.

Chapter 2. Literature Review

Flystrike is a major disease of sheep in NZ. An animal is considered flystruck or fly-blown when it is infested with the eggs or larvae of blowflies (Calliphoridae), which are characterised by the ability of their larvae to develop on the flesh of living vertebrates (Stevens, 2003). Blowfly larvae require a protein-rich substrate to complete their development (Stevens, 2003), and animal tissues provide this substrate, particularly sheep with dags or urine accumulation in the breech area, or fleecerot on their back (Norris *et al*. 2008; Tellam and Bowles, 1997).

There are several common farm practices used to reduce the incidence of flystrike. These include the strategic use of shearing and crutching, and the use of chemical dips (James, 2006; Tellam and Bowles, 1997). However, there is an increasing consumer demand to reduce chemical residue in the wool (Horton *et al*. 1997; Levot, 1995), and therefore research has begun to focus on the genetic improvement of sheep to reduce flystrike occurrence.

The main fly species causing flystrike in NZ

In NZ, the main species of blowfly are *L. cuprina, L. sericata*, and *C. stygia*. These are the 'primary strike species', meaning they are able to initiate flystrike (Levot, 1995). *Chrymosmya rufifacies* is a secondary strike species, and can only infest sheep once another species has initiated the flystrike (Lang *et al*. 2001). Each of these species has distinguishing geographical, phenotypic and life-cycle characteristics, and these are outlined below.

Lucilia cuprina is commonly called the 'Australian blowfly', although it was accidentally introduced into Australia from South Africa in the late 19th to the early 20th century (Wardhaugh *et al*. 2001; Watts *et al*. 1979). It was then accidentally introduced into NZ in the late 1970s, but not formally detected until 1988 (Heath and Bishop, 1995). *Lucilia cuprina* is also found in many other parts of the world, but it prefers a warm environment with soil temperatures above 15 ºC, air temperatures above 17 ºC, but below 40 ºC, and mean wind speeds below 30 km/h (FlyBoss, 2017).

The adult *L. cuprina* blowfly (Figure 2.1a) is characterised by having a shiny green or green-bronze abdomen with bronze-coppery reflections, large reddish eyes, two pairs of wings (membranous wings and halters), a round to oval body shape, and a length of between 4.5 to 10.0 mm (Stevens and Wall, 1996). Figure 2.2 shows the typical life cycle of *L. cuprina*. Adult females lay their eggs on soiled wool, and in warm moist conditions, the eggs hatch in 8 to 36 hours. In the first maggot stage (the first larval instar), the maggot has no mouth parts and cannot damage healthy tissue. However, the skin affected with fleecerot or soiling, by urine or faeces, can weep fluid and this provides nutrients for the maggot. This creates conditions that are ideal for the larvae to establish. The second and third instar larvae (older maggots, Figure 2.1b) attack and damage the skin tissues causing myiasis (Levot, 2016). The third instar larvae can fall off the sheep and burrow into the soil to pupate, and this is where they

spend a major part of their life (Molyneux and Bedding, 1984). The duration of this stage is dependent on soil temperature. At 30 ºC pupation takes about six days, whereas at 15 ºC it takes 25 days (Foster *et al*. 1975). If the soil temperature is too cold, the third instar larvae will enter a state of diapause. They will remain in the soil until the following spring when soil temperatures reach a base temperature of 9.2 ºC for 30 days, and then go through pupation and emergence (Pitts and Wall, 2005; Wall *et al*. 1992). After pupation, the adult fly emerges from the soil and feeds on plant and animal material; the females requiring protein nutrition before they can complete their sexual development and lay eggs (Levot, 2016).

Lucilia cuprina's closest relative is *L. sericata,* also known as the 'common green bottle blowfly' (Gleeson and Heath, 1997). *Lucilia sericata* has a brilliant, metallic, blue-green or golden colouration with black markings, black bristle-like hair and three cross-grooves on its thorax. These are between 10 and 14 mm long. Their wings are clear with light brown veins, and their legs and antennae are black (Figure 2.3a). While globally *L. sericata* is a common species, it is found mainly in the southern hemisphere and prefers coastal terrain, and warm, moist climates (Gleeson and Heath, 1997). Like most fly species, *L. sericata* has three larval stages (Figure 2.3b shows the third instar) in their life cycle (Levot, 2016; Stevens and Wall, 1996). Adult female *L. sericata* can lay up to 200 - 250 eggs at a time (Cruickshank and Wall, 2002; Smith and Wall, 1998). They will often lay their eggs on animal or human corpses, the infected wounds of humans or animals, and in animal excrement. The larvae of *L. sericata* feed mostly on decomposing tissue (Valachová *et al*. 2013).

Calliphora stygia, also known as the "brown blowfly", is native to Australia and is characterised by having gold - brown hairs that cover its body. It has a grey thorax and yellow-brown mottled abdomen (Figure 2.4a) (Pickering, 2013). Prior to the introduction of *L. cuprina* to NZ, *C. stygia* was one of the major species involved in the initiation of flystrike on sheep and the earliest reports of *C. stygia* being involved in flystrike were written in 1841 (Phillips, 2009). *Calliphora stygia* is more adapted to lower temperatures than other species of blowflies, and it will often strike sheep in cooler months when *L. cuprina*, *L. sericata* and *C. rufifacies* are in low abundance (Norris, 1965). *Calliphora stygia* can be present in large numbers in the spring and autumn, and can be found on sunny winter days. These flies are less common during the heat of summer (Levot, 2016). Figure 2.4b shows the third instar larvae of *C. stygia.*

Figure 2.1 a) Adult female *Lucilia cuprina* and b) third instar larvae.
(Source https://upload.wikimedia.org/wikipedia/commons/thumb/e/e0/Australian_sheep_blowfly. jpg/800px-Australian_sheep_blowfly.jpg, and http://agriculture.vic.gov.au/__data/assets/image/ 0010/226666/AG0081b.jpg, respectively).

Figure 2.2 The life cycle of *Lucilia cuprina*, the 'Australian blowfly'.
(Adapted from http://www.thefarmpage.com/wordpress/wp-content/uploads/2011/06/blowfly-2-300x205.jpg 5/6/2017)

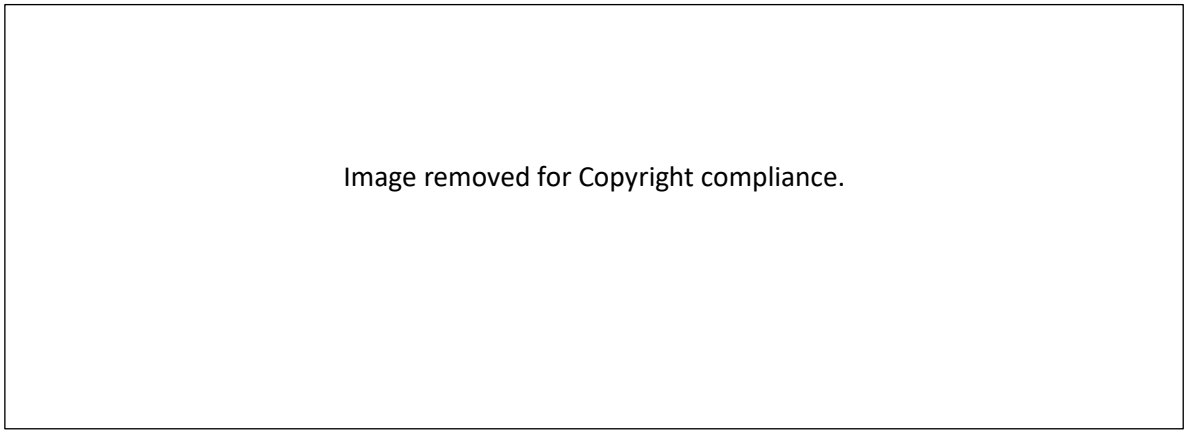


Figure 2.3 a) Adult female *Lucilia sericata*, and b) a third instar larvae.
(Adapted from http://www.terrain.net.nz/friends-of-te-henui-group/local-flies/fly-green-bottle-lucilia-sericata.html and https://digitalinsectcollection.wikispaces.com/Common+Green+Bottle+Fly, respectively 5/6/2017)

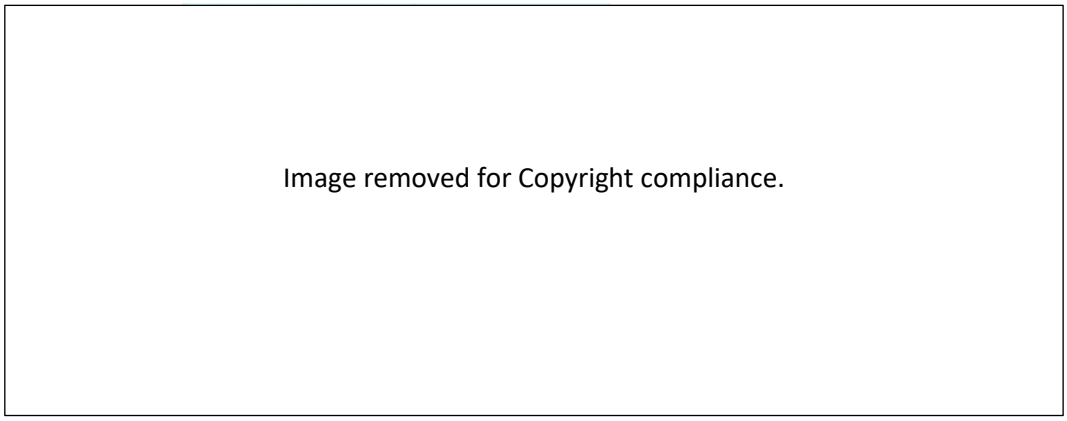


Figure 2.4 a) Adult female *Calliphora stygia*, and b) third instar larvae.
(Both images adapted from http://agriculture.vic.gov.au/agriculture/pests-diseases-and-weeds/pest-insects-and-mites/sheep-blowflies-in-victoria 5/6/217).

Chrymosmya rufifacies is also referred to as the 'hairy maggot blowfly'. It has a shiny metallic blue-green colour, and is about 6 to 12 mm in length (Figure 2.5a). It is very similar in colour to *L. cuprina*, but has a smaller and white coloured anterior thoracic spiral (Lang *et al*. 2001). Unlike *L. cuprina, C. rufifacies* cannot initiate flystrike, but once other species have, it can become involved in a secondary strike. Once *C. rufifacies* is involved, the damage to the sheep increases within a matter of hours. This is because the life cycle of this fly is short and it takes from 190 to 598 hours depending on the temperature. *Chrymosmya rufifacies* larvae are much larger than the larvae of the other flies, and they have more vigorous mouth parts than those of other species, which they can out-compete and even devour (Lang *et al*. 2001). The larvae of this species are easily identified as they have sharp fleshy tubercles aligned along their bodies, and are about 14 mm in length when mature. They have a dirty-yellowish colouration (Figure 2.5b) (Levot, 2016). The involvement of *C. rufifacies* in an infestation, can lead to the death of the sheep.

Figure 2.5 a) Adult female *Chrymosmya rufifacies*, and b) a third instar larva.
(Both images adapted from http://www.terrain.net.nz/friends-of-te-henui-group/local-flies/fly-hairy-maggot-blow-fly-chrysomya-rufifacies.html 5/6/2017).

Flystrike in sheep

Strike can occur on most parts of the sheep, provided there is sufficient moisture to protect the eggs and young larvae of the flies from desiccation (Sandeman *et al*. 1987; Wardhaugh *et al*. 2001). As a consequence, flystrike often occurs in association with other diseases and conditions, including: dermatophilosis, footrot, tick infestations and bites, lice infestation, uterine prolapse, scouring and internal parasitism (Heath and Bishop, 2006), and fleecerot and mycotic dermatitis (Karlsson and Greeff, 2012).

Fleecerot, also known as bacterial dermatitis, is a predisposing condition for flystrike. It occurs in sheep that have fleece characteristics and a body conformation that allows the fleece to hold moisture after wetting (Norris *et al*. 2008). Fleecerot and flystrike can occur together as a disease 'complex', and with a strong interdependence for where the strike occurs on the body of the sheep (i.e. the shoulders, back and flanks) (Colditz *et al*. 2000). Fleecerot causes inflammation and ulceration of the skin, which can attract flies to lay their eggs, and provides moisture for the eggs to hatch, along with a soluble protein source for the hatched larvae to feed on (Smith *et al*. 2010). In addition to areas with fleecerot, and the presence of urine and faecal stains in the wool, other 'attractive' areas for flies to strike include the eye, areas of skin damage caused by grass seeds or other agents, footrot affected hooves, wear around the horns on rams that have been fighting, mulesing wounds and lambing stain on ewes (Levot, 1995). Ovi-positing female flies release a pheromone that entices other females to lay, thus existing flystrike is an attractant for other gravid female blowflies (Levot, 1995)

While strike can occur on most parts of the sheep, most flystrike occurs in the breech area because this area is often warm and moist (Figure 2.6) (D'Arcy, 1990). Breech-strike can be further divided into crutch-strike, which occurs from the tail base to the border of the udder or scrotum, and tail-strike, which usually occurs around the stump or sides of the tail (Phillips, 2009). The occurrence of breech-strike is influenced by gender, age, breed, season, wool length, tail length and the conformation of the crutch area (Raadsma, 1987).

Body-strike occurs over the shoulder and back regions of the sheep. Outbreaks often develop after a period of prolonged rainfall during the warmer months of the year. Young sheep, regardless of gender are the most susceptible (Broughan and Wall, 2007; Raadsma, 1987). Body-strike is typically preceded by the bacterial dermatitis that can develop when the fleece and skin become wet after extended wet periods (Colditz *et al*. 2006). The normal defence barriers of the skin appear to breakdown under bacterial challenge, and this provides an area that is more attractive to the female fly to lay her eggs on. The moist, bacteria-laden skin is also an ideal environment for the larvae to survive and grow on, once hatched.

Other areas where flystrike can occur include the poll, pizzle and feet. Poll-strike predominantly occurs in rams due to the presence of horns and wrinkles at the horn site. The majority of strike occurs

at the base of the horns, but strike can also occur in wounds sustained during fighting (Karlsson and Greeff, 2012). Poll-strike is the most common type of strike in rams, accounting for 55 % of the strike in Australian studies (Raadsma, 1987; Watts *et al*. 1979).

Pizzle-strike occurs more commonly in young rams and wethers, when the long wool around the preputial opening becomes soiled with urine, or possibly because of the presence of pizzle rot (Raadsma, 1987). There is also an association between the risk of flystrike and footrot (Horton and Champion, 2001). Footrot has been found to be a factor predisposing sheep to flystrike, but the prevalence of flystrike in the feet is not well documented, as it is often covert and goes unnoticed (Heath and Bishop, 2006).

Figure 2.6 Regions of the sheep that are commonly struck by flies in New Zealand. 1: Breech-strike accounting for 81.2 % of strike, 2: Body-strike accounting for 9.3 % of strike, 3 and 4: Shoulder and head/poll-strike respectively, accounting for 7.1 % of strike, 5 and 6: Belly/pizzle and foot-strike, accounting for 2.5 % of strike.
(Adapted from Fleming 2003; Pickering, 2013).

In NZ, flystrike has been reported since 1890s (Heath, 1994), but it was not until the 1920s that it became of major concern to farmers (Heath and Bishop, 2006). A survey at the time found that the primary fly species involved were *L. sericata* and *C. stygia*. Up until the 1980's, flystrike in NZ was a seasonal disease, but after the introduction of *L. cuprina* the prevalence and period of occurrence of flystrike increased (Heath and Bishop, 2006; Holloway, 1991).

In NZ, flystrike challenges vary both regionally and seasonally. Flystrike is more prevalent in the North Island of NZ, where weather conditions are typically more humid, especially when compared with prevalence in the lower half of the South Island, where flystrike is less common (Heath and Bishop, 2006). Flystrike does not typically occur in cooler temperatures, such as those occurring in winter. Typically, flies 'over-winter' as pupae or as adults. Once soil temperatures rise above 12 ºC in the spring, the pupae hatch and the adult flies become active (Heath and Bishop, 1995; West *et al*.

2009). Most flystrike, therefore occurs from November to March, during warm and humid climatic conditions. In some years and regions, flystrike can occur from October to May, if not longer with both *L. cuprina* and *C. stygia* striking sheep all year round if conditions are favourable (Hacker, 2010; Hughes and Levot, 1987; West *et al*. 2009). *Lucilia sericata* usually strikes from December to March and *C. rufifacies* strikes between January and March (Heath and Bishop, 1995).

During the peak strike season, flies attack in waves. In pastoral zones, these have been classified as the primary, secondary and tertiary fly waves (Bates, 2007). Fly waves often occur after spring and summer rainfall, resulting in the rapid occurrence of flystrike (Hughes and Levot, 1987). Strike that occurs in the peak of the flystrike season can be more severe, because affected sheep can be re-struck by the large population of gravid female blowflies present during this period (Wardhaugh and Morton, 1990). Sheep need to remain wet for long periods to favour flystrike, so during the summer in Australia, where the sheep can dry within hours of being wet, flystrike is not as prevalent (Hacker, 2010; Levot, 1995). In NZ though, it is possible for flystrike to occur throughout the year, especially if the winter weather conditions are mild, or warm and moist during the rest of the year (Heath, 1994).

The pathological effects of flystrike

The numerous pathological effects of flystrike on sheep are largely a result from the feeding activity of the larvae. These effects are both mechanical and chemical (Heath and Bishop, 2006). Larval infestation results in reduced wool quality and growth, as well as a reduction in ewe fertility, with heavy infestations sometimes causing death (Broadmeadow *et al*. 1984a). Decreased wool staple strength results from both the decreased feed intake of the sheep and from having increased cortisol levels. This leads to the narrowing of wool fibres, and subsequently a decrease in the tensile strength of the wool (O'Sullivan *et al*. 1984; Colditz *et al*. 2005). Indeed, wool growth is impaired for 30 days after a larvae challenge has been removed (Broadmeadow *et al*. 1984a).

Struck sheep have an increased rectal temperature and respiration rate, and a decreased feed intake (Colditz *et al*. 2005; O'Sullivan *et al*. 1984). Anorexia can occur soon after the larval challenge begins and continues progressively during the infestation, resulting in live-weight loss. It is not until a larval challenge is over that sheep feed-intake increases and live-weight can increase (O'Sullivan *et al*. 1984). Temperature and respiration rates increase as a result of a systemic reaction to the larval challenge, and when the number of larvae on the sheep decrease the sheep's rectal temperature and respiration rate also decline (O'Sullivan *et al*. 1984).

At the third larval stage, larvae release ammonia which can elicit an acute-phase response in the sheep resulting in rapid progression of disease, and death within three days (Karlsson and Greeff, 2012). Death from flystrike is usually a consequence of the toxaemia caused by the large quantity of ammonia released by the larvae (Broadmeadow *et al*. 1984b). For example, one hundred *L. cuprina* larvae are capable of producing and excreting 80 mg of ammonia each day, thereby increasing the pH

of the skin. *L. cuprina* larvae thrive in alkaline environments, with the optimum pH for larval collagenase growth and survival being pH 8.0 to 9.0. The pH of the skin and fleece of infested sheep can rise to 8.5 ± 0.3, and skin temperatures during myiasis can reach 53 ^{o}C, which favours further larval growth and further ammonia production (Guerrini, 1988).

Immune responses to flystrike

Sheep that are infested with larvae mount both a humoral and cellular immune response (Sandeman *et al*. 2014). A key finding was that sheep produce Immunoglobulin G (IgG) in response to infection with blowfly larvae (O'Donnell *et al*. 1980), while other studies describe the presence of immunoglobulin isotypes including, IgG1, IgG2, IgA and IgM (O'Meara *et al*. 1997), and IgE (MacDiarmid *et al*. 1995). IgG1 has been identified as the most abundant isotype present during repeated infection (Seaton *et al*. 1992).

Bowles *et al*. (1992) described the cellular immune response of sheep struck by flies. They observed that within 48 hours of infection the cellular infiltrate was comprised primarily of leucocytes. Neutrophils and eosinophils made up the major leucocyte types found at the wound surface; the increased presence of neutrophils resulting from the physical damage done to the skin. The cellular damage to the skin, including the wool follicles, results in loss of wool in the area of active strike (Sandeman *et al*. 1987).

Methods to prevent or reduce the impact of flystrike

Changing the physical characteristics of sheep fleece/skin

There are a range of management strategies commonly used on farm to prevent and reduce the impact of flystrike. Several of these strategies focus on manipulating the sheep's physical characteristics and include the use of shearing and crutching, along with tail docking (the removal of the sheep's tail) and the mulesing of lambs (Rammell *et al*. 1988).

Shearing decreases the likelihood of flystrike because short wool dries faster than long wool, and this reduces the time available for larval establishment (Tellam and Bowles, 1997). Shearing also improves the effectiveness of insecticides when they are applied to sheep, as it allows greater penetration of the chemical through the wool and onto the skin (Rothwell, 2005).

Crutching mid-way between successive shearing's is a common preventive measure to prevent breech strike (Bell, 2010). Crutching is also used as a way of removing dags and urine stained wool from around the breech area, thus reducing the attractiveness of the area to flies (Tellam and Bowles, 1997). Changing the date of shearing and crutching can reduce the risk of un-mulesed sheep getting flystrike, but the dates set for shearing are determined by multiple factors, such as the availability of shearers, lambing dates, market factors, the risk of wool contamination and the risk of flystrike occurring (Horton and Champion, 2001).

To provide a more permanent solution to breech-strike, a procedure called 'mulesing' was developed. Mulesing involves the surgical removal of both the wool and skin from around the breech area. This results in scarring when the wound heals. This scarred area is devoid of wrinkles and/or skin folds, hence there is less wool available for contamination with either urine or faeces. As a result, this area becomes less attractive to the female flies (Plant and Coombes, 1988; Tellam and Bowles, 1997). Mulesing reduces the occurrence of breech-strike, and increases the survival rate of sheep that get breech-strike (Plant and Coombes, 1988).

Although there are clear benefits from mulesing, there is also considerable farmer and public opposition to this procedure, as it is stressful for the sheep (James, 2006; Lee and Fisher, 2007; Tellam and Bowles, 1997). Australia has undertaken a phased withdrawal of mulesing, and this presents particular challenges for the pastoral wool industry, as the mustering and treatment of sheep is more difficult and expensive than the practice of mulesing in some farming situations. There is no accepted alternative to mulesing, although there are several options under evaluation (Bell, 2010; James, 2006), such as breeding sheep that have increased breech bareness and reduced dag accumulation (Scobie *et al*. 2010).

Chemical and hormonal methods for controlling flystrike

Insecticides are currently relied on for controlling flystrike. Not only are they applied as a method to prevent flystrike, but they can also be used as a dressing to treat struck areas (Tellam and Bowles, 1997). Historically, a wide range of chemicals and insecticides have been used to control flystrike. For example, the earliest treatment for flystrike used mixtures of arsenic trioxide, copper sulphate, sulphur and cresylic acid (Levot, 1995). In the late 1940s, organochlorines were introduced and used extensively, but these were withdrawn from use in 1958 due to residue problems caused by the persistence of these insecticides in the adipose tissue of sheep (Levot, 1995). In this time fly larvae also developed resistance to this class of insecticide, particularly the product Dieldrin (Levot, 1995). The organochlorines were superseded by a range of organophosphorus insecticides. These have been very successful as they can be used to control both lice infestations and flystrike on sheep (Tellam and Bowles, 1997). In the last 50 years, organophosphorus insecticide use has become widespread, due to the low cost of production and their double benefit (Tellam and Bowles, 1997). However, resistance has also become a problem with this type of flystrike prevention/treatment (Levot, 1993).

The most recent chemical/hormonal methods for preventing flystrike are the use of insect growth regulators (IGRs). These work by inhibiting larval moulting (West *et al*. 2009). In the early 1980s, Cyromazine, a triazine derivative with IGR activity, was introduced to NZ and Australia for the prevention of flystrike (Hart *et al*. 1982; Nottingham *et al*. 2001). Since then other IGRs have been developed, including the benzoylureas, diflubenuron and diflubenzuron (Nottingham *et al*. 2001). The IGR class of insecticides can give up to 24 weeks of protection against blowflies, and this is twice as long as other insecticides (Tellam and Bowles, 1997).

There are several concerns around the use of insecticides, with the major concern being the detection of chemical residues in the wool and lanolin (Russell, 1994). Insecticide residues can be removed from the wool during scouring and subsequent wet processing, and may not be present in the clean wool. However, the insecticides removed during scouring remain in the wool wax, dirt and effluent water. The insecticide can remain in the wool wax through the lanolin refining process, and they have been found in pharmaceutical grades of lanolin (Russell, 1994).

The effluent water from wool scouring contains a high concentration of insecticide and this can be damaging to the environment (Horton *et al*. 1997). When applied to long wool there is an increase in the retention of chemical residue (Horton, *et al*. 1997; Plant *et al*. 1999), thus withholding periods for wool shorn after dipping have been recommended to be 60 days for crossbred sheep, 100 days for mid-micron sheep and 180 days for fine wool sheep (Anon, 2005).

Another major concern is the growing resistance to insecticides in the fly population. *Lucilia cuprina* has been changed by, and become resistant to, various insecticides with different modes of action (Batterham *et al*. 2006). The species quickly became resistant to organochlorines and it has now developed resistance to the organophosphorus insecticides, in particular Diazinon (Levot, 1993, 1995; Tellam and Bowles, 1997). Organophosphorus is still used, but its protection period has reduced from 14 weeks, to 4 - 6 weeks (Levot, 1993). More recently, *L. cuprina* has developed moderate resistance to the IGR diflubenzuron (Levot and Sale, 2002). This resistance to diflubenzuron is a good example of why farmers need to rotate insecticide groups and not rely solely on one class of insecticide for flystrike prevention (Levot and Sales, 2002). In 2008, diflubenzuron was deregistered for the prevention of flystrike on sheep, due to the high levels of resistance observed in the blowfly population (Flyboss, 2017).

Chemical drenching is another common farm practice that aids in the prevention of flystrike. Drenching can be used to reduce scouring and dag formation by controlling gastrointestinal worm burdens for internal parasites such as *Trichostrongylus colubriformis* and *Ostertagia spp* (Gogolewski, 1997; Sneddon and Rollin, 2010; Waghorn *et al*. 1999).

Morley *et al*. (1976) investigated the relationship between gastrointestinal helminth infections and breech strike in weaned lambs. Their study revealed that fewer sheep where struck when drenched at weaning (23 %), compared with those that were not drenched at weaning (50 %). The incidence of flystrike was also lower in sheep weaned onto clean pastures, when compared with those weaned onto parasite infected pastures (30 % versus 44 %). In NZ, there has been a report of a phenotypic correlation of 0.97 between the presence of dagginess and the incidence of breech-strike (Leathwick and Atkinson, 1995).

There are several other factors that result in scouring and dag formation with sheep, such as the occurrence of bacterial infections, the weather conditions, nutrition changes at weaning, and the

presence of lower neutral detergent fibre (NDF) levels in spring pastures (Waghorn *et al*. 1999). Given that some sheep within a flock will develop dags, while others will have none, it is clear that there are traits that are associated with individual sheep that affect the initiation and accumulation of dags. These may also include gender, tail length, wool type and length, and other physiological differences (Waghorn *et al*. 1999).

Fly-trapping

Fly-trapping has been shown to reduce the density of the fly population and the strike incidence. The placement of bait-bins on a sheep property that kept quantitative historical records of fly numbers and strike incidence, indicated that flystrike and *L. cuprina* numbers were lowered by their presence (Anderson *et al*. 1990; Cook, 1990; Urech *et al*. 2004). Chemical attractants lure flies into the devices, which then trap them. The trapped flies then die from starvation and dehydration (Tellam and Bowles, 1997). Such control methods could reduce the use of insecticides, and this would accordingly decrease the levels of residues in wool products (Urech *et al*. 2004).

Studies over the last 60 years have focussed on the types of volatile components emitted by natural sources, and which attract flies. These studies have found that *L. cuprina* migrates towards bacterial strains that produce chemical attractants and volatile compounds from the myiatic lesions of sheep (Khoga *et al*. 2002; Urech *et al*. 2004). Liver and sodium sulphate have also been found to be suitable attractants *for L. cuprina* (Urech *et al*. 2004). These two substances are often used to bait LuciTraps (Bioinsectaries SA [BISA]), which are commercially available fly traps that have been shown to reduce *L. cuprina* populations and the incidence of flystrike when used at a recommended rate of one trap per 100 sheep (Urech *et al*. 2009). The area to fly-trap ratio can vary, and it is recommended to have fly traps in areas where sheep may congregate (for example along creeks or near water troughs). Mackerras *et al*. (1936) showed that a density of one trap per 40 Ha resulted in a significant reduction in flystrike. The current cost of LuciTraps is $138.50 Australian dollars for five traps (https://bugsforbugs.com.au/product/sheep-blowfly-trap-kit-lucitrap/), which includes the bait, or $27.70 per individual trap.

Immunisation

Vaccine development studies have shown that the sheep's immune system is able to recognise components of the larvae of flies as foreign, and that it is able to generate immune responses (Colditz *et al*. 2006). It was speculated that immunity may develop following exposure to blowfly larvae, and result in a proportion of adult sheep developing an adaptive resistance to strike (Baron and Colwell, 1991).

There have, however, been inconsistent results as regards the acquisition of immunity to *L. cuprina*, and the resistance that does develop, appears to be ineffective in the face of a large challenge (French *et al*. 1995). Bowles *et al*. (1987) showed that when sheep are repeatedly immunised in such a way as

to induce a hypersensitivity reaction to the larvae, a diminishing level of immune protection results. This diminishing immunity to repeated larval challenges contrasts the increase in immune response observed for other pathogens, such as viruses and bacteria (Colditz *et al*. 2006). This is believed to occur because when sheep that have been vaccinated are challenged with live larvae, the larvae are able to modulate the sheep's immune system, and prevent it from recognising the larvae as a foreign invader (Colditz *et al*. 2006). With the ongoing study of the host-immune response to myiasis and larval-host interactions, it is thought that an effective vaccine against flystrike will be designed in the future (Otranto, 2001; Sandeman e*t al*. 2014).

Breeding for genetic resilience to flystrike

Many heritable traits predispose sheep to flystrike. These factors include susceptibility to fleecerot, having increased numbers of breech wrinkles, and the formation of dags. Flystrike and fleecerot occurrences have been found to have a high genetic correlation (r = 0.9), and therefore they can be, and have been selected for concurrently when breeding to obtain flystrike resilient sheep (McGuirk *et al*. 1978; Morris, 2009; Raadsma, 1991).

In the 1970s a breeding programme for resistance to fleecerot and flystrike was established by the New South Wales Department of Primary Industries (NSW DPI) at Trangie in NSW, Australia. The DPI developed experimental conditions for inducing fleecerot and flystrike using artificial wetting with overhead sprinklers on sheep that were temporarily housed indoors (Colditz *et al*. 2001; McGuirk *et al*. 1978). These sheep were scored for fleecerot and flystrike susceptibility in the weeks following the artificial wetting. Separate lines of sheep were then bred for resistance (although this should more accurately be called resilience) and susceptibility, using an index that included susceptibility scores for both natural, and induced fleecerot and flystrike (Colditz *et al*. 2006).

For Merino sheep, the susceptibility to body-strike is moderately heritable, and the heritability was estimated by Raadsma *et al*. (1989) to be 0.3 ± 0.22. It has also been suggested that a major gene may account for 20 % of the phenotypic variation in fleecerot and 15 % of the variation in body-strike for Merino sheep (Mortimer *et al*. 2001). In NZ, Beef and Lamb Genetics has calculated that a 10 % genetic improvement in flystrike resilience would be worth $6.02 million (Ludemann *et al*. 2010; Linscott. E, personal communication, August 30, 2016), and hence identifying a major gene would be of substantial value to the industry.

Breech-strike is the most common type of flystrike that occurs in NZ. Seddon *et al*. (1931) found that the susceptibility to breech-strike in sheep was a repeatable trait; with the same individual sheep being likely to be re-struck each season (Seddon *et al*. 1931; Scholtz *et al*. 2010). Traits such as increased breech wrinkles, decreased breech bareness and increased dag formation have been identified as contributing traits to breech-strike susceptibility (Scobie and O'Connell, 2010; Seddon *et al*. 1931). The association between breech wrinkles and susceptibility to breech-strike was identified in the early

1930s (Seddon *et al*. 1931). Increased breech wrinkles results in increased urine and faecal staining of the breech wool, and this provides a moist environment, thus increasing the risk of flystrike.

Breech-wrinkle is correlated to breech-strike (r = 0.22), with an estimated heritability for wrinkling of 0.36 (Smith *et al.* 2009). Breech-wrinkle can be selected against, and bred out to produce a more plain bodied sheep, that is less susceptible to flystrike (Figure 2.7).

Breech-strike prevalence also increases with the increased accumulation of dags (Scobie and O'Connell, 2010). Breech bareness (Figure 2.8) has been shown to reduce the accumulation of dags and decrease the incidence of flystrike (Scobie *et al*. 2002). Scobie *et al*. (2007) showed that breech bareness has a heritability of 0.33 ± 0.06. Like breech bareness, sheep can also be 'scored' on their dag accumulation with high repeatability (Figure 2.9). This makes 'dag-score' a good indicator trait for breech-strike. Dag-score has a heritability of 0.23, and breech-strike has a high genetic correlation with dag-score (0.71) (Scobie and O'Connell, 2010; Pickering *et al*. 2015) and low correlation with breech bareness, where a breech bareness of 4 or 5 is sufficient to protect lambs against breech-strike (Scobie and O'Connell, 2010).

Figure 2.7 Wrinkle scores. Increasing score indicates more wrinkles.
(https://www.agric.wa.gov.au/livestock-parasites/managing-non-mulesed-sheep?page=0 %2C2 24/8/2017).

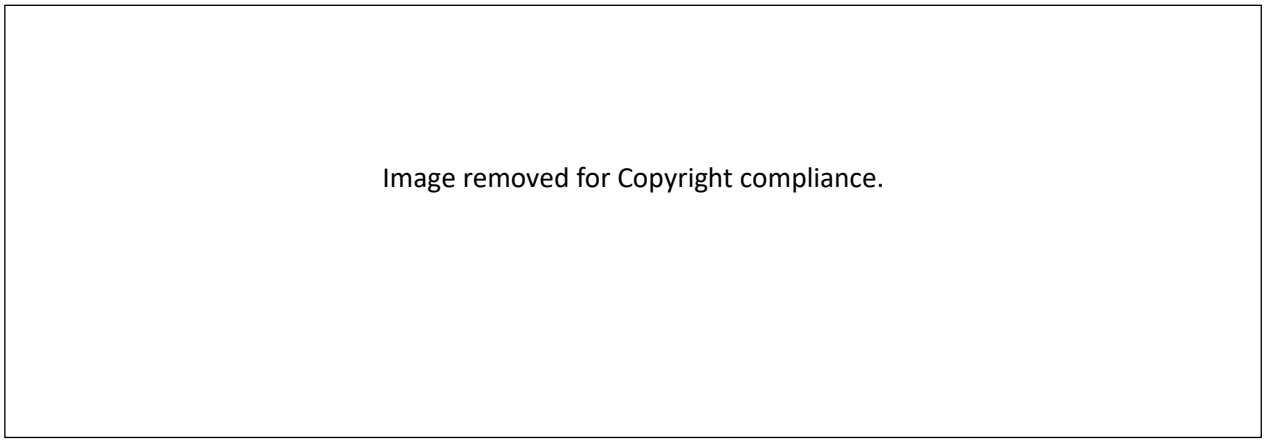


Figure 2.8 Breech bareness scores. Increasing score indicates more breech area wool.
(https://www.agric.wa.gov.au/livestock-parasites/managing-non-mulesed-sheep?page=0 %2C2 24/8/2017).

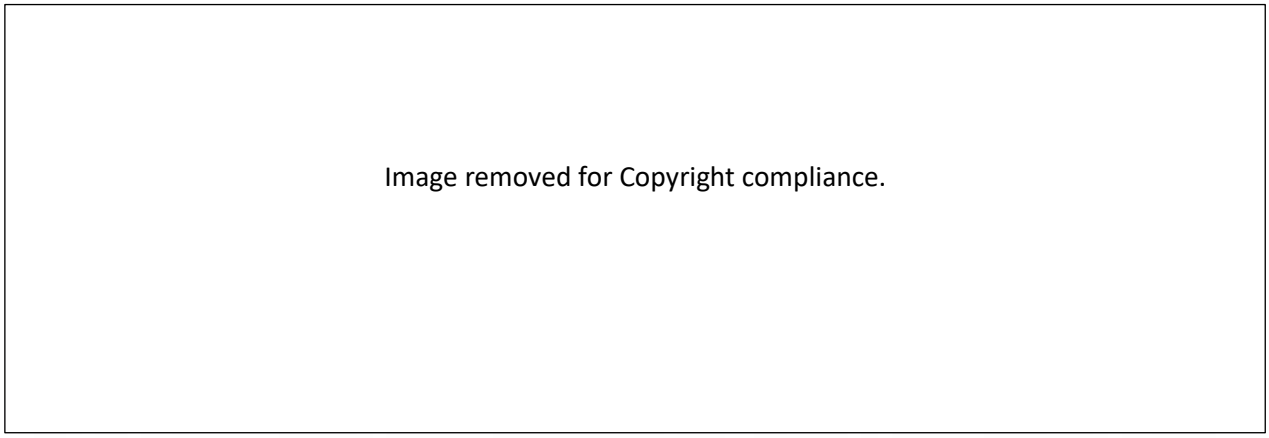


Figure 2.9 Dag scores. A score of 1 represents no dags and score 2 to 5 indicate increasing dag accumulation.
(https://www.agric.wa.gov.au/livestock-parasites/managing-non-mulesed-sheep?page=0 %2C2 24/8/2017).

Fleece characteristics are another crucial factor influencing flystrike susceptibility. The fleece is composed of the wool fibre, wax, suint (sweat) and extraneous matter such as soil, plant material, water, fungi and bacteria. Wool fibres are composed predominantly of protein, namely the keratins and the keratin-associated proteins, and these are coated with wax and suint (Henderson, 1965). Wool wax is secreted by the sebaceous (oil) glands and is a mixture of cholesterol, lanosterol, fatty-acids (e.g. palmitic and iso-stearic acid) and hydroxy fatty-acids (Emmens and Murray, 1982).

Suint is produced by the sudoriferous (sweat) glands and contains a range of water-soluble compounds, such as electrolytes (e.g. potassium, carbonate and sulphate), fatty-acids, organic acids, amino acids, urea and other nitrogenous compounds (Emmens and Murray, 1982). These compounds from suint provide nutrients for a variety of bacteria that survive in the fleece, including *Pseudomonas aeruginosa, Bacillus subtilis, Enterobacter cloacae and Proteus mirabilis* (Emmens and Murray, 1982). Wool wax and suint mix together to form wool grease, which functions as a barrier to protect the skin and wool from external damage (Henderson, 1965; Collins and Davidson, 1997; Norris *et al*. 2008).

During periods of prolonged rainfall, suint acts as a detergent and aids in the removal of wax from around the wool fibres (James *et al*. 1984). During this time, the chemical composition of the wool wax is also altered, with an increase in the content of cholesterol and lanosterol and an associated increase in the quantity of a variety of *Pseudomonas spp* (Merritt and Watts, 1978; James *et al*. 1984). Cholesterol is a powerful emulsifying agent in wool wax, and it seems likely that the physical breakdown of the skin-wax layer is caused by the *Pseudomonas spp.* (James *et al*. 1984). The changes in wool wax observed in wool wetting experiments include an increase in the hydrophilic character of the wax (Hay and Mills, 1982; James *et al*. 1984) and these conditions can give rise to both yellowing of the wool, and fleecerot.

Resistance to yellowing and fleecerot has been described in sheep that have higher wool wax contents (Aitken *et al*. 1994; Evans and McGuirk, 1983; Lipson *et al*. 1982). The amount of wax and suint in a fleece varies between breeds of sheep. Strong wool types of sheep have a lesser amount of wax in their wool compared to fine wool breeds (Collins and Davidson, 1997; Daly and Carter, 1956), and thus the skin of Merino sheep is often more difficult to wet than strong wool, open-fleeced sheep. Merinos also have a much greater ratio of follicles per unit of skin area than strong wool sheep, hence strong wool sheep have a reduced sebaceous wax film that is less effective in protecting them from fleecerot (Roberts, 1963).

Candidate genes for flystrike resilience in sheep

There are few direct clues indicating the genes or gene activities that contribute to variation in susceptibility or resilience to flystrike. However, it is likely that susceptibility to flystrike is contributed to by variation in many different genes that control fleece, skin and immune system

characteristics (Raadsma, 1987). There have been very few studies looking at the genetics of flystrike susceptibility, but some potential candidate genes are described below.

Smith *et al*. (2010) identified that variation in the genes for fatty-acid binding protein 4 (FABP4) and fibulin-1 (FBLN1) were associated with fleecerot resistance in the divergent lines of Merino sheep farmed at Trangie (Trangie Agricultural Research Centre, NSW, Australia), and that were selected on the basis of fleecerot and flystrike resistance, when compared to a separate flock of outbred Merino X Romney-cross ewes from the CSIRO mapping flock (Armidale, NSW, Australia). Single nucleotide polymorphisms in *FBLN1* were associated with both pre-wetting and post-wetting fleecerot score in the Trangie resistant line. Single nucleotide polymorphisms in *FABP4* were also associated with fleecerot score in the Trangie resistant line pre-wetting, and in the susceptible line post-wetting.

The fatty acid binding protein 4 gene (FABP4)

The FABPs come from a family of small, cytoplasmic proteins, which are around 14-15 kDa in size, and that are conserved throughout evolution from *Drosophila* to humans (Tuncman *et al*. 2006). *FABP4* has been observed to have a conserved structure; with four exons, interrupted by three introns (Yan *et al*. 2012).

Adipocytes and macrophages express the same two FABP genes, *FABP4* and *FABP5*, and these are expressed at similar levels in the activated macrophages. They are regulated by both metabolic and inflammatory mediators (Tuncman *et al*. 2006) and FABP4 acts to coordinate functional interactions between macrophages and adipocytes in the adipose tissue (Furuhashi and Hotmaisligil, 2008). These cells play roles in metabolic inflammatory diseases, which could suggest FABP4 could have a role in the inflammatory response to flystrike.

Fatty acid binding protein 4 has also been suggested to have a role in sebaceous gland differentiation (Tsuda *et al*. 2009). In sheep, the sebaceous glands have a key role in protecting skin and wool from the development of fleecerot, by secreting wax into the fleece. The amount of wax and suint in a fleece varies between breeds of sheep; with strong wool sheep typically having less wax in their wool than fine wool breeds (Collins and Davidson, 1997; Daly and Carter, 1956).

The fibulin-1 gene (FBLN1)

The fibulin (FBLN) family of extracellular-matrix (ECM) proteins have a role in the early stage of tissue differentiation and organ development, and function as intramolecular bridges that stabilise the organisation of supra-molecular ECM structures, such as elastic fibres and basement membranes (Argraves *et al*. 2003; Chu and Tsuda, 2004).

In humans, *FBLN1* is expressed in various types of skin cells and skin structures, including the basal cells of the epidermis, the fibroblasts, the dermal-epidermal junction regions, the elastic fibres, the sebaceous glands, and the sweat gland epithelium (Roark *et al*. 1995). *Fubulin-1* has also been shown

to be expressed around the hair follicle and dermal/epidermal border in the skin of rats (Aspberg *et al*. 1999). *Fibulin-1* is likely to have similar expression in the sebaceous and sweat glands of sheep, as well as around the wool follicle, which might be how it contributes to fleecerot and flystrike resilience.

The toll-like receptor 4 gene (TLR4)

The immune response to flystrike involves both the innate and adaptive immune response. Blowfly larval antigens stimulate an innate immune response in sheep, and this activates natural killer cells, eosinophils, macrophages, granulocytes, monocytes and dendritic cells (Otranto, 2001). The process of pathogen recognition involves the coordinated action of several protein families, including the family of toll-like receptors (TLRs) (Bochud *et al*. 2007).

Among the TLRs, TLR4 recognises the lipopolysaccharides (LPS) of Gram-negative bacteria, mannans from fungal pathogens, a soluble component of *Mycobacterium tuberculosis*, and other endogenous ligands such as fibronectin and some heat-shock proteins (Ferwerda *et al*. 2008). Lipopolysaccharides (sometimes referred to as endotoxin), can promote the release of inflammatory cytokines that trigger innate immune responses, and they can cause shock, and even death. It was thought originally that LPS acted through many different cell receptors, but a study in mice revealed that the LPS sensor is dependent solely on TLR4 (Sultzer, 1968; Watson *et al*. 1977), while a nonsense mutation in *TLR4* causes insensitivity to LPS (Beutler, 2000).

In humans, single nucleotide polymorphisms (SNPs) in *TLR4* have been associated with the incidence or development of various inflammation-related diseases and cancer (Mantovani *et al*. 2008; Noreen *et al*. 2012). Furthermore, SNPs in the leucine rich repeats (LRR) region of human *TLR4* have been found to reduce TLR signalling (Arbour *et al*. 2000; Smirnova *et al*. 2001) and change the ligand-binding properties of TLR4 (Rallabhandi *et al*. 2006). In this respect, variations in the ovine pathogen-associated molecular patterns (PAMP) recognition regions of TLR4 may result in functional and structural differences to the TLR4 molecule, which could lead to variation in the way TLR4 responds to bacterial and parasitic infections (Ferwerda *et al*. 2008; Lin *et al*. 2016). This could lead to variability in the immune response of sheep challenged with fleecerot and/or flystrike.

Ras guanyl-releasing protein 1 (RASGRP1)

As described above, there is a strong immune response to flystrike, and therefore it is likely that immune response genes may have a role in flystrike resilience. Ras guanyl-releasing protein 1 (RASGRP1) functions as a nucleotide exchange factor that activates Ras through the exchange of bound GDP for GTP (Roose, *et al*. 2005). This in turn activates enzymes in several signalling pathways, including mitogen-activated protein kinases (MAPKs) (Stone, 2011), extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 (Priatel *et al*. 2002). In the immune system, expression of *RASGRP1* is most abundant in T cells, but it is also expressed in B cells, natural killer cells and mast cells (Ebinu *et al*. 2000; Lee *et al.* 2009; Liu *et al*. 2007). RASGRP1 regulates T

and B cell development and function (Coughlin *et al*., 2005; 2006; Ebinu *et al*. 2000; Priatel *et al*. 2002), regulates the cytotoxicity of natural killer cells (Lee *et al.* 2009), and regulates mast cell activity in some allergic responses (Liu *et al*. 2007). Many of these cells that express *RASGRP1* are activated in the immune response to flystrike.

In response to flystrike, the innate immune system is stimulated to activate natural killer (NK) cells, eosinophils (E), mast cells (M), neutrophils (N), and γδ+ T cells (γδ+), while the adaptive immune response interleukin 12 (IL-12), Th1 and Th2 T cells, interleukin 2 (Il-2), and interferon - γ (IFN – γ) (Otranto, 2001).

ATP-binding cassette C11 protein gene (ABCC11)

The ATP-binding cassette (ABC) C11 protein gene (*ABCC11*) was identified by the SNP analysis conducted by Smith *et al*. (2010), as having a potential role in fleecerot resistance in Merino sheep. The ATP-binding cassette family are involved in the intracellular transport of various lipids, cholesterol and fatty-acids. *ATP-binding cassette C11* encodes an ATP-driven efflux pump for amphipathic anions (Ota *et al.* 2010), and there is evidence suggesting that variation in human *ABCC11*, a SNP that results in an amino acid substitution, determines earwax type (Toyoda and Ishikawa, 2010) and axillary osmidrosis (Nakano *et al*. 2009); as well as being associated with apocrine colostrum secretion from the mammary gland (Miura *et al*. 2007; Rodriguez *et al*. 2013). If variation in *ABCC11* had a similar function in sheep as it does humans, it could result in different odours that are differentially attractive to flies.

Technologies used to study variation in genes

The use of molecular markers to describe variation at the DNA level is a valuable tool for the investigation of animal genetics. There are a number of methods used to investigate variation, and the choice of method used is often based on the utility, simplicity and cost-effectiveness of the technology (Vignal *et al*. 2002). Methods used include Random Amplified Polymorphic DNA (RAPD) analysis, Amplified Fragment Length Polymorphism (AFLP) analysis, Restriction Fragment Length Polymorphism (RFLP) analysis, PCR-SSCP analysis, and SNP analysis. The last two methods will be discussed below.

Single Nucleotide Polymorphism (SNP) analysis

Single Nucleotide Polymorphisms (SNPs) are single nucleotide changes in a DNA sequence (Vignal *et al*. 2002). There have been a large number of SNPs identified in the human genome, some of which are used for disease diagnosis (Gupta *et al*. 2001). Single nucleotide polymorphisms also have a promising application in the agricultural industries, with the emerging use of genome-wide association studies (GWAS) to select and breed for production and disease-resistant traits in farm animals (Fan *et al*. 2010).

The rapid development of 'next generation' sequencing and the availability of reference genomes has allowed for the whole-genome sequencing of different species by modifying these technologies for that particular species (Fan *et al*. 2010). This technology has enabled the whole-genome sequencing of many farm animals including sheep. Average read lengths of 228 base pairs (bp) were assembled into sequences of 800 bp thus giving the virtual sheep genome (Dalrymple *et al*. 2007). From this, the International Sheep Genomics Consortium (ISGC) has assembled a sheep reference genome. The total length of the assembled genome, Sheep Genome v3.1, has reached 2.61 Gb (Jiang *et al*. 2014).

Mapping and sequencing of the sheep genome has allowed for ovine SNP chips to be designed (Oddy *et al*. 2007). Over 20,000 sheep world-wide have been genotyped with a 50K Ovine SNP chip (Ovine SNP50 BeadChip, Illumina), and in NZ, approximately 15,000 animals have been genotyped using this chip. Most of these are sires, while others include selection lines, sheep with extreme phenotypes and progeny tested animals (Pickering, 2013).

There are still some limitations in SNP arrays. For example, the coverage of the arrays for some species is low and uneven. Some genomic regions do not have many SNPs. Another limitation is that some SNPs are still unmapped in some of the commercially released SNP arrays. An annotation issue with SNP arrays is that most of the trait-associated SNPs are in genes that do not have an obvious biological significance on the studied phenotype. Lastly, there is a challenge in the statistical analysis of SNP array data. The SNP array produces a large volume of data, which requires sophisticated statistical models for analysis (Fan *et al*. 2010).

Polymerase Chain Reaction - Single-Strand Conformation Polymorphism (PCR-SSCP) analysis

Single-strand conformation polymorphism (SSCP) can be used to detect SNPs along with various other forms of nucleotide variation at any position in a fragment of DNA (Orita *et al*. 1989). Defined fragments of DNA can be produced using the polymerase chain reaction (PCR) and nucleotide variation in the DNA amplicon can result in a different conformation, or secondary structure of single-strands, after denaturation. This can change the electrophoretic mobility of the strands and this can be detected on a polyacrylamide gel (Orita *et al*. 1989; Gupta *et al*. 2001). Single strand conformation polymorphism gel analysis to detect nucleotide variation is better suited to shorter fragments of DNA, typically fragments of less than 430bp (Orita *et al*. 1989; Hayashi, 1999).

For the present study, PCR-SSCP was chosen as the preferred method of DNA analysis because it is a rapid, accurate, cost-effective and easily interpreted method for genotyping large numbers of sheep (Byun *et al*. 2008). This method also allows for the screening of cloned amplicons and the identification of the desired amplicon prior to sequencing. This allows for variants and different loci to be determined before sequencing. The technique is relatively inexpensive, very reliable and a simple technique for the determination of the number of amplicons required for sequencing, but it is typically limited to fragments of 100 to 400 bp in size (Zhou and Hickford, 2008). The method is a powerful

tool for identifying known variants of a specific gene region, as well as detecting new sequence variants (Byun *et al*. 2008).

Chapter 3. Ovine *FABP4* variation and its association with flystrike susceptibility

The concept of breeding sheep that are less susceptible to flystrike is an attractive proposition for reducing its impact on the NZ sheep industry. If genetic variation associated with increased or decreased susceptibility to flystrike is identified and characterised, then this could provide greater accuracy for making breeding selections to reduce the incidence of flystrike in flocks.

In 2010, a SNP study was undertaken by Smith *et al.* (2010) on sheep from the fleecerot and flystrike resistant and susceptible flocks at Trangie (Agricultural Research Centre, NSW, Australia) and sheep from the CSIRO AB78 mapping flock (CSIRO, Armidale, NSW, Australia). In total 581 Merinos from the Trangie flocks and 206 sheep from the Armidale flock 1997 lamb drop, were genotyped for 16 SNPs. This led to the identification of several candidate genes that may underpin fleecerot and flystrike resilience; the validation of which could lead to 'vital tests' for marker-assisted selection, and that would ultimately increase the natural fleecerot and flystrike resilience of sheep (Smith *et al*. 2010).

Among the genes identified was the FABP4 gene (*FABP4)*, which was differentially expressed in the phenotypic extremes, and several SNPs within the gene were associated with fleecerot score. Given that fleecerot has been identified as an important predisposing factor to flystrike (Norris *et al*. 2008) and that a study by (Raadsma, 1991) reported a very strong correlation (r = 0.9) between the traits, there is sound justification for testing whether genetic variation in *FABP4* is also associated with the occurrence of flystrike.

The fatty-acid binding proteins (FABPs) are hydrophobic ligand-binding cytoplasmic proteins, which are thought to be involved in lipid metabolism through the binding and intracellular transport of long-chain fatty-acids (Furuhashi and Hotamisligil, 2008; Tsuda *et al*. 2009). Studies also imply roles for FABP family proteins in cell-signalling (Furuhashi and Hotamisligil, 2008), the modulation of cell growth (Chmurzyńska, 2006), and cellular differentiation (Furuhashi and Hotamisligil, 2008).

Fatty-acid binding protein four (FABP4) has specifically been shown to be expressed in adipocytes and macrophages (Furuhashi and Hotamisligil, 2008). In relation to skin, FABP4 has been shown to be localised in the sebaceous glands (Watanabe *et al*. 1997), and FABP4 has been suggested to regulate sebaceous gland activity by modulating lipid-signalling and/or lipid metabolism in sebocytes (Lin & Khnykin, 2014).

Tsuda *et al.* (2009) showed that *FABP4* is strongly expressed in phosphatase and tensin (*Pten*) - null keratinocytes. In this context, FABP4 has been suggested to selectively enhance the activities of peroxisome proliferator-activated receptor gamma (PPARγ), which is a member of the nuclear

hormone receptor family, and that regulates genes involved in sebaceous tissue differentiation (Michalik and Wahli, 2007). Keratinocyte-specific *Pten* - null mice display distinct phenotypes, which includes having wrinkled skin; and ruffled, shaggy, and curly hair. Histological examination revealed that these mice have acanthosis, sebaceous gland hyperplasia, and accelerated hair follicle morphogenesis (Suzuki *et al*. 2003). *FABP4* is therefore implicated as having a role in the development of these phenotypes.

The loss of waxes and hydrophobicity is thought to be a major contributing factor in the development of fleecerot in sheep (Norris *et al*. 2008), therefore *FABP4*'s role in sebaceous gland activity may also have direct implications for fleecerot.

In humans, *FABP4* has been shown to have an impact on metabolic inflammatory diseases such as obesity, insulin resistance, type II diabetes and fatty-liver disease. Adipocytes and macrophages have an important role in these pathways (Furuhashi and Hotamisligil, 2008), suggesting that *FABP4* may have a specific role in the control of lipid signalling pathways and inflammatory responses. Accordingly, FABP4 could also play a role in the inflammatory response observed with fleecerot and flystrike.

Yan *et al*. (2012) studied ovine *FABP4* variation within two regions of the gene. In the first region, which spanned parts of exon 2 and intron 2, they found five unique sequences (named A_1-E_1), which came about as a consequence of three nucleotide substitutions and one deletion in intron 2. In the second region of the gene, which spanned parts of exon 3 and intron 3, four different sequences named A_2-D_2 were detected, and which came about as a consequence of four nucleotide substitutions. Yan *et al*. (2012) described 14 *FABP4* haplotypes based on the variation in these two regions.

In order to ascertain whether variation in *FABP4* is associated with variation in susceptibility to flystrike in NZ sheep, animals with and without flystrike were identified at shearing time from farms distributed throughout the Canterbury region. Genetic variation within ovine *FABP4* was analysed using PCR-SSCP and associations with the occurrence of fleecerot and flystrike explored statistically. Any associations identified could provide the basis of a genetic test that could be used in marker-assisted selection for decreased susceptibility to flystrike, and thus potentially reduce its prevalence.

Materials and Methods

Animals

A total of 890 sheep were blood sampled over a period five years (2013 – 2017). Of these sheep, 470 had flystrike or were recovering from flystrike. These sheep were from both commercial and stud farms throughout NZ (Appendix A.1) and they were of different breeds, including: Merino, Corriedale, Perendale, Romney, Lincoln, Coopworth, Poll Dorset, Texel, Finn, Dorset Down, Suffolk,

South Suffolk, Shropshire and various crossbreds and composites. The sheep were from farms where the farmers had used a variety of management practices to control flystrike.

DNA samples from another six breeds (n = 48) that were not selected based on flystrike occurrence, were also tested to ensure the methods from Yan *et al.* (2012) could be replicated and to develop a PCR-SSCP protocol for typing a third region of ovine *FABP4*. These samples, along with samples from the flystrike research flocks, were used to determine the breed-specific frequencies of the variants of *FABP4* in each of the three regions. In total 18 different breeds, including crossbreeds and composites were used.

Identification of flystrike

Sheep with flystrike, and those that had had flystrike, were identified either during shearing or in the yard. These sheep were diagnosed as having active flystrike when larvae could be seen on the skin, and their wool was discoloured and bad smelling. Sheep that had had flystrike were identified through having areas of pink skin with no wool, obvious scar tissue from maggot damage and flaky, dry skin, which had been damaged by larvae, and sheep that had recovered from flystrike, were identified through having shorter wool in the area struck and often having flaky skin in this area too. (Figure 3.1).

Figure 3.1 Damaged skin and lack of wool growth on sheep that has had flystrike and still has active flystrike in the shoulder area with discoloured wool (left photo) and healed breech flystrike (right photo).
(Photos taken by Lucy Burrows, 2017)

Blood collection

Blood samples were collected onto FTA cards (Whatman, Middlesex, UK) by nicking the lower part of the ear with side-cutters (Figure 3.2). This sampling method did not require animal ethics approval as ear nicking is a common farming practice. Samples were labelled with year, farm, breed, gender, age, the presence or absence of flystrike, and the region of the sheep's body that had been struck. The blood was left to dry and was stored in darkness at room temperature.

Figure 3.2 Sheep Blood Collection on FTA Card. Electrical side cutters are used to make a cut in the tip of the ear.
(Source: Instruction for Sheep Blood Collection on FTA Card).

PCR primers used for ovine FABP4 amplification

Blood samples were analysed at the Lincoln University Gene-Marker Laboratory. Three sets of PCR primers, two of which were designed by Yan *et al.* (2012), were used.

Yan *et al*. (2012) designed a set of primers to amplify exon 2 and part of intron 2 (Region 1, Figure 3.3) and another set to amplify exon 3 and part of intron 3 (Region 2, Figure 3.3). A new set of primers was also designed to amplify part of intron 2 and part of exon 3 (Region 3, Figure 3.3, Appendix A.2). All primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA).

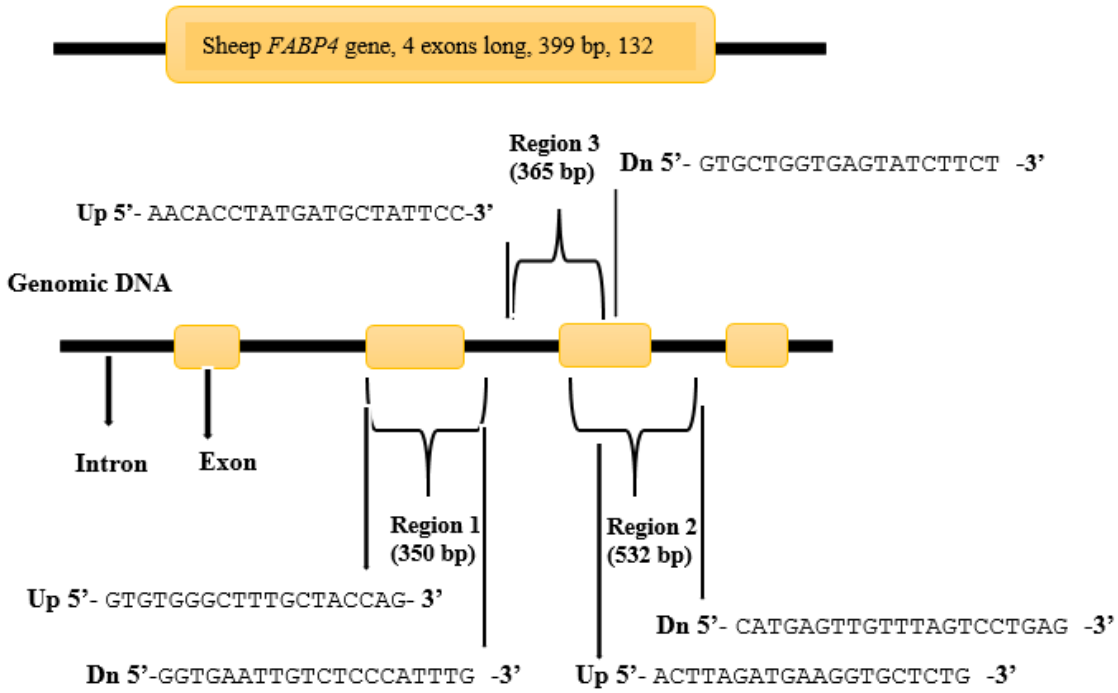


Figure 3.3 Location of *FABP4* PCR primers. Three sets of primers were designed in order to amplify a 350 bp region spanning exon 2-intron 2, 365 bp region spanning intron 2-exon 3, and 532 bp region spanning exon 3–intron 3. Primers design was based on the ovine *FABP4* sequence NW_001493222.2).

Developing the PCR-SSCP protocol for FABP4 Region 3

DNA samples (n = 20) from several breeds were used to develop a PCR-SSCP protocol for analysis of the third region of ovine *FABP4*. Different PCR protocols were tested, starting with an annealing temperature of 58 ºC, which was optimised to a temperature of 60 ºC. The amplicons were visualised by electrophoresis in 1% agarose (Quantum Scientific, Queensland, Australia) gels, using 1× TBE buffer (98 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA) containing 200 ng/mL of ethidium bromide. When the agarose gels produced a satisfactory result, several samples were then run on 14% acrylamide gels, and at various temperatures, to determine the best conditions for band separation and resolution.

PCR-SSCP analysis and genotyping of ovine FABP4

PCR amplification was performed in a 15 µL reaction containing the genomic DNA on one 1.2 mm punch of FTA card, 0.25 µM of each primer, 150 µM dNTPs (Bioline, London, UK), 2.5 mM of Mg^{2+}, 0.5 U of Taq DNA polymerase (Qiagen, Hilden, Germany) and 1× the reaction buffer supplied with the enzyme. The thermal profile consisted of an initial denaturation step of 2 min at 94 ºC, followed by 35 cycles of 30 s at 94 ºC, 30 s at 60 ºC and 30 s at 72 ºC, and with a final extension of 5 min at 72 ºC. Amplification was carried out in S1000 Thermal Cyclers (Bio-Rad, Hercules, CA, USA). This protocol was used for all three regions of *FABP4* investigated in this study.

Amplicons were visualised by electrophoresis in 1% agarose (Quantum Scientific, Queensland, Australia) gels, using 1 x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA) containing 200 ng/mL of ethidium bromide.

A 0.7 µL aliquot of each amplicon was mixed with 7 µL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol). After denaturation at 95 ºC for 5 min, samples were rapidly cooled on wet ice and then loaded on 16 cm × 18 cm, 14 % acrylamide:bisacrylamide (37.5:1) (Bio-Rad, California, USA) gels.

For Region 1, amplicons were loaded onto 16 cm x 18 cm, 10% acrylamide:bisacrylamide (37.5:1) (Bio-Rad) gels and electrophoresis was performed using Protean II xi cells (Bio-Rad), at 180 V for 19 h at 11 ºC in 0.5× TBE buffer.

For Region 2, amplicons were loaded onto 16 cm x 18 cm, 10% acrylamide:bisacrylamide (37.5:1) (Bio-Rad) gels and electrophoresis was performed using Protean II xi cells (Bio-Rad), at 300 V for 19 h at 7.5 ºC in 0.5× TBE buffer.

For Region 3, amplicons were loaded onto 16 cm x 18 cm 14% acrylamide:bisacrylamide (37.5:1) (Bio-Rad) gels and electrophoresis was performed using Protean II xi cells (Bio-Rad), at 250 V for 19 h at 7.5 ºC in 0.5× TBE buffer.

The gels were silver-stained using the method of Byun *et al*. (2009b).

Sequencing of the ovine FABP4 Region 3 variants

Yan *et al*. (2012) had sequenced Region 1 and 2 of the ovine *FABP4*, (JX 290313-JX 290317 and JX409931-JX409934), hence only Region 3 was sequenced in this study. Homozygous PCR amplicons were identified using PCR-SSCP. These amplicons were sequenced at the Lincoln University DNA Sequencing Facility.

The computer program DNAMAN (version 5.2.10, Lynnon BioSoft, Canada) was used for sequence alignment and comparisons. Sequence alignments, translations and comparisons were carried out using DNAMAN (version 5.2.10, Lynnon BioSoft, Canada). The BLAST algorithm was used to search the NCBI GenBank database (http://blast.nci.nlm.nih.gov/) for homologous sequences.

Statistical analyses

Variant frequencies were calculated for different breeds of sheep, along with the frequencies of sheep with flystrike and without flystrike, with different variants of ovine *FABP4*.

All analyses were performed using IBM SPSS Statistics version 24 (Chicago, IL, USA). For each *FABP4* region, the presence or absence of a variant in each sheep's genotype was coded with a 1 or 0, respectively. Variables considered in the analyses included: age, gender, breed, geographical region, and year. For some variables the categories were combined, and several variables were also merged (Appendix A.3). Age (lamb < 2, 2, 3, and 4 years) and gender (ewe, ram) were merged into a new variable called age_gender consisting of: Lamb, Ewe 2, Ewe 3+, Ram 2 and Ram 3+. The 20 breeds were combined into four categories; black-faced sheep, Merino-cross sheep, crossbred sheep, and purebred sheep. The six geographical locations were combined into four: Mid Canterbury, North Canterbury and South Canterbury. The combining of variables into groups was done to avoid having small cell sizes when performing cross tabulations (Appendix A.5).

To determine which of the variables would be included in subsequent multivariate binary logistic regression models, univariate Pearson chi-square tests were performed to explore the association between the variables and the presence or absence of each of the gene region variants (Appendix A.5).

For each gene variant, a Pearson chi-square test along with a binary logistic regression was performed to explore whether the presence or absence of the variant was associated with the presence or absence of flystrike, and to determine the odds-ratio, respectively (Appendix A.5). A multivariate binary logistic regression was then performed to determine the independent effects of the gene variants on the incidence of flystrike when year, breed, geographical location, age and gender were taken into account (Appendix A.6). If appropriate, an additional binary logistic regression model containing more than one of the gene variants was performed, and which included the variables from the previous models (year, breed, geographical location, and age_gender), along with any gene variant from the previous

univariate gene variant models where P < 0.200, indicating a potential impact on the presence or absence of flystrike.

For Region 1 a copy-number analysis was performed on *FABP4* variant A_1, as it was the only variant to produce a significant result, Appendix A.8. Copy number analysis was performed using cross-tabulations (with Chi-square analysis), and if significant, followed with a binary logistic regression containing year, breed, geographical location, and age_gender along with number of copies (0, 1 or 2)

The univariate and multivariate analysis was repeated for *FABP4* Region 2.

No Pearson chi-square analysis was performed for *FABP4* Region 3, as no trend in flystrike association was observed and there was a lack of variation in this region.

Results

Summary of flystrike data

Overall, flystrike had an occurrence of 3.0% in the flocks studied over the five years of investigation. The breech was the most common area struck (Table 3.1 and Figure 3.4). Prevalence was calculated by dividing the number of flystruck sheep by the total of all mob sizes from each year. Overall prevalence was the average of all the individual annual prevalences (Appendix A.3).

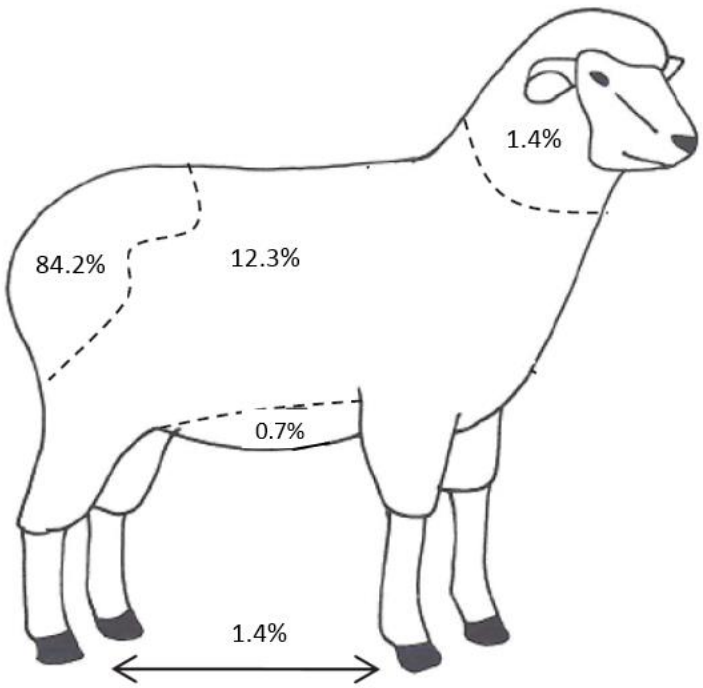


Figure 3.4 Percentage of strike in each area. Belly 0.72%, Breech 84.2%, Foot 1.44%, Poll: 1.44%, Shoulder 12.23%.

Table 3.1 Summary number of sheep struck and prevalence of strike for each year.

	2013	2014	2015	2016	2017	Grand Total
Not struck	138	106	56	81	39	420
Flystruck[a]	143	80	34	99	40	396
Total mob size[b]	4,405	3,245	1,276	2,680	1,385	12,991
Prevalence	**3.2%**	**2.5%**	**2.7%**	**3.7%**	**2.9%**	**3.0%**

[a]The number of sheep with flystrike did not include sheep from farms where the mob size was unknown.
[b]The total of all the mob sizes studied in that year.

Sequence variation in *FABP4*

The previously identified A_1, B_1, C_1 and D_1 variants of the exon 2-intron 2 region (Region 1; Yan *et al*. 2012) of *FABP4* were identified in the sheep studied (Figure 3.4, a, Table 3.2). The frequencies observed for these variants are shown in Table 3.3. Although there is an E_1 variant (Yan *et al*. 2012), it was not detected in the sheep studied here.

The previously identified A_2, B_2, and C_2 variants of the exon 3-intron 3 (Region 2; Yan *et al*. 2012) of *FABP4* were identified in the sheep studied (Figure 3.3). The D_2 variant (Yan *et al*. 2012) was not detected and very few sheep with the C_2 variant were identified. The frequency of the Region 2 variants was determined for 435 sheep from 18 different breeds (Table 3.2). In Region 2, the A_2 variant was found at the highest frequency of 64.3%, and the B_2 variant at a frequency of 33.0%. The C_2 variant was observed at a frequency of 2.7%.

Two variants A_3, and B_3, were found in Region 3 of *FABP4* in the sheep studied, with B_3 present at a frequency of 9.0% (Figure 3.5c). Frequencies were only determined for 223 sheep of 12 different breeds, and the A_3 variant was found at a much higher frequency than B_3, in all the breeds typed.

Table 3.2 Breed specific frequencies for each of the *FABP4* variants.

		Region 1 (exon 2-intron 2)				Region 2 (exon 3-intron 3)				Region 3 (intron 2-exon 3)		
Breed	***N***	***A*$_1$**	***B*$_1$**	***C*$_1$**	***D*$_1$**	***n***	***A*$_2$**	***B*$_2$**	***C*$_2$**	***n***	***A*$_3$**	***B*$_3$**
Merino	59	0.43	0.25	0.28	0.04	57	0.60	0.40	0.01	54	0.97	0.03
Corriedale	50	0.23	0.38	0.35	0.04	50	0.82	0.16	0.02	37	0.84	0.16
Coopworth	31	0.08	0.31	0.58	0.03	23	0.54	0.46	-	26	0.79	0.21
Romney	37	0.12	0.24	0.60	0.04	25	0.64	0.36	-	11	0.71	0.29
Perendale	54	0.11	0.42	0.38	0.09	26	0.56	0.44	-	9	0.89	0.11
Texel	4	0.25	0.25	0.50	-	3	0.50	0.50	-	5	0.70	0.30
Lincoln	7	0.14	0.21	0.57	0.07	6	0.92	0.08	-	8	0.94	0.06
Poll Dorset	15	0.23	0.40	0.37	-	11	0.82	0.18	-	7	1.00	-
Dorset Down	30	0.27	0.28	0.33	0.12	22	0.34	0.55	0.11	18	0.75	0.25
Shropshire	25	0.02	0.44	0.46	0.08	23	0.57	0.41	0.02	8	1.00	-
Suffolk	31	0.24	0.26	0.37	0.13	37	0.58	0.31	0.11	-	-	-
South Suffolk	8	0.25	0.19	0.44	0.13	6	0.67	0.17	0.17	-	-	-
Merino X	9	0.28	0.44	0.28	-	5	0.90	0.10	-	-	-	-
Corriedale X	52	0.22	0.37	0.36	0.06	26	0.69	0.31	-	-	-	-
White face X	55	0.16	0.34	0.43	0.07	50	0.68	0.31	0.00	-	-	-
Black face X	49	0.17	0.37	0.39	0.07	33	0.64	0.35	0.02	-	-	-
Composite	21	0.19	0.38	0.33	0.10	18	0.47	0.53	-	20	0.95	0.05
Dorper	23	0.04	0.41	0.54	-	14	0.29	0.42	0.29	20	0.93	0.08
Total	560	0.19	0.33	0.42	0.06	435	0.62	0.34	0.04	223	0.91	0.09

Not all samples collected were used to determine frequency of FABP4 in the three different regions, there were no dorpers in the flystrike research sheep but were tested for frequency variation.

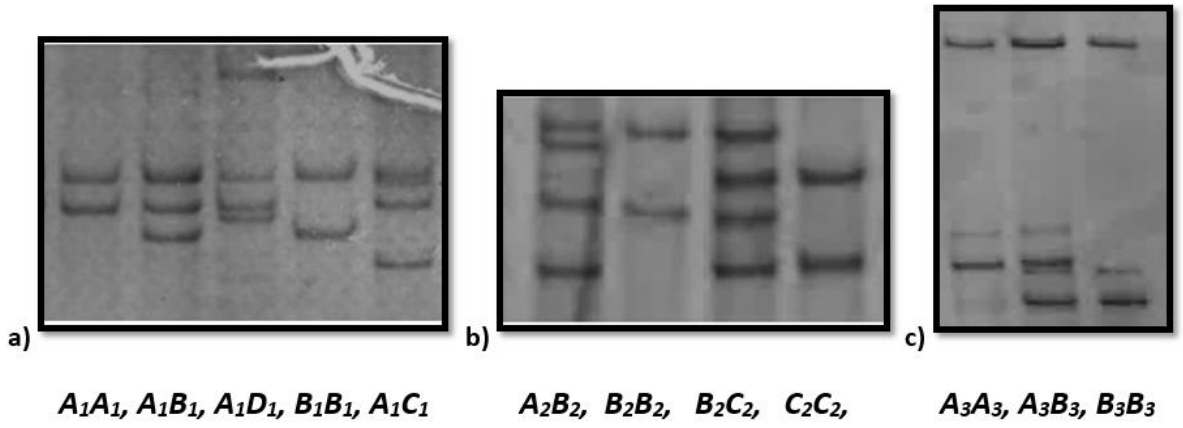


Figure 3.5 SSCP-PCR patterns of the variants of *FABP4* in the three regions investigated.

Frequencies of FABP4 variants

The frequencies of the *FABP4* variants for sheep with flystrike within each region investigated are shown in Table 3.3.

Table 3.3 Frequencies of *FABP4* variants for sheep with flystrike, within each region.

FABP4	*n*	Total frequency (%)	Number with flystrike	Frequency of flystrike in those animals containing the variant (%)
Region 1				
A_1	344	20.4	154	45
B_1	587	34.8	314	53
C_1	656	38.8	361	55
D_1	101	6.0	53	52
Region 2				
A_2	795	66.8	393	49
B_2	373	31.2	194	52
C_2	24	2.0	11	46
Region 3				
A_3	343	86.6	184	54
B_3	52	13.4	28	54

Variation in FABP4 Region 1 and flystrike

The results of the univariate analyses exploring the effect of the different independent effects on associations between the presence or absence of each Region 1 variant and flystrike presence and absence are shown in Table 3.4 and Appendix A.5. Associations existed for each variable with at least one of the variants, and hence all the variables were retained in the subsequent multivariate analyses.

Table 3.4 Pearson chi-square analyses exploring associations[1] between the presence or absence of each *FABP4* Region 1 variant and flystrike.

Variable	*FABP4* variants				P- Value
	A_1	B_1	C_1	D_1	
Age_Gender	**0.001**	0.782	0.232	0.215	**< 0.001**
Breed	**< 0.001**	0.535	**0.004**	0.332	**0.050**
Region	0.584	0.406	**0.002**	0.635	0.545
Year	**0.001**	**< 0.001**	**< 0.001**	0.868	0.132

[1]Significant associations (P ≤ 0.05) are in bold.

The *FABP4* Region 1 variants tended to be associated with the presence/absence of flystrike (P = 0.089). The absence of flystrike was associated with the presence of *FABP4* A_1 (Table 3.5). The presence of C_1 tended (P < 0.100) to be associated with the presence of flystrike (Appendix A.6). The association of the presence of *FABP4* A_1 with the absence of flystrike persisted in the multivariate analyses, thus the effect was independent of any of the other variables included in the model (Table 3.6; Appendix A.7).

Table 3.5 The univariate association of the presence of each *FABP4* Region 1 variant with the occurrence of flystrike.

Gene variant	Odds ratio1	95% Confidence Interval[1]		P value[2]
		Upper	Lower	
A_1	0.651	0.492	0.862	**0.003**
B_1	1.094	0.833	1.437	0.519
C_1	1.267	0.959	1.673	0.095
D_1	0.948	0.617	1.456	0.807

[1]Derived from a binary logistic regression with presence or absence of flystrike as the dependent variable, and presence or absence of the gene variant as the independent variable.
[2]Derived from both the Pearson chi-square and the binary logistic regression. Significant associations are in bold.

Table 3.6 The odds[1] of flystrike occurrence given the presence of a particular *FABP4* Region 1 variant

Gene variant	Odds ratio	95% Confidence Interval		P value
		Upper	Lower	
A_1	0.689	0.512	0.927	**0.014**
B_1	1.110	0.832	1.480	0.478
C_1	1.209	0.899	1.625	0.210
D_1	1.001	0.638	1.570	0.996

[1] The odds ratios and confidence intervals were derived from a multivariate binary logistic regression in which the presence or absence of flystrike was the dependent variable and the independent variable were presence or absence of the gene variant, year, breed, geographical region, and the combined variable of age_gender.

The copy number analysis (correcting for year, breed, geographical location, and age_gender) revealed there was a 30.5% reduction in flystrike for each additional copy of *FABP4* A_1 (P = 0.006, Odds Ratio = 0.695; 95% CI 0.537-0.900; Appendix A.7).

FABP4 Region 2

Univariate analyses of *FABP4* Region 2 variants did not reveal any associations with flystrike (Table 3.7; Appendix A.9). This result was confirmed in the binary logistic regression analysis (Table 3.8; Appendix A.10).

Table 3.7 The univariate association of the presence of each FABP4 Region 2 variant with the occurrence of flystrike.

Gene variant	Odds ratio[1]	95 % Confidence Interval		P value[2]
		Upper	Lower	
A_2	1.080	0.693	1.683	0.734
B_2	1.247	0.900	1.727	0.184
C_2	0.897	0.359	2.240	0.816

[1]Derived from a binary logistic regression with presence or absence of flystrike as the dependent variable and presence or absence of the gene variant as the independent variable
[2]Derive from both the Pearson chi-square and the binary logistic regression. Significant associations are in bold

Table 3.8 The odds[1] of flystrike occurrence given the presence of a particular *FABP4* Region 2 variant.

Gene variant	Odds ratio	95% Confidence Interval		P value
		Upper	Lower	
A_2	1.100	0.687	1.762	0.691
B_2	1.161	0.814	1.655	0.409
C_2	1.075	0.402	2.872	0.886

[1]The odds ratios and confidence intervals were derived from a multivariate binary logistic regression in which the presence or absence of flystrike was the dependent variable and the independent variable were presence or absence of the gene variant, year, breed, geographical region, and the combined variable of age_gender

FABP4 Region 3

Two variants were found with PCR-SSCP for Region 3 of *FABP4*. Sequencing revealed that the two unique PCR-SSCP patterns were a result of one SNP (g/a) in intron 2 at position c.246+388. Pearson chi-square analysis was performed for variants in Region 3 and flystrike and the results are shown in Table 3.9. Due to lack of variation and association in this region, no further testing was undertaken.

Table 3.9 Pearson chi-square analyses exploring variable associations with the presence or absence of each *FABP4* Region 3 variant along with flystrike.

FABP4 Region 3	With Flystrike	Without flystrike	Pearson chi-square	P-Value
$\boldsymbol{A_3}$	343	184	0.211	0.646
$\boldsymbol{B_3}$	52	28	0.063	0.801

Discussion

This study found the overall prevalence of flystrike over the five-year collection period to be 3.0 %. Historically flystrike had an estimated overall prevalence of 3-5 % in the NZ sheep flock (Heath and Bishop, 1995), with a range of prevalence from 0.5 % to 7.3 % (Brandsma and Blair, 1997). More recent work has reported a range of prevalence from 3.4 % to 24 % (Scobie and O'Connell, 2010), with an average prevalence across NZ of 2.15 % from 2009 to 2011 (Pickering, 2013).

A recent study found the prevalence of flystrike to be 2.15 %, with 88 % of strike occurring in the breech region (Pickering 2013). In this study the majority (81.2 %) of flystrike occurred in the breech area, followed by the body (12.3 %). Ewes and lambs were found to be the most susceptible to flystrike. Roberts, (1963) suggested that the reason why younger sheep have a higher incidence of fleecerot and flystrike, is because of the greater penetrability for water of their more open fleeces.

Older sheep are more resistant to *Dermatophilus congolensis* infection and fleecerot because they can better maintain the integrity of the wool wax layer that gives a waterproofing effect to the wool (Roberts, 1963). Ewes are often more susceptible to flystrike than rams, especially in the breech region as flies are attracted to urine stains in this area (Tellam and Bowles, 1997).

Smith *et al*. (2010) identified five SNPs in ovine *FABP4* that are associated with fleecerot. Three of these SNPs were also identified by Yan *et al*. (2012, 2018). This study identified one more of the remaining two SNPs found by Smith *et al*. (2010), along with one novel ovine *FABP4* SNP that had not been reported previously (Table 3.10).

Table 3.10 Comparison of SNPs in ovine *FABP4* and traits they are associated with from Yan *et al.* (2012) [a], Smith *et al.* (2010) [b], Yan *et al.* (2018, unpublished manuscript) [c] and the current study[d].

Smith *et al.* 2010[b]	Yan *et al.* 2012[a c]	SNP	Current study[d]	Association
-	c.246 + 33	g/a	Found B_1	
-	c.246 + 37	c	Found A_1	Fat sheep*, decreased leg LMY[#] & total LMY decrease flystrike & increased WY in lambs[d]
-	c.246 + 46	t/c	Found D_1	
-	c.246 + 47	g/a	Not Found E_1	
-	-		Found C_1	Lean sheep[a]
FABIn20115	-	c/a	Not Found	Fleecerot[b]
FABIn20237	-	g/a	A_3 and B_3	Fleecerot[b]
-	c.317	A/G	Not Found D_2	
FABIn30227	c.348 + 166	t/c	Found C_2	Fleecerot[b]
FABIn30360	c.348 + 298	t/c	Found B_2	Fleecerot[b]
FABIn30420	c.348 + 356	t/c	Found A_2	Fleecerot[b], decrease in WW & pre-WGR[c]

#LMY is lean meat yield, WY is wool yield, WW is weaning weight and WGR is weaned growth rate.

In total, Yan *et al.* (2012) detected five variant sequences of *FABP4* (named A_1 - E_1) spanning an exon 2 – intron 2 fragment (Region 1) and four variant sequences (A_2 - D_2) in a fragment spanning exon 3 – intron 3 (Region 2). In this study four of the *FABP4* Region 1 variants were detected (A_1 - D_1), with E_1 not being found. The E_1 variant described by Yan *et al*. (2012) is rare and was found at a frequency of 1% in the sheep they studied, so it is perhaps not surprising that it was not found in this study. The C_1 variant was not found in all the breeds investigated and overall was at a much lower frequency, than the 15.1% frequency that it was found for the sheep studied by Yan *et al*. (2012). Yan *et al*. (2012) also reported that some of the variants they described were not observed in some of the breeds, and the frequencies of the variants differed in different breeds. For example, the D_1 variant was not found in Poll Dorset sheep in either Yan *et al*. (2012) and the present study. D_1 was also not found in Texel or Shropshire sheep in the present study, but these breeds were not investigated by Yan *et al*. (2012). In both studies Merino sheep had a higher frequency of A_1, than any other variant, this suggesting that FABP4 may play a role regulating wax secretion in fine wool breeds. Merino sheep also have a higher follicle number per unit area of skin, giving them a higher wax content in their fleece (Collins and Davidson, 1997; Daly and Carter, 1956).

The SNPs described in *FABP4* in this study were all found in introns, making it difficult to analyse their influence in FABP4 function. Although introns do not code for amino acids, some SNPs in

introns, silent substitutions in coding sequences and variation in regions flanking coding sequences can have a functional role, and affects gene expression. For example, some intronic SNPs affect mRNA splicing efficiency, message stability, RNA processing and translation (Le Hir *et al*. 2003), and silent substitutions in coding regions have been shown to affect mRNA stability and pre-mRNA splicing (Duan *et al*. 2003; Supek *et al*. 2014). The SNPs identified did not fall into any known RNA splicing sites. It is possible that the intronic variation is linked to sequence variation in other regions of *FABP4* that is of structural or functional importance, as has been suggested by Yan *et al*. (2012; 2018) and Smith *et al*. (2010).

Variation in FABP4 and its association with flystrike susceptibility

The aim of this study was to determine if there was an association between the presence of flystrike and variation in ovine *FABP4*. The results suggested that sheep with the A_1 variant were less likely to have flystrike than those without A_1. However, it is still impossible to say if any given sheep carrying the A_1 variant will, or will not get flystrike, as some sheep in this study carrying A_1 also had flystrike and some sheep carrying two copies of A_1 were also struck, with them accounting for 2.5% of the struck genotypes.

The first associations between SNPs in or near *FABP4* and fleecerot were detected in fine wool Merinos, and therefore, they could be peculiar to fine wool sheep (Smith *et al*. 2010). There is a high correlation (r = 0.9) between fleecerot and flystrike on the body of sheep making, it possible to breed sheep that are resistant to fleecerot and, in turn, less susceptible to flystrike (i.e. body-strike, Raadsma, 1991). Smith *et al.* (2010) was the first to report gene expression (including *FABP4*) changes in the skin of sheep before, during and after the induction of a fleecerot challenge. They identified five SNPs in *FABP4* that had a significant association with fleecerot resistance. Four of those SNPs were also identified in this study, three in Region 2 and one in Region 3, but none of these variants had a significant association with flystrike and fleecerot resistance was not investigated in this study.

The susceptibility of wool to fleecerot and flystrike can also be assessed through the measurement of indicator traits, such as some physical characteristics of the wool, wool colour and chemical characteristic of the wool, including wax and suint levels that affect the wettability of the wool (Belschner, 1937; Norris *et al*. 2008). Bright, high yielding greasy wool that is white in colour, is the most consistent wool type associated with fleecerot resistance and it is highly heritable (Hayman, 1953; James *et al*. 1984; Raadsma, 1987). Fibre diameter is another highly heritable trait that has been associated with resistance to fleecerot (Raadsma, 1993), with a lower fibre diameter resulting in a greater resistance in Merino sheep (James and Ponzoni, 1992). Wax levels in the wool are possibly influenced by *FABP4* variation, and this could be how FABP4 contributes to fleecerot and flystrike resilience. Exploring the relationship between FABP4 and wool wax levels, along with other wool traits could shed light on FABP4 role in flystrike resilience. However, the phenotypic correlations between fleecerot, flystrike and other fleece characteristics are typically low (Raadsma, 1987).

Fatty acid binding protein 4 has a role in adipose tissue metabolism. It could, therefore, be assumed that differences in flystrike susceptibility and resilience in sheep carrying different variants of *FABP4* could also be associated with the differences in fat deposition, this also being reflected in breed differences.

Bakhtiarizadeh *et al*. (2013) used Lori-Bakhtiari and Zel sheep to investigate the differences in *FABP4* expression in fat-tail tissue and visceral adipose tissue. Their results suggested that the expression of *FABP4* was higher in the fat-tail of Lori-Bakhtiari sheep, than expression in either the fat-tail or visceral tissue of the Zel sheep. The higher level of expression of *FABP4* in the fat-tail tissue of Lori-Bakhtiari sheep was related to there being more fatty-acid transportation into the fat-tail compared with the Zel sheep. If fat is a factor that affects flystrike resilience in sheep, then increased expression of *FABP4*, leading to more fatty-acid transport into different areas of the body could be an underlying mechanism for the variation in resilience. It would be interesting to look at the frequency of *FABP4* variants A_1-E_1 in Lori-Bakhtiari and Zel sheep to see if they have a higher frequency of the A_1 variant, especially as this variant has also been found to occur at a high frequency in a line of sheep that were deliberately bred for increased fatness (Yan *et al*. 2012). The Lori-Bakhtiari and Zel sheep could also be investigated to ascertain their resilience to flystrike in the context of *FABP4* variation.

Yan *et al*. (2012) found a vast difference between fat and lean lines of sheep and variation in *FABP4*. Sheep carrying the *FABP4* A_1 variant was associated with the fat line, while sheep carrying the C_1 variant was associated with the lean line. In the present study, sheep with the A_1 variant were the least likely to get flystrike, and yet Yan *et al*. (2012), were the sheep predominant found in the fat line. This could be due to the higher carcass fat content of these sheep, which means these sheep may have higher lipid content in their wool. Wool wax contains fatty acids, which coats the wool and skin and inhibits bacterial growth by lowering the pH of the skin surface (Lambers *et al*. 2006). This would need further investigation to be confirmed.

If the *FABP4* A_1 variant decreases flystrike susceptibility and increases the fat content in meat, then farmers would have to decide if they want to selectively breed sheep for both these traits. This could conflict with consumer demands, especially as an issue affecting the consumption of red meat is the need for improvement of lean meat yield, eating quality and human nutritional value. Current consumer demand is for leaner meat due to the common perception that fat is linked to obesity and cardiovascular disease (Pethick *et al*. 2011; Volk, 2007) and this demand is most efficiently achieved by producing leaner slaughter animals on farm (Pethick *et al*. 2011).

Merino meat has been shown to be leaner and have similar eye muscle area to meat breed crosses, when compared at the same carcass weight and grown under good conditions (Fogarty *et al*. 2000; 2003). Traditionally Merino breeding programs have placed a higher emphasis on reduced wool fibre diameter, with less emphasis placed on carcass traits (Fogarty *et al*. 2003). However, with the increasing prices of sheep meat, relative to wool in recent years, farmers are needing to place more

emphasis on carcass traits (Greeff *et al*. 2008). Future work on *FABP4* should investigate any interaction between carcass traits, wool traits and flystrike susceptibility. Ideally the whole gene would be investigated in association with the traits that it appears to be influencing.

Fatty acid-binding protein 4 has been shown to modulate inflammatory responses in macrophages (Furuhashi and Hotmaisligil, 2008). In macrophages lacking FABP4 (*FABP4 -/-)* there are several signalling pathways suppressed, including the production of cytokines such as tumour-necrosis factor –α (TNF-α), interleukin 1β (ILβ) and IL6 (Makowski *et al*. 2005; Furuhashi and Hotmaisligil, 2008). It is of interest that inflammatory cytokines and T cell – dependant cytokine IL-2 and IFN –γ are produced in the skin during flystrike (Bowles *et al*. 1994). FABP4 also acts to coordinate functional interactions between macrophages and adipocytes in the adipose tissue (Furuhashi and Hotmaisligil, 2008). The adipocytes sit near the skin surface, thus these cells could play a role in the inflammatory response to flystrike in the skin. The 'fatter' type sheep, as shown by Yan *et al*. (2012) to have a high frequency of A_1 variant, may have a greater resilience to flystrike by having a great inflammatory response to fleecerot and flystrike, than leaner sheep.

The results of this study suggest that variation in *FABP4* may be used as a gene-marker for flystrike resilience in sheep. Breeding from sheep that carry the *FABP4* A_1 variant, may decrease the prevalence of flystrike in the flock. However, *FABP4* has been shown to be a highly pleiotropic gene and, therefore, its association with other production traits such as wool and meat quality would need further investigation, as selection for one desirable trait may in turn compromise another.

Chapter 4. Ovine *FBLN1* variation and its association with flystrike susceptibility

Rainfall and wetting of the fleece and skin of sheep can increase vascular permeability, cause dermatitis and lead to leucocytic invasion of the epidermis, resulting in the development of fleecerot (Chapman *et al*. 1984). Fleecerot causes further inflammation and ulceration, which is attractive to gravid blowflies, as it provides moisture for the eggs to hatch, and bacteria and protein for the freshly hatched larvae to feed on (Burrell *et al*. 1982; Norris *et al*. 2008). Neutrophils and eosinophils accumulate in the skin lesions on flystruck sheep (Bowles *et al*. 1992) and there is extensive production of inflammatory cytokines (Colditz *et al*. 2005; Elhay *et al*. 1994). The skin, therefore, is likely to play a key role in the host response to fleecerot and flystrike.

Four SNPs in the fibulin-1 (FBLN1) gene (*FBLN1*) were found in associated with fleecerot and flystrike resistance in the Trangie selection lines. These SNPs occurred in exon 10 FBLIn100090 and in intron 12 FBLIn120135, FBLIn120280, FBLIn120995 (Smith *et al*. 2010). Fubulin-1 is a 90 kDa calcium-binding protein and is found in the extracellular matrix (ECM) and in blood plasma (Argraves *et al.* 1989). *Fibulin-1* appears to contribute to the physical barrier of the skin, and thus potentially acts at the interface of host and bacterial interactions (Argraves *et al*. 2003). FBLN1 also functions to stabilise the ECM and has various roles in organ development and tissue differentiation (De Vega *et al*. 2009). *Fibulin-1* knockout mice have been used to look at various biological roles of FBLN1. These mice display massive haemorrhaging in their skin, muscle and peri-neural tissue. This begins in mid-gestation and results in the death of most homozygous individuals at birth (Kostka *et al*. 2001). The haemorrhaging is caused by having dilated and irregular-shaped capillaries, although the large blood vessels appear to be unaffected. *FBLN1*-null mice also have abnormalities in their lung tissue, with the presence of thick immature alveolar septal walls, and inadequate expanded sacculi. This suggests *FBLN1* has a role in ECM re-modelling in the lung interstitial tissue (Bouchey *et al*. 1996; Chu and Tsuda, 2004).

In the context of infectious disease, *FBLN1* has been found to be expressed at higher levels in the bed of non-healing ulcers in humans, when compared to the bed of healing ulcers (Charles *et al*. 2008). It is therefore possible that *FBLN1* may be expressed at various levels in sheep that have ulcers perhaps caused by fleecerot, or in the lesions caused by flystrike. *Fibulin-1* is, therefore, a candidate gene for further study into fleecerot and flystrike resilience in sheep.

Materials and methods

Animals

A total of 890 sheep were sampled from over five strike seasons 2013 to 2017. These sheep were from both commercial and stud farms and were of various breeds (as described in Chapter 3). As in Chapter 3, DNA samples (n = 20) from eight breeds (not from the flystrike research sheep) were used to develop a PCR-SSCP protocol for each of the three regions of ovine *FBLN1* to be investigated. These samples, along with those from the flystrike research sheep, were used to determine the frequencies of *FBLN1* variants in different breeds.

Blood collection

Blood samples were collected using the methods described in Chapters 3. This sampling method did not require animal ethics approval as ear nicking is considered to be an accepted farming practice. The blood samples were analysed at the Lincoln University Gene-Marker laboratory. Genomic DNA was purified using a two-step washing procedure as described by Zhou *et al*. (2006).

Primer design for ovine FBLN1

Three sets of primers were designed to amplify three regions of *FBLN1*, based on the ovine reference sequence, XM0150950641. Region 1 was located spanning exon 2, which makes up the anaphylatoxin-like motifs. Region 2 was located at exon 10 and Region 3 was located at intron 12. Region 2 and 3 corresponded to the regions Smith *et al*. (2010) found SNPs associated with fleece-rot resistance (Table 4.1, Figure 4.1). The primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA).

Table 4.1 Primers used to amplify three regions of the ovine FBLN1 gene (*FBLN1*).

FBLN1 Region[1]	Bp	Up Primer	Down Primer
1	320	5′-CCTGGGATGCTGTGAGGTG-3′	5′-GGAGAGGAAGGGAGAGTGC-3′
2	315	5′-CTTCCTTGCCTTCCGTTCTG-3′	5′-CCTCTGACAATCCTGAGT-3′
3	378	5′-CTACCAGCTCAGCGACGTG-3′	5′-CATCTGGGTTCATCTTGCAC-3′

[1]**See Figure 4.1**

Developing the PCR-SSCP protocols

Different PCR protocols were tested, starting with an annealing temperature of 58 ºC, which was after optimisation set at 59 ºC and 60 ºC, see below. Amplicons were visualised by electrophoresis in 1% agarose (Quantum Scientific, Queensland, Australia) gels, using 1 x TBE buffer (98 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA) and containing 200 ng/ml of ethidium bromide. When the agarose gels produced a well resolved band, then several samples were run on 14% acrylamide gels at various temperature to determine the conditions that would best allow for separation of the banding patterns, and in order to determine all variants.

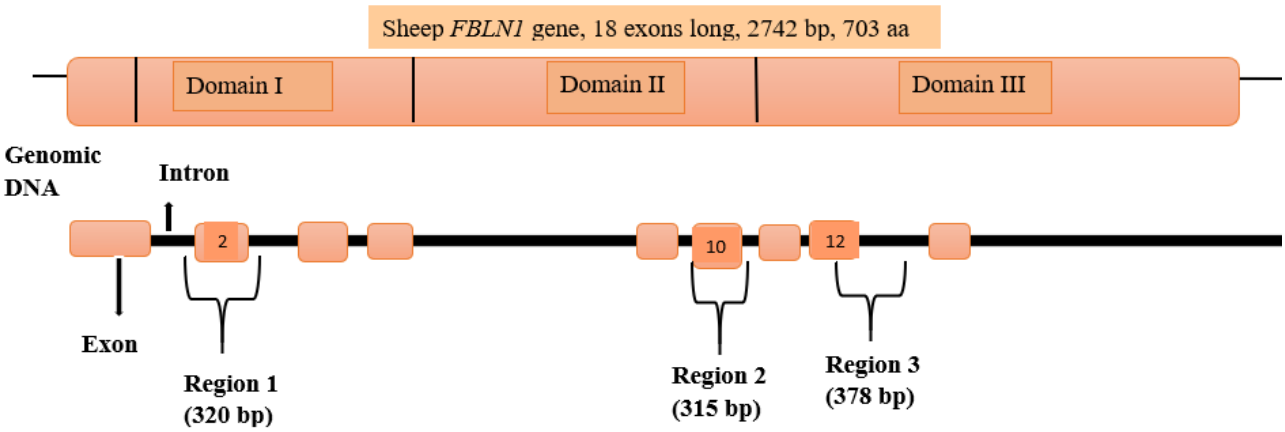


Figure 4.1 Location of PCR primers designed to amplify three regions of the ovine fibulin-1 gene (*FBLN1*). Region 1, 320 bp region of intron 2 – exon 2; Region 2, 3315 bp region of intron 10 – exon 10; Region 3, 378 bp region of exon 12 –intron 13. Gene structure based on the ovine *FBLN1* sequence, not drawn to scale, ovine *FBLN1* is 18 exons long. (GenBank access numbers MG704148 – MG704151, MG704152-MG704155).

PCR amplification and SSCP analysis

PCR amplification was performed in a 15 µL reaction containing the genomic DNA on one 1.2 mm punch of FTA card, 0.25 µM of each primer, 150 µM dNTPs (Bioline, London, UK), 2.5 mM of Mg^{2+}, 0.5 U of Taq DNA polymerase (Qiagen, Hilden, Germany) and 1× the reaction buffer supplied with the enzyme. The reaction was the same for all three regions investigated in this study. The thermal profile for each primer set had an initial denaturation for 2 min at 94 ºC, followed by a specified number of cycles as described in Table 4.2, with a final extension of 5 min at 72 ºC. Amplification was carried out in S1000 thermal cyclers (Bio-Rad, Hercules, CA, USA).

Table 4.2 PCR cycling parameters for each of the *FBLN1* regions being amplified.

FBLN1 Region[1]	Number of cycles	Cycle
1	37	30 s at 94 ºC, 30 s at 60 ºC and 40 s at 72 ºC
2	37	30 s at 94 ºC, 30 s at 60 ºC and 40 s at 72 ºC
3	35	30 s at 94 ºC, 30 s at 60 ºC and 40 s at 72 ºC

[1]See Table 4.1

A 0.7 µL aliquot of each amplicon was mixed with 7 µL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol). After denaturation at 95 ºC for 5 min, the samples were rapidly cooled on wet ice and then loaded on 16 cm × 18 cm, 14% acrylamide:bisacrylamide (37.5:1) (Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad) in 0.5× TBE buffer at 220 V for 19 h at 33 ºC for primer set 1, 250 V for 19 h at 33 ºC for primer set 2, and 200 V for 19 hours at 25 ºC for primer set 3. The gels were silver-stained according to the method of Byun *et al*. (2009a).

Sequencing of the ovine FBLN1 variants and sequence analysis

Homozygous amplicons were identified for the three regions of *FBLN1* by PCR-SSCP analysis. These amplicons were then sequenced at the Lincoln University DNA Sequencing Facility. The computer program DNAMAN (version 5.2.10, Lynnon BioSoft, Canada) was used for sequence alignment, translations and comparisons. The BLAST algorithm was used to search the NCBI GenBank database (http://blast.nci.nlm.nih.gov/) for homologous sequences.

Statistical analysis

Variant frequencies were calculated for the different breeds of sheep, along with the frequencies of sheep with flystrike and without flystrike with variants from the three regions of ovine *FBLN1* investigated.

All analyses were performed using SPSS version 24 (Chicago, IL, USA), as described in Chapter 3, Appendix A.3. To determine which of the variables would be included in subsequent multivariate binary logistic regression models, univariate Pearson chi-square tests were performed to explore the association between the variables, and the presence or absence of each of the gene region variants.

For each *FBLN1* variant, a Pearson chi-square test along with a binary logistic regression was performed to explore whether the presence or absence of the variant was associated with the presence or absence of flystrike, and to determine the odds ratio, respectively. A multivariate binary logistic regression was then performed to determine the independent effects of gene variant on incidence of flystrike when year, breed, geographical location, age and gender were considered. If appropriate, an additional binary logistic regression model containing more than one of the gene variants was performed which included the variables from the previous models; year, breed, geographical location, and age_gender (age; lamb < 2, 2, 3, and 4 years and gender: ewe or ram, were merged into a new variable called age_gender consisting of: Lamb, Ewe 2, Ewe 3+, Ram 2 and Ram 3+), along with any gene variant from the previous univariate gene variant models with $P < 0.200$ (indicating a potential impact on the presence or absence of flystrike).

Copy number analysis was performed for *FBLN1* B_1 and C_1, using cross-tabulations (with Chi-square analysis), and if significant, followed with a binary logistic regression containing year, breed, geographical location, and age_gender along with number of copies (0, 1 or 2)

Results

PCR-SSCP typing of FBLN1

Four unique PCR-SSCP patterns representing four unique sequences (*A, B, C*, and *D*) were detected in each of the three regions (designated by the subscript, Figure 4.2).

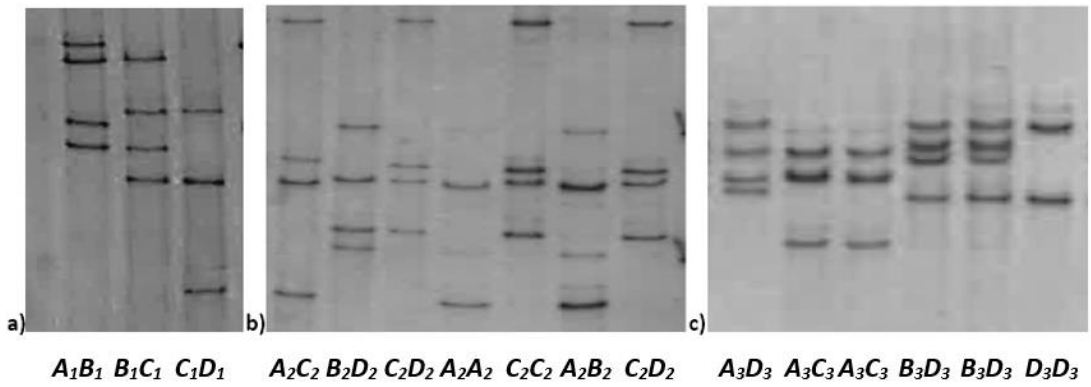


Figure 4.2 PCR-SSCP banding patterns for the three regions of ovine fibulin-1 gene (FBLN1) investigated. a) Region 1, intron 2 –exon 2 , b) Region 2, intron 10 –exon 10, and c) Region3, exon 12 –intron 12**.**

Four sequences (A_1 - D_1) were found in Region 1, corresponding to exon 2. In this region of *FBLN1* the A_1 and C_1 variant were found at the highest frequency of 30.3% and 49.6% respectively (Table 4.2). The B_1 variant was found at a much lower frequency 17.6%, and not at all in Poll Dorset sheep. The D_1 variant was not found in Lincoln, Dorset Downs, Shropshire and South Suffolks, and it had the lowest overall frequency of 2.5%.

In Region 2, A_2 was found at the highest frequency of 59.4% and in all the breeds investigated. The C_2 variant was found at the lowest frequency of 6.6% and was not found in Perendale, Romney, South Suffolk, Poll Dorset, and composite breeds. In Region 3, A_3 and C_3 were found at a similar frequency of 39.2% and 38.9% respectively, and these two variants were found in all breeds investigated. Variants B_3 and D_3 were found at a lower overall frequency of 14.3% and 7.6% respectively, and were not found in Lincoln or Poll Dorset sheep. Variant D_3 was not found in composite sheep (Table 4.3).

Table 4.3 Breed specific frequency (expressed as a percentage) for each of the regions of ovine fibulin-1 gene (*FBLN1*).

	***FBLN1* variant**														
	Region 1 (Exon 2)					**Region 2 (Exon 10)**					**Region 3 (Exon 12 – Intron 12)**				
Breed	n	A_1	B_1	C_1	D_1	*n*	A_2	B_2	C_2	D_2	*n*	A_3	B_3	C_3	D_3
Merino	55	17.7	8.1	73.3	0.9	69	64.5	11.6	8.0	15.9	57	20.2	26.3	40.4	13.1
Corriedale	51	44.5	8.5	46.8	0.2	62	88.0	7.0	2.3	2.7	39	23.1	5.1	70.5	1.3
Coopworth	45	24.5	30.0	42.5	3.0	30	58.3	15.0	10.0	16.7	17	37.5	15.6	37.5	9.4
Romney	50	35.1	32.8	26.1	6.0	50	49.0	50.8	-	0.2	43	57.0	3.5	33.7	5.8
Perendale	48	45.8	18.8	27.1	8.3	24	64.5	29.2	-	6.3	19	42.1	7.9	47.4	2.6
Texel	30	20.0	23.3	55.8	0.9	12	58.3	20.8	8.4	12.5	12	50.0	25.0	20.8	4.2
Lincoln	7	28.6	35.7	35.7	-	7	28.6	71.4	-	-	7	64.3	-	35.7	-
Poll Dorset	5	30.0	-	60.0	1.0	9	44.4	55.6	-	-	9	72.2	-	27.8	-
Dorset Down	29	13.8	19.0	67.2	-	23	21.7	50.5	17.4	10.4	19	5.3	15.7	73.7	5.3
Shropshire	12	33.3	4.2	62.5	-	39	74.4	19.2	2.6	3.8	21	7.1	50.0	31.0	11.9
Suffolk	44	41.4	2.3	51.5	4.8	25	48.0	6.0	32.0	14.0	38	40.8	15.8	39.5	3.9
South Suffolk	7	28.6	21.4	50.0	-	5	60.0	20.0	-	20.0	8	50.0	18.8	12.6	12.6
Dorper	23	30.4	10.9	54.3	4.3	23	65.2	19.6	10.9	4.3	23	37.0	15.2	26.1	21.7
White face Crossbreed	49	35.3	25.3	36.4	3.0	23	59.5	22.6	1.2	16.7	40	30.0	13.8	40.0	16.2
Black face Crossbreed	46	25.0	18.5	52.2	4.3	8	87.5	-	12.5	-	52	34.6	11.5	42.3	11.5
Composite	21	23.8	23.8	47.6	4.8	21	78.6	21.4	-	-	20	55.0	2.5	42.5	-
Total	538	30.3	17.6	49.6	2.5	430	59.4	26.3	6.6	7.7	444	39.2	14.3	38.9	7.6

Fewer samples were analysed for *FBLN1* Region 2 and 3 because these were the last genes studied, and some of the blood samples were no longer useable.

Sequence variation in ovine FBLN1

Using NCBI GenBank, a reference sequence for ovine *FBLN1* (XM0150950641. Appendix B.1) was used to describe the variants found in this study (Table 4.4). There were several base pair changes between the A_1, B_1, C_1 and D_1 sequences. At position c.698 the C/T bp change results in an amino acid change from alanine to valine. The SNP at position c.1821 in Region 2 investigated is found in a splice region (http://asia.ensembl.org) but does not result in an amino acid change.

Table 4.4 Nucleotide variation in three ovine fibulin-1 gene (FBLN1) regions.

Region	*Position*	*Gene Variant*			
		A	***B***	***C***	***D***
1	c. 59 - 98	c	t	c	t
	c. 59 – 30	a	g	g	g
	c.59	C	T	T	T
	c.141	C	T	C	C
2	c.1127	G	A	G	A
	c.1133	G	A	G	G
	c.1134 + 9	g	g	t	g
	c.1134 + 42	a	g	a	g
3	c. 1383 + 67	a	g	g	g
	c. 1383 + 124	t	c	t	t
	c. 1383 + 203	c	t	t	t
	c. 1383 + 239	t	t	t	c
	c.1383 + 294	c	c	c	t
	c. 1383 + 313	t	c	c	c

The capital letter represents coding nucleotides, while non-coding nucleotides are represented by lower case letters.

Association between ovine FBLN1 and flystrike

The frequencies of *FBLN1* variants for sheep with flystrike within each region investigated are shown in Table 4.5.

Table 4.5 Variant frequencies (expressed as a percentage) in the three *FBLN1* regions typed, including frequencies in sheep with flystrike.

FBLN1	*N*[1]	Total frequency (%)	Flystruck (n)	Frequency of flystrike for those animals containing the variant (%)
Region 1				
A_1	538	35.3	274	35.8
B_1	269	17.7	127	16.6
C_1	667	43.8	339	44.1
D_1	50	3.2	28	3.6
Total	*1524*	*100*	*768*	*100*
Region 2				
A_2	661	65.5	330	62.7
B_2	221	21.9	120	22.8
C_2	52	5.1	32	6.1
D_2	76	7.5	44	8.4
Total	*1010*	*100*	*526*	*100*
Region 3				
A_3	343	33.3	173	31.7
B_3	135	13.1	72	13.2
C_3	433	41.9	236	43.2
D_3	121	11.7	65	11.9
Total	*1032*	*100*	*546*	*100*

[1]Total number of variant copies. Note that sheep carry a maternally and paternally inherited variant, so this number is double the number of sheep tested.

Results for FBLN1 region 1

The results of the univariate analyses exploring variable associations with the presence or absence of each *FBLN1* Region 1 variant and flystrike are shown in Table 4.6. The presence of flystrike was associated with *FBLN1* C_1 (Table 4.6).

Table 4.6 The univariate association of the presence of each *FBLN1* Region 1 variant with the occurrence of flystrike

Gene variant	Odds ratio[1]	95% Confidence Interval[1]		P value[2]
		Upper	Lower	
A_1	0.924	0.696	1.226	0.582
B_1	0.799	0.587	1.089	0.156
C_1	1.472	1.093	1.982	**0.011**
D_1	1.088	0.606	1.956	0.777

[1]Derived from a binary logistic regression with presence or absence of flystrike as the dependent variable and presence or absence of the gene variant as the independent variable.
[2]Derived from both the Pearson chi-square and the binary logistic regression. Significant associations are in bold.

All the variables were retained in all the subsequent multivariate analyses (Appendix A.5). The association of the presence of *FBLN1* C_1 with the absence of flystrike persisted in the multivariate analyses, thus the effect was independent of any of the other variables included in the model (Table 4.7). The analyses showed that the presence of *FBLN1* B_1 variant was associated with less flystrike (P = 0.029), but it was found at a low level in the total sheep population (17 %). In contrast, the *FBLN1* C_1 variant has the highest frequency out of the four variants described for Region 1.

Table 4.7 The odds[1] of flystrike occurrence given the presence of a particular *FBLN1* Region 1 variant

Gene variant	Odds ratio	95% Confidence Interval		P value
		Upper	Lower	
A1	0.948	0.703	1.280	0.728
B1	0.686	0.489	0.961	**0.029**
C1	1.687	1.221	2.333	**0.002**
D1	1.049	0.571	1.927	0.878

[1]The odds ratios and confidence intervals were derived from a multivariate binary logistic regression in which the presence or absence of flystrike was the dependent variable and the independent variable were presence or absence of the gene variant, year, breed, geographical region, and the combined variable of age-gender.

Copy number analysis did not detect any additive effect of the *FBLN1 B_1* or *FBLN1 C_1* variants (P = 0.109 and P = 0.093, respectively; Appendix B.2).

Results for FBLN1 Region 2

The univariate analyses of *FBLN1* Region 2 revealed no associations between the variants and flystrike, (Table 4.8).

Table 4.8 The univariate association of the presence of each *FBLN1* Region 2 variant with the occurrence of flystrike

Gene variant	Odds ratio[1]	95% Confidence Interval[1]		P value[2]
		Upper	Lower	
A2	0.766	0.502	1.168	0.215
B2	1.225	0.838	1.789	0.295
C3	1.466	0.798	2.691	0.217
D3	1.087	0.649	1.823	0.751

[1]Derived from a binary logistic regression with presence or absence of flystrike as the dependent variable and presence or absence of the gene variant as the independent variable.
[2]Derived from both the Pearson chi-square and the binary logistic regression.

Multivariate analyses

FBLN1 C_2 trends towards an association with increased flystrike susceptibility (P = 0.060) (Table 4.9), but was also found at the lowest frequency of the four variants in this region (5.1%).

Table 4.9 The odds[1] of flystrike occurrence given the presence of a particular *FBLN1* Region 3 variant

Gene variant	Odds ratio	95% Confidence Interval		P value
		Upper	Lower	
A2	0.702	0.443	1.111	0.131
B2	1.313	0.853	2.022	0.216
C2	1.957	0.973	3.938	0.060
D2	1.051	0.599	1.843	0.862

[1] The odds ratios and confidence intervals were derived from a multivariate binary logistic regression in which the presence or absence of flystrike was the dependent variable and the independent variable were presence or absence of the gene variant, year, breed, geographical region, and the combined variable of age_gender.

In Region 2 (exon 10), *FBLN1* variant *C_2* trended towards having an association with flystrike.

Results for FBLN1 Region 3

Variants described in *FBLN1* Region 3 did not show an association with flystrike susceptibility (P= 0.832), (Table 4.10).

Table 4.10 The univariate association of the presence of each *FBLN1* Region 3 variant with the occurrence of flystrike

Gene variant	Odds ratio[1]	95% Confidence Interval[1]		P value[2]
		Upper	Lower	
A₃	1.170	0.753	1.818	0.484
B₃	1.022	0.679	1.537	0.918
C₃	1.058	0.740	1.513	0.758
D₃	0.823	0.580	1.168	0.276

[1]Derived from a binary logistic regression with presence or absence of flystrike as the dependent variable and presence or absence of the gene variant as the independent variable.
[2]Derive from both the Pearson chi-square and the binary logistic regression.

Multivariate analyses

The lack of associations between *FBLN1* variation in Region 3 and flystrike susceptibility continued in the binary logistic regression model, (Table 4.11).

Table 4.11 The odds[1] of flystrike occurrence given the presence of a particular *FBLN1* Region 3 variant

Gene variant	Odds ratio	95% Confidence Interval		P value
		Upper	Lower	
A₃	1.105	0.690	1.770	0.678
B₃	1.010	0.647	1.578	0.963
C₃	1.017	0.697	1.485	0.929
D₃	0.960	0.652	1.414	0.835

[1] The odds ratios and confidence intervals were derived from a multivariate binary logistic regression in which the presence or absence of flystrike was the dependent variable and the independent variable were presence or absence of the gene variant, year, breed, geographical region, and the combined variable of age-gender.

Discussion

This study set out to determine if there is an association between flystrike resilience in sheep and variation in ovine *FBLN1*. Both *FBLN1* B_1 and C_1 had an association with flystrike prevalence. Interestingly, the *FBLN1* C_1 variant was found at the highest frequency in the sheep studied, but it was associated with susceptibility to flystrike. This might mean that breeding sheep to reduce the frequency of C_1 would reduce the propensity to getting flystrike, but this assumes that C_1 does not have some other beneficial pleiotropic effect in the sheep studied, and thus it is not being actively selected for when breeding for some other trait. Nevertheless, the results presented here and the evidence provided by Smith *et al*. (2010), reinforce the idea that *FBLN1* is associated with susceptibility to fleecerot and flystrike in sheep. Smith *et al.* (2010) did not give a clear explanation of how *FBLN1* may be involved in resistance to fleecerot.

The SNP c.698 C/T found in Region 1 of ovine *FBLN1* resulted in an amino acid change from alanine to valine. This SNP occurs at the very first nucleotide of exon two, and sits next to a G/A splice region variant (that was not detected in this study). Fibulin-1 is made up of three structural domains. Domain I has, an N-terminus and makes up the Anaphylatoxin-like motifs. Domain II is a tandem repeat of calcium-binding epidermal growth factor like (cbEGF), followed by domain III, which consists of a

globular C-terminal, also known as “fibulin-like” or “FC domain” (Argraves *et al*. 2003; De Vega *et al*. 2009; Segade, 2010). Exons 2 – 4 make up three Anaphylatoxin-like motifs (Pan *et al.* 1999).

Region 2 and 3 of this study correspond to *FBLN1* exon 10 and intron 12, where Smith *et al*. (2010) described SNPs associated with fleecerot susceptibility. Three of the SNPs reported by Smith *et al*. (2010), FBLIn100090, FBLIn120135 and FBLIn120280, were also identified in this study. Region 2 variant B_2 corresponded to FBLIn100090, with a single base change from a G to an A at position c.1821, but this change was synonymous and would not lead to amino acid change if expressed. Although this SNP did not lead to an amino acid change, synonymous SNPs can impacts mRNA secondary structure and impact mRNA stability (Duan *et al*. 2003). It is of interest that the *FBLN1* C_2 variant trends towards an association with increased flystrike susceptibility. This variant was cased by a t/g SNP that is found close to the end of exon 10 but is not near the mRNA splice site.

The Region 3 fragment encompassed FBLIn120135 and FBLIn120280 (Smith *et al*. 2010), with A_3 variant having the corresponding A/G and C/T bp chance. Six SNPs were found in this region, indicating *FLBN1* is a very polymorphic gene. These SNPs were not found near mRNA splicing sites and were also not found to be associated with flystrike. Exon 14- 16 encode the nine epidermal growth (EG) modules of Domain II (Pan *et al* 1999).

It is well documented that *FBLN1* is an important gene controlling the physical barrier provided by skin (Argraves *et al*. 2003; Aspberg *et al*. 1999). Skin provides an obstacle to invading pathogens and it has been suggested that skin integrity is important in protecting sheep from both fleecerot and flystrike (Norris *et al*. 2008; Smith *et al*. 2010). Indeed the adhesion of bacteria to the host is mediated by interactions with the extracellular matrix (Courtney *et al.* 2009). *Fibulin-1* has been shown to interact with *Streptococcus pyogenes* bacteria, through its major serum opacity factor (SOF) receptor (Courtney *et al*. 2009). Therefore *FBLN1* could have a role in the adhesion of other bacteria that are involved in fleecerot.

Roark *et al*. (1995) found *FBLN1* to be expressed in a variety of human tissue, most notably in various skin cells including, the basal cells of the epidermis and fibroblasts, and at the dermal-epidermal junction, in elastic fibres, in sebaceous glands and sweat gland epithelia. Aspberg *et al*. (1999) found that *FBLN1* is also expressed around the hair follicle and at the dermal-epidermal border in rat skin. These findings in humans and rodents suggest that *FBLN1* is important to skin integrity and may be expressed in the various skin tissues including the sebaceous glands, sweat gland epithelia, and around the wool follicle of sheep. However, studies to confirm this have not been undertaken yet.

The bacterial infections associated with fleecerot, can result in blood plasma leaking on to the skin of sheep, as a consequence of damage to the wool follicle. The plasma can provide nutrients for proliferating bacteria and the subsequent release of chemical attractants from those bacteria that could attract blowflies (Emmens & Murray, 1982; Merrit and Watts, 1978). Colditz *et al*. (1992) found that

the Trangie resistant sheep had less plasma leakage than the susceptible line. When infected with flystrike and fleecerot, the degree of plasma leakage into the skin was influenced by the individual sheep's ability to generate permeability mediators (Colditz *et al*. 1992). Mediators used in their study included histamine, bradykinin, platelet-activating factor, serotonin and activated complement. It has also been revealed that *FBLN1* is expressed at a higher level in the bed of non-healing ulcers, compared to healing ulcers (Charles *et al*. 2008). This could have implications for the development and healing of ulcerations caused by fleecerot and flystrike. Due to the role of *FBLN1* in the ECM of the skin it is possible that variants in *FBLN1* act differently to reduce plasma leakage in more resistant sheep.

From the results shown here, *FBLN1* is playing a role in flystrike in sheep. While the mechanisms that underpin this role are not yet known, it would be a good canididate gene for future investigation. The ovine *FBLN1* consists of 18 exons and is 2742 bp long. This study only looked at regions of exon 2, exon 10 and intron 13. In future, it would be better to sequence the whole of ovine *FBLN1*. Numerous studies in humans and mice highlight the importance of FBLN1 in the basal cells of the epidermis and fibroblasts, and at the dermal-epidermal junction, in elastic fibres, in sebaceous glands and sweat gland epithelia. Future research should focus on the role of *FBLN1* in sheep skin, specifically looking at variation between sheep with flystrike and those without it. It would also be interesting to compare *FBLN1* expression in the skin of sheep that get flystrike compared with sheep that have never been struck, with emphasis on the skin, the sebaceous glands, the sweat gland epithelia and other tissue in and around the wool follicle.

Chapter 5. Ovine *TLR4* variation and its association with flystrike susceptibility

Three host-specific factors are probably involved in the development of sheep flystrike resilience: wool characteristics, skin characteristics and the immune response (Smith *et al*. 2010). With regards to the latter, the immune system of animals recognises foreign bodies (antigens) and destroys or inactivates them using a variety of processes (Takeda *et al*. 2003). In the context of flystrike, it has been confirmed that the immune system of sheep can recognise components of the fly larvae as being foreign, and generate an immune response to them (Figure 5.1, MacDiarmid *et al.* 1995; Colditz *et al.* 2006). Immune response genes are, therefore, likely to play an important role in flystrike resilience and susceptibility, especially those genes that underpin the generation of inflammatory and hypersensitivity responses.

These responses have been suggested to be important for the rejection of larvae by resistant hosts (Tellam and Bowles, 1997; Colditz *et al.* 2006; Norris *et al*. 2008). In other investigations, it was revealed that a sheep's immune system responded to both the larvae, and the bacteria that causes fleecerot on the wound surface (O'Meara *et al*. 1992; 1997). In the Trangie selection lines, the resistant line of sheep have been shown to produce a higher level of antibodies to *Pseudomonas aeruginosa*, which is one of the main bacteria associated with fleecerot (Chin and Watts, 1991), while other studies suggest that resistant sheep produce a larger inflammatory skin reaction than susceptible sheep (Sandeman *et al*. 1986; MacDiarmid *et al*. 1995).

The toll-like receptors (TLRs) are a group of proteins that play a key role in innate and adaptive immune responses. They are membrane-spanning receptors and are usually expressed in sentinel immune cells such as macrophages, monocytes and dendritic cells (Akira, 2003). The TLRs recognise structurally conserved molecules derived from infecting microbes. Once these microbes have breached physical barriers such as the skin or mucosa of the intestinal tract, they are recognised by the TLRs, and this then activates subsequent immune cell responses. The known TLRs include TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, TLR11, TLR12 and TLR13, although the latter two have not been found in humans (Werling *et al*. 2007; Chang, 2009).

The presence of microbial antigens activates dendritic cells, leading to their maturation and the expression of many of the TLRs (Banchereau and Steinman, 1998; e Sousa, 2001). Dendritic cells contribute to the activation of the adaptive immune system, as they have a high capacity for endocytosis, which facilitates the uptake of foreign antigens. They then migrate to the draining lymph nodes where they attract T and B cells by releasing chemokines in order to initiate an adaptive immune response (Akira *et al*. 2001; Banchereau and Steinman, 1998; Kawai and Akira, 2010; Lim and Staudt, 2013; e Sousa, 2001). This process then induces the production of inflammatory cytokines

from myeloid cells (Newton and Dixit, 2012) such as interleukin 6 (IL-6), tumour necrosis factor-α (TNF-α) and interleukin -1β (IL-1β) (Chemonges *et al*. 2014).

Aside from bacteria, the TLRs recognise glycoinositol phospholipids produced by parasites and viral structural proteins (Kawai and Akira, 2011). The interaction occurs with input from other soluble or membrane-bound co-receptors, which give the receptors diversity in ligand recognition and allows them to play a greater role in establishing immunity to diseases (White *et al*. 2003; Chang *et al*. 2009).

Among the TLRs, TLR4 recognises the lipopolysaccharides (LPS) of Gram-negative bacteria, mannans from fungal pathogens, a soluble component of *Mycobacterium tuberculosis*, and other endogenous ligands such as fibronectin and some heat-shock proteins (Ferwerda *et al*. 2008). Lipopolysaccharides (sometimes referred to as endotoxin), can promote the release of inflammatory cytokines that trigger innate immune responses, and can cause shock and even death. It was thought originally that LPS acted through many different cell receptors, but a study in mice revealed that the LPS sensor is dependent solely on TLR4 (Sultzer, 1968; Watson *et al*. 1977), while nonsense mutation in *TLR4* causes insensitivity to LPS (Beutler, 2000).

Toll-like receptor 4 is expressed on many different immune cells, including the classical antigen-presenting cells (dendritic cells, monocytes and macrophages), and also the B and T cells (Takeda *et al*. 2003). It is therefore possible that TLR4 is directly involved in the inflammatory response, along with other immune responses to flystrike.

The ovine TLR4 gene (*TLR4*) has been characterised and found to be associated with different diseases that affect sheep, such as interdigital dermatitis, footrot and Johnes disease (Taylor *et al*. 2008; Davenport *et al*. 2014). Variation in *TLR4* has been associated with variation in faecal egg count (FEC) in lambs parasitized by the *Nematodirus spp*., a genus of gastro-intestinal (GI) parasites (Lin *et al*. 2016). High GI parasite burdens can cause scouring and dag formation, which can then predispose sheep to flystrike (Waghorn *et al*. 1999), with one study reporting a correlation of 0.97, between dagginess and breech strike (Leathwick and Atkinson, 1995). Accordingly, sheep that have a greater resistance to internal parasites, or dagginess, may also be more resistant to breech strike.

Research has revealed that exon 3 of ovine *TLR4* encodes the putative ligand-binding region (Zhou *et al*. 2007). To date, based on the analysis of exon 3 of *TLR4* in sheep, seven variants of the gene have been identified (Byun *et al*. 2009b). These variants contain numerous SNPs, with most of these SNPs resulting in amino acid changes. These genetic variants may therefore create structural and functional differences in the TLR4 receptors, which could then lead to differences in immune response when sheep are challenged by the occurrence of flystrike. In this respect, no one has studied the possible role of TLR4 in flystrike and so an investigation to ascertain if there is an association between variation in ovine *TLR4*, and susceptibility to flystrike, was undertaken.

Figure 5.1 A diagram illustrating the interaction between *L. cuprina* larvae and the host immune system. In response to flystrike both innate and adaptive immune responses are activated by the larval antigens on the wound surface. The innate immune system is stimulated to activate natural killer (NK) cells, eosinophils (E), mast cells (M), neutrophils (N), and γδ+ T cells (γδ+), as well as the Alternative Complement Pathway. The NK cells produce interferon γ (IFN-γ), which triggers T helper type 0 (Th0) cells. The interaction between larval antigens (Ag) and major histocompatibility complex class II (MHC II) molecules on antigen-presenting cells (APC), then induces an adaptive immune response. In this response, the release of interleukin 12 (IL-12) by the APCs, stimulates the differentiation of Th0 into Th1 and Th2 T cells. The Th1 cells enhance the cellular immune response by releasing the cytokines interleukin 2 (IL-2) and IFN-γ. These cytokines stimulate the cellular immune response through the recruitment of cytotoxic T cells (CD8+). The Th2 cells release the cytokines IL-4, IL-5, IL-6 and IL-10 and these activate B cells leading to the production of immunoglobulins (Igs). IgE then triggers eosinophils and mast cell recruitment at the site of larval infestation. The APCs also release the pro-inflammatory cytokines IL-1 and tumour necrosis factor α (TNF-α).
(Adapted from Otranto, 2001).

Material and Methods

Animals

A total of 890 sheep were sampled from over five strike seasons 2013 to 2017. Sheep were from both commercial and stud farms and were of various breeds as described in Chapter 3. Of the amplified samples, 422 samples (frequency of breeds was calculated over several years so not all samples from later years were included) from 14 different breeds, or breed crosses, were used to determine breed frequencies for the *TLR4* variants, along with 23 samples from the Dorper breed that were not part of the flystrike study.

Blood collection

Blood samples were collected onto FTA cards (Whatman, Middlesex, UK), using methods described in Chapter 3. This sampling method did not require animal ethics approval as ear nicking is considered a common farming practice. Genomic DNA for analysis was purified using a two-step washing procedure as described in Zhou *et al*. (2006).

Primer design for ovine TLR4

PCR primers were the same primers described by Byun *et al*. (2009b), that amplify a 256 bp fragment within exon 3 of ovine TLR4 (Figure 5.2). The primers were TLR4-up2 (5'-TCTTTAGGAAGTCTACAAGC-3') and TLR4-dn2 (5'-AATGCTTCAGGTTGGTTGTC-3'). These primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA). Previously typed genomic DNA samples were run as standards to confirm the PCR-SSCP typing (see Byun *et al.* 2009b).

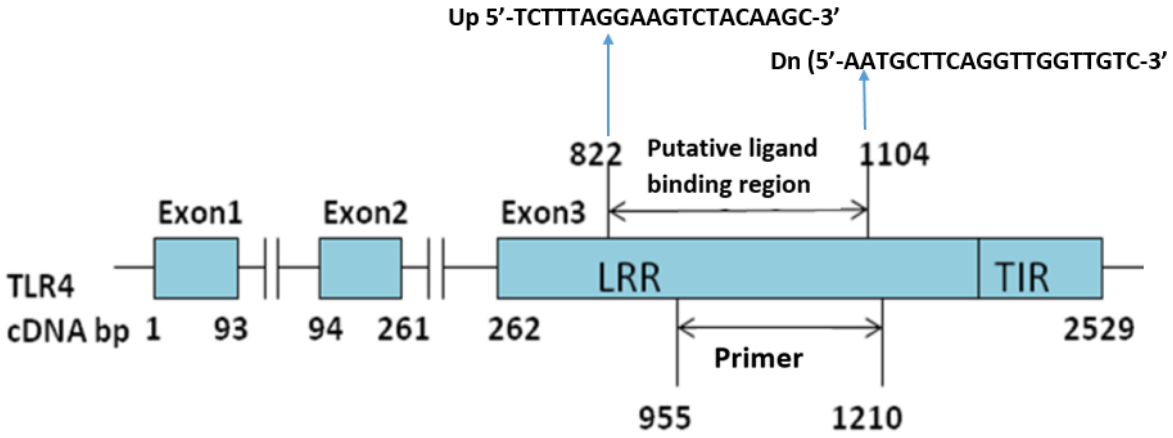


Figure 5.2 Location of PCR primers and the portion of ovine TLR4 amplified. LRR= leucine rich region; TIR= Toll-interleukin 1 receptor domain.
(Adapted from Byun, 2012).

PCR-SSCP analysis and genotyping of ovine TLR4

PCR amplification was performed in a 15 µL reaction containing the genomic DNA on one 1.2 mm punch of FTA card, 0.25 µM of each primer, 150 µM dNTP's (Bioline, London, UK), 2.5 mM of Mg^{2+}, 0.5 U of Taq DNA polymerase (Qiagen, Hilden, Germany) and 1× the reaction buffer supplied with the enzyme. The thermal profile consisted of 2 min at 94 ºC, followed by 35 cycles of 30 s at 94 ºC, 30 s at 60 ºC and 30 s at 72 ºC, with a final extension of 5 min at 72 ºC. Amplification was carried out in S1000 thermal cyclers (Bio-Rad, Hercules, CA, USA).

A 0.7 µL aliquot of each amplicon was mixed with 7 µL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol). After denaturation at 95 ºC for 5 min, samples were rapidly cooled on wet ice and then loaded on 16 cm × 18 cm, 14% acrylamide:bisacrylamide (37.5:1) (Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad), at 300 V for 19 h at 5 ºC in 0.5 × TBE buffer. Gels were silver-stained according to the method of Byun *et al.* (2009a).

Statistical analysis

Variant frequencies were calculated for different breeds of sheep, along with the frequencies of the variants in the sheep with flystrike and without flystrike.

All analyses were performed using SPSS version 24 (Chicago, IL, USA), and as previously described in Chapter 3, Appendix A.3.

To determine which of these variables would be included in subsequent multivariate binary logistic regression models, univariate Pearson chi-square tests were performed to explore the association between the variables and the presence or absence of each of the gene region variants. A Pearson chi-square test was used to ascertain whether *TLR4* variants were associated with the incidence of flystrike.

For each gene variant, a Pearson chi-square test along with a binary logistic regression was performed to explore whether the presence or absence of the variant was associated with the presence or absence of flystrike and to determine the odds ratio, respectively. A multivariate binary logistic regression was then performed to determine the independent effects of the gene variants on the incidence of flystrike when year, breed, geographical location, age and gender were taken into account. If appropriate, an additional binary logistic regression model containing more than one of the gene variants was performed which included the variables from the previous models (year, breed, geographical location, and age_gender, along with any and gene variant from the previous univariate gene variant models with $P < 0.200$ (this indicating a potential impact on the presence or absence of flystrike).

Results

Variation in TLR4

Four of the previously seven identified variants of the TLR4 gene were found in this study, and these matched the *01* to *04* variants identified by Zhou *et al.* (2007) (Figure 5.3). The *05* to *07* variants were not found in this study.

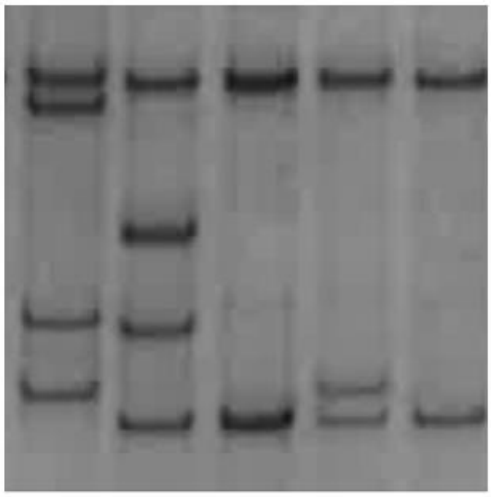


Figure 5.3 SSCP-PCR gel illustrating the four variants of ovine *TLR4*.

The frequency of the variants in *TLR4* in 15 different breeds of sheep is shown in Table 5.1.

Table 5.1 Frequency of *TLR4* variants (*01 - 04*) in the sheep breeds investigated.

Breed	***n***	***01***	***02***	***03***	***04***
Merino	59	63.6	34.7	-	1.7
South Suffolk	8	56.3	25.0	-	18.7
Corriedale	54	54.4	30.3	11.7	3.6
Dorper	23	47.8	13.0	23.9	15.2
Dorset Down	26	46.2	40.4	1.9	11.5
Shropshire	12	41.7	33.3	4.2	20.8
Suffolk	31	43.5	25.8	14.6	16.1
Black face Crossbred	46	32.7	41.3	15.2	10.8
Perendale	32	23.4	53.4	14.1	3.1
Lincoln	7	21.4	57.1	14.3	7.2
Poll Dorset	15	26.7	40.0	23.3	10.0
White face Crossbred	47	25.5	44.5	21.7	8.3
Composite	21	9.5	66.7	23.8	-
Total	445	36.1	39.9	14.4	9.6

The frequencies of *TLR4* variants for sheep with flystrike is shown in Table 5.2.

Table 5.2 Frequencies (expressed as a percentage) of variants in *TLR4* and the occurrence of flystrike.

	n	Total frequency	*n* with fly-struck	Frequency with flystrike
01	582	37.4	299	35.5
02	672	40.8	357	41.7
03	238	14.5	126	14.9
04	118	7.3	67	7.9
Total	*1604*	*100*	*844*	*100*

Univariate analyses for TLR4

The results of the univariate analyses exploring variable associations with the presence or absence of each *TLR4* variant and flystrike are shown in Table 5.3. *TLR4* variants showed no association with flystrike, (P= 0.308).

Table 5.3 The univariate association of the presence of each *TLR4* variant with the occurrence of flystrike.

Gene variant	Odds ratio[1]	95% Confidence Interval[1]		P value[2]
		Upper	Lower	
01	0.835	0.630	1.107	0.211
02	0.970	0.726	1.296	0.837
03	0.879	0.640	1.206	0.424
04	1.077	0.716	1.622	0.721

[1]Derived from a binary logistic regression with presence or absence of flystrike as the dependent variable and presence or absence of the gene variant as the independent variable.
[2]Derived from both the Pearson chi-square and the binary logistic regression.

Multivariate analyses for TLR4

There was no statistically significant association between flystrike and variations of *TLR4* in the binary logistic regression model, (Table 5.4).

Table 5.4 The odds[1] of flystrike occurrence given the presence of a particular *TLR4* variant.

Gene variant	Odds ratio	95% Confidence Interval		P value
		Upper	Lower	
01	0.901	0.664	0.114	0.507
02	0.941	0.693	1.276	0.694
03	0.828	0.593	1.154	0.265
04	1.077	0.705	1.645	0.731

[1] The odds ratios and confidence intervals were derived from a multivariate binary logistic regression in which the presence or absence of flystrike was the dependent variable and the independent variables were presence or absence of the gene variant, year, breed, geographical region, and the combined variable of age_gender.

Discussion

This chapter aimed to investigate whether variations in *TLR4* were associated with variation in flystrike susceptibility in sheep. Out of the 10 reported TLR genes found in sheep (Chang *et al*. 2009), this study chose to focus on the leucine rich repeats (LRR) region of exon 3 of *TLR4*. The human, cattle and sheep TLR4 extracellular domain consists of 23 LRRs that interact with different ligands (Ohara *et al*. 2006). In humans, LRR 9 to LRR14 of TLR4 make up the central region of the extra-cellular domain, which binds putative co-receptors and ligands (Bell *et al*. 2003). This region of the LRR is the most variable region of TLR4, with nucleotide sequence variation in *TLR4* causing

variation in the motif structure of the protein (Mucha *et al*. 2009). Arbour *et al.* (2000) have shown that variation in this region is associated with varying responses to LPS and suscepti bility to various diseases (Hawn *et al*. 2005; Mockenhaupt *et al*. 2006).

Previously, variation in this gene has been associated with variation in disease susceptibility and altered immune responses to pathogens in mammals (Lin *et al*. 2016; Smirnova *et al.* 2001; and Coffey, 2007). For example, variation in ovine *TLR4* has been associated with faecal egg count (FEC) in sheep infected with gastrointestinal parasites (Lin *et al*. 2016), and altered expression of *TLR4* has been associated with susceptibility to Johnes disease (Taylor *et al*. 2008), and footrot (Davenport *et al*. 2014).

For the four common ovine *TLR4* variants (*01 – 04*) identified in the sheep studied here, and that had been described by Byun *et al.* (2009b) previously, the frequencies of the variants were comparable in the two studies. However, in Byun *et als'* (2009b) study, *TLR4 05, 06* and *07* were found at a combined frequency of 0.3%. At this low frequency, it is perhaps unsurprising that these variants were not found in the sheep in this study. The *TLR4* variation identified in this study did not have an association with flystrike susceptibility. It should, however, be noted that *TLR4* is an immune response gene that is activated when the immune system is challenged. Thus, it might be expected to have an effect on how different sheep respond to flystrike, rather than preventing them from getting flystrike in the first instance. Flystrike is an external parasitic infection, in contrast with previous studies that have found association with variation in TLR4 and internal parasites. Toll-like receptor 4 is well characterised for its response to LPS of gram-negative bacteria (Akira and Takeda, 2004; Ferwerda *et al*. 2008), but has also involved in response to gastrointestinal parasites (Gopal *et al*. 2008; Helmby & Grenise, 2003; Kosik-Bogacka *et al*. 2012, 2013, Lin *et al*. 2016). Toll-like receptor 4 is highly expressed in the muscle layer of the large intestine, where internal parasites are often found. The large intestine is also the most colonised (by bacteria) region of the gut, and a combination of bacteria and tissue-damaging gastrointestinal parasites could result in LPS leakage, trigging an innate immune response from TLR4 (Helmby & Grenise, 2003). There is a strong correlation between the occurrence of dags and breech strike (r = 0.97) (Leathwick and Atkinson, 1995), and high internal parasite burden and dag formation (Waghorn *et al*. 1999). This could be how TLR4 has a role in internal parasite resistance, but not external parasite resistance. If TLR4 had a role in flystrike resilience it is possible that it would be a secondary effect through its role in internal parasite resistance.

This study did not look at the association between variation in *TLR4* and fleecerot in sheep, but future work involving TLR4 should explore this relationship. TLR4 responds to Gram-negative bacteria, such as *P. aeruginosa*, the main bacterial species involved in fleecerot (Chin and Watts, 1991; Norris, 2008). In Australian Merinos it has been shown that flystrike infected animals generate wool and skin substances in response to the moisture and bacteria from fleecerot (Raadsma, 2000). The immune responses to *P. aeruginosa* (Chin and Watts, 1991) and immune-inflammatory responses (Colditz *et*

al. 1992; 1994; O'Meara *et al*. 1995) have been well documented in response to both fleecerot and flystrike. Fleecerot is often caused by multiple species of bacteria, of which some are gram-negative and some are gram-positive. While TLR4 is known to respond to Gram-negative bacteria, TLR2 responds to Gram-positive bacteria (Takeuchi *et al*. 1999), and these proteins have previously been found to work together to activate an immune response to invading bacteria (Weiss *et al*. 2004). It is therefore possible that both TLR2 and TLR4 are required to mount an immune response to the bacteria causing fleecerot and perhaps flystrike resilience in sheep.

This idea is supported by Davenport *et al*. (2014) who reported a strong correlation between the expression of *TLR2* and *TLR4* (R^2= 0.81) in the interdigital skin of the hooves of sheep infected with footrot, supporting the potential that these two genes may work together in response to bacterial infections in sheep.

It is also noteworthy that TLR3 and TLR8 were identified in the differentially expressed (DE) list of 155 genes in Smith *et al.* (2010). Toll-like receptor 3 was found to have increased expression, but it is typically associated with viral infections with the ability to recognise dsRNA in mice (Kawai and Akira, 2006; Takeda *et al*. 2003). Furthermore, TLR9 has been shown to recognise DNA from parasites, bacteria, viruses and fungus (Kawai and Akira, 2011). Thus, it is also conceivable that other TLRs may have a role in the immune response to flystrike, and accordingly this group of genes probably requires further investigation.

In conclusion, resilience to flystrike is likely to be a result of a combination of genes rather than one individual gene. In terms of the immune response to flystrike, this too is likely to be the result of various genes, not just *TLR4* alone. Further investigation into TLR4 and other TLRs in the future could prove useful in identifying sheep that have a better immune response when challenged with fly larvae. Future research could also look at the whole TLR4 gene, rather than just the exon 3 region.

Chapter 6. Ovine *RASGRP1* variation and its association with flystrike susceptibility

The immune response plays a role in the susceptibility of sheep to flystrike and flies are often attracted to unhealthy sheep with predisposing conditions such as bacterial infections from fleecerot and dermatophilosis (Eisemann, 1988; Morris *et al*. 1998; Norris *et al*. 2008b). As mentioned previously, fleecerot and flystrike susceptibility are highly correlated and sheep mount an immune response to both the bacteria that cause fleecerot and the fly larvae causing flystrike (O'Meara *et al.* 1992).

When infested with *L. cuprina* larvae, both neutrophils and eosinophils are found on the wound surface (Bowles *et al*. 1992; Broadmeadow *et al*. 1984b), and CD4+, CD8+ and γδ T lymphocytes (T cells) are found in the dermis (Bowles *et al*. 1992; Elhay *et al*. 1994). This suggests that T cell polyclonal activation and selective recruitment occurs at the lesion site (Bowles *et al*. 1992). Genes that have a role in the maturation and signalling of these immune cells could have a role in the immune response to flystrike.

Recently, Pickering (2013) identified Ras guanyl release protein 1 (*RASGRP1*) within 100kbp of the most strongly associated SNP (OAR7_35146905) to flystrike resilience in sheep. This was the first indication that *RASGRP1* might play a role in flystrike resilience in sheep. The Ras family of proteins are involved in many signalling pathways, where they regulate cell functions including: proliferation, the differentiation of thymocytes into mature T cells and the activation of T cells (Dower, 2000; Fuller *et al*. 2012).

The RASGRP1 gene is expressed at elevated levels in T cells, but it is also expressed in B cells, neuronal cells and mast cells (Ebinu *et al*. 2000; Liu *et al*. 2007). Ras guanyl release protein 1 is specifically involved in the T cell receptor (TCR) signalling pathway. It enables the generation of a diverse collection of TCRs, and these allow a diversity of response to a vast range of foreign peptides presented by the major histocompatibility complex (MHC) (Priatel *et al*. 2002). In terms of flystrike, there is an interaction between larval antigens and MHC class II molecules on antigen-presenting cells, and this induces an immune response (Bowles *et al*. 1994; Kerlin and East, 1992).

In humans, variation in *RASGRP1* has been associated with autoimmune disorders including type I and type II diabetes, systemic lupus erythematosus, Crohn diseases, multiple sclerosis, and rheumatoid arthritis (Yasuda et *al*. 2007; Zhou *et al*. 2016). Taneera *et al*. (2012) found diabetic Islets have a decreased expression of *RASGRP1*. Zhou *et al*. (2016) found an intronic variant of *RASGRP1* to be associated with IgA nephropathy, which is a kidney inflammation disease caused by an immune response that often results in renal failure. They suggested that *RASGPR1* variants are likely to influence disease onset by regulating gene expression. They found that the *RASGRP1* variants were

pleiotropic, likely affect susceptibility to multiple autoimmune diseases. Yasuda *et al*. (2007) identified 13 new isoforms of human *RASGRP1* that arise due to alternate splicing in the exon 5 to 17 region. They found that splice variant A, which occurs with the deletion of exon 11, is associated with systemic lupus erythematosus.

Overexpression of RASGRP1 in mice results in an increased number of CD8+ T cells (Norment, 2003). In contrast, *RASGRP1* null (*RASGRP1*$^{-/-}$) mice have a block in thymocyte development and diminished numbers of CD4+ T cells and CD8+ T cells (Dower, 2000). Mice deficient in *RASGRP1* therefore have diminished T cell responses and delayed pathogen clearance, this response being functionally similar to the 'exhausted' memory T cell response found during chronic infections (Priatel *et al*. 2007). Accordingly ovine *RASGRP1* could have a role in flystrike susceptibility in sheep through the occurrence of a diminished T cell response to the invading larvae.

Materials and Methods

Animals

A total of 890 sheep were sampled from over five strike seasons 2013 - 2017 as described in Chapter 3. Of the samples typed, 460 samples from 13 different breeds and crossbreeds were used to determine the frequency of the *RASGRP1* variants in different breeds. These breeds included Dorper sheep (n = 18) that were not from the flystrike affected flocks. The frequency of breeds was calculated over several years, so not all samples from later years were included.

Blood collection

Blood samples were collected onto FTA cards (Whatman, Middlesex, UK), as described in Chapter 3. This sampling method did not require animal ethics approval as ear nicking is considered a common farming practice. DNA was purified using a two-step washing procedure as described in (Zhou *et al*. 2006).

Primer design and location for ovine RASGRP1

Two PCR primers were designed based on the predicted sequence of ovine *RASGRP1* (XM 012181173.2) to amplify a 446 bp fragment spanning exon 16 of the gene (Figure 6.1). Exon 16 was targeted because it is the largest exon of RASGRP1. Exon 16 and 17 make up the C – terminal domain which has an important role in RASGRP1 function.

The primers were *RASGRP1*-up (5'ACAGCACCTGAGGAAGGA-3') and *RASGRP1*-dn (5'-ATGTTCTTCCCTTGTTAGCC-3'). These primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA), Appendix C.1.

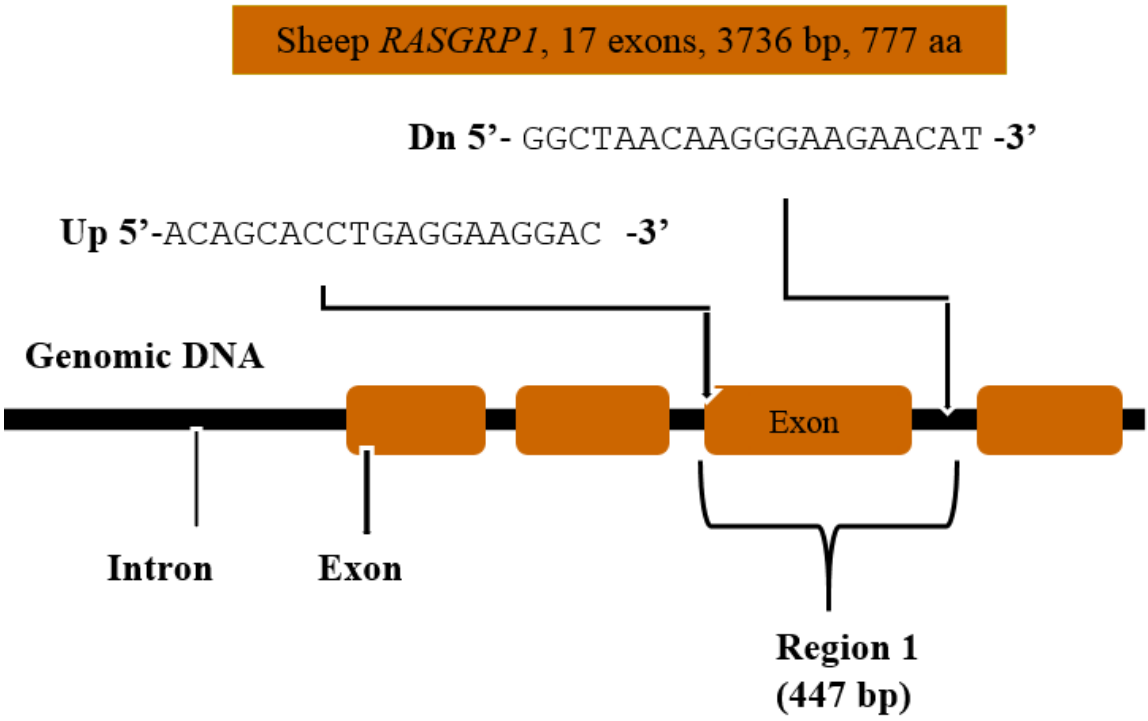


Figure 6.1 Location of PCR primer designed to amplify 447 bp of ovine *RASGPR1* region studied. Gene structure based on the ovine *RASGRP1*, not drawn to scale.

Developing the PCR-SSCP protocols

DNA samples (n = 20) from several breeds were used to develop a PCR-SSCP protocol for ovine *RASGRP1*. Different PCR protocols were tested, starting with an annealing temperature of 58ºC, with this being optimised at 60ºC. Amplicons were visualised by electrophoresis in 1% agarose (Quantum Scientific, Queensland, Australia) gels, using 1 x TBE buffer (98 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA) and containing 200 ng/ml of ethidium bromide. When the agarose gels produced a good result, several samples were then run on 14% acrylamide gels at various temperatures to produce the best resolution of bands.

PCR-SSCP analysis and genotyping of ovine RASGRP1

PCR amplification was performed in a 15 µL reaction containing the genomic DNA on one 1.2 mm punch of FTA card, 0.25 µM of each primer, 150 µM dNTP's (Bioline, London, UK), 2.5 mM of Mg^{2+}, 0.5 U of Taq DNA polymerase (Qiagen, Hilden, Germany) and 1× the reaction buffer supplied with the enzyme. The thermal profile consisted of 2 min at 94 ºC, followed by 35 cycles of 30 s at 94 ºC, 30 s at 60 ºC and 30 s at 72 ºC, with a final extension of 5 min at 72 ºC. Amplification was carried out in S1000 thermal cyclers (Bio-Rad, Hercules, CA, USA).

A 0.7 µL aliquot of each amplicon was mixed with 7 µL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol). After denaturation at 105 ºC for 5 min, samples were rapidly cooled on wet ice and then loaded on 16 cm × 18 cm, 14% acrylamide:bisacrylamide (37.5:1) (Bio-Rad) gels. Electrophoresis was performed using Protean II xi

cells (Bio-Rad), at 300 V for 19 h at 18 ºC in 0.5 × TBE buffer. Gels were silver-stained according to the method of (Byun *et al*. 2009a).

Sequencing of the ovine RASGRP1 variant and sequence analysis

Homozygous amplicons were identified for ovine *RASGRP1* by PCR-SSCP. These amplicons were then sequenced at the Lincoln University DNA Sequencing Facility. The computer program DNAMAN (version 5.2.10, Lynnon BioSoft, Canada) was used for sequence alignment, translations and comparisons. The Blast algorithm was used to search the NCBI GenBank database (http://blast.nci.nlm.nih.gov/) for homologous sequences.

Statistical analyses

All analyses were performed using SPSS version 24 (Chicago, IL, USA), data was categorised as described in Chapter 3, Appendix A.3.

To determine which of these variables would be included in subsequent multivariate binary logistic regression models, univariate Pearson chi-square tests were performed to explore the association between the variables and the presence or absence of each of the gene region variants.

A Pearson chi-square test was undertaken to ascertain whether *RASGRP1* genotype was associated with the incidence of flystrike.

For each gene variant, a Pearson chi-square test along with a binary logistic regression was performed to explore whether the presence or absence of the variant was associated with the presence or absence of flystrike and to determine the odds ratio, respectively. A multivariate binary logistic regression was then performed to determine the independent effects of gene variant on incidence of flystrike when year, breed, geographical location, age and gender were considered. If appropriate, an additional binary logistic regression model containing more than one of the gene variants was performed which included the variables from the previous models (year, breed, geographical location, and age_gender, along with any gene variant from the previous univariate gene variant models with $P < 0.200$ (indicating a potential impact on the presence or absence of flystrike).

Copy number analysis was performed for *RASGRP1 A*, using cross-tabulations (with Chi-square analysis), and if significant, followed with a binary logistic regression containing year, breed, geographical location, and age_gender along with number of copies (0, 1 or 2).

Results

Three unique banding patterns were found in the region of *RASGPR1* studied, Figure 6.2.

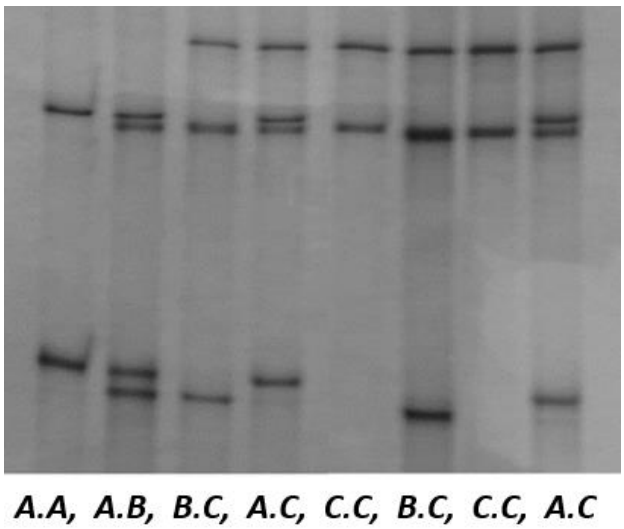


Figure 6.2 SSCP gel for variants in *ovine* RASGRP1.

The frequency of the three variants in RASGRP1 for the different breeds investigated is shown in Table 6.1.

Table 6.1 Frequency (expressed as a percentage) of *RASGPR1* variants (A_1 – C_1) in a New Zealand population of sheep.

	n	***A_1***	***B_1***	***C_1***
Breed				
Composite	13	88.5	11.5	-
Perendale	47	64.9	19.1	16.0
White face Crossbred	52	63.1	17.9	19.0
Coopworth	29	62.1	14.2	20.7
Poll Dorset	10	60.0	40.0	-
Dorper	18	58.3	33.4	8.3
Merino	59	55.1	29.7	15.2
Corriedale	67	52.7	24.2	20.1
Dorset Down	26	50.0	19.2	30.8
Shropshire	25	50.0	16.0	34.0
Black face Crossbred	53	41.5	34.0	24.5
Suffolk	31	37.7	36.1	26.2
Romney	30	31.7	53.3	15.0
Total	*460*	*55.0*	*26.8*	*18.2*

The SNPs and their position resulting in the three variants found in *RASGPR1* are shown in Table 6.2.

Table 6.2 Three single nucleotide polymorphisms associated with the three unique PCR-SSCP patterns observed for exon 16 of ovine *RASGRP1* (variants *A*-*C*).

Position	*A*	*B*	*C*	GenBank Reference sequence
c.2002 C<T	T	T	C	MF925341
c. 2122 T<C	C	T	T	MF925342
c.2146 T<C	C	T	T	MF925343

The frequencies of *RASGPR1* variants for sheep with flystrike is shown in Table 6.3.

Table 6.3 Frequencies (expressed as a percentage) of variants in *RASGRP1* and the occurrence of flystrike.

RASGRP1	*n*	Total frequency	Flystruck (n)	Frequency with flystrike
A	853	53.9	468	55.6
B	393	24.8	212	25.2
C	336	21.3	162	19.2
Total	*1582*	*100*	*842*	*100*

Univariate analyses

The results of the univariate analyses exploring variable associations with the presence or absence of each *RASGRP1* variant and flystrike are shown in Table 6.4. *RASGRP1* A genotype tended to be associated with the presence of flystrike (P = 0.049), while the *RASGRP1 C* variation was associated with the absence of flystrike (P = 0.049).

Table 6.4 The univariate association of the presence of each *RASGRP1* variant with the occurrence of flystrike.

Gene variant	Odds ratio[1]	95% Confidence Interval[1]		P value[2]
		Upper	Lower	
A	1.404	1.002	1.968	**0.049**
B	1.163	0.874	1.547	0.299
C	0.745	0.556	0.998	**0.049**

[1]Derived from a binary logistic regression with presence or absence of flystrike as the dependent variable and presence or absence of the gene variant as the independent variable.
[2]Derive from both the Pearson chi-square and the binary logistic regression. Significant associations are in bold.

Multivariate analyses

The association of the presence of *RASGRP1 A* with flystrike susceptibility persisted with the binary logistic regression model, (P = 0.015). The *RASGRP1 C* variant association with flystrike also persisted in the model (P = 0.047), Table 6.5.

Table 6.5 The odds[1] of flystrike occurrence given the presence of a particular *RASGRP1* variant.

Gene variant	Odds ratio	95% Confidence Interval		P value
		Upper	Lower	
A	1557	1.091	2.221	**0.015**
B	1.236	0.914	1.671	0.169
C	0.733	0.539	0.996	**0.047**

[1] The odds ratios and confidence intervals were derived from a multivariate binary logistic regression in which the presence or absence of flystrike was the dependent variable and the independent variable were presence or absence of the gene variant, year, breed, geographical region, and the combined variable of age_gender.

The presence of the *A* variant of *RASGRP1* was associated at the level of a trend with an increase in the prevalence of flystrike, while the presence of the *C* variant had a trend of association with a decrease in flystrike. No additive effect of the *A* variant was detected in the copy number analysis (Chi-square, P = 0.207), Appendix C.2. The *C* variant only makes up 18.9 % of the total frequency, with sheep carrying *CC* genotype still being affected with flystrike.

Discussion

The aim of this study was to investigate whether variation in *RASGRP1* was associated with the occurrence of flystrike. Three sequence variants were identified in the sheep studied, and these occurred because of three nucleotide changes in exon 16 of the gene. These three SNPs are synonymous, but could play a role in mRNA processes and the resulting protein structure of RASGRP1. Two of these variant sequences (*A* and *C*) were found to be associated with the occurrence of flystrike. The *A* variant was present at a high frequency in sheep, indicating that it could have a strong influence in flystrike susceptibility. Although the *C* variant was only just significant. This suggest that the *C* variant is not having a large effect on flystrike in sheep. Selectively breeding sheep that carry the *C* variant could reduce flystrike. These results are consistent with the genome-wide association study (GWAS) undertaken by Pickering (2013), which identified *RASGRP1* within 100kbp of the SNP OAR7_35146905 that was strongly associated with flystrike resistance in sheep.

Sheep mount an immune response when challenged with flystrike. Neutrophils and eosinophils concentrate on the surface of the skin, where the strike has occurred, while CD4+, CD8+ and γδ T cells are found in the dermis (Bowles *et al*. 1992, Elhay, *et al*. 1994). In mice, it has been found that an over-expression of *RASGRP1* in thymocytes results in an increased number of CD8+ T cells (Norment, 2003). In contrast *RASGRP1* null (*RASGRP1*$^{-/-}$) mice have a block in thymocyte development and a diminished number of CD4+ and CD8+ T cells (Dower *et al*. 2000). Thymocytes from *RASGRP1*$^{-/-}$ mice lack TCR- and diacylycerol (DAG)-induced activation of RAS-ERK signalling (Dower *et al*. 2000) and young *RASGRP1*$^{-/-}$ mice display a defect in positive selection and diminished ERK phosphorylation in their thymocytes (Dower *et al*. 2000). It is therefore conceivable that variation in *RASGRP1*, such as has been described in this study, may be associated with variation in the expression or function of RASGRP1, and that this affects immune response. RASGRP1 may therefore potentially play a role in regulating T cell response to both the bacteria associated with

fleecerot (which predisposes sheep to flystrike) and to the invading larvae that result from being flystruck.

Mice deficient in *RASGRP1* have diminished T cell responses and delayed pathogen clearance, this response being functionally similar to the 'exhausted' memory T cell responses found during chronic infections (Priatel *et al*. 2007). The deletion of 200 amino acids of the C-terminal of *RASGRP1* in mice, has been revealed to diminish T cell development, although the effect is not as severe as in *RASGRP1* $^{-/-}$ mice (Fuller *et al*. 2012). In this context, exon 16 and 17 of the RASGRP1 gene were targeted for investigation in this chapter, as that region of the gene encodes most of the C-terminal tail domain of the protein.

In humans, Yasuda *et al*. (2007) found 13 new splice variants of *RASGRP1*, as a result of alternative splicing in exon 5 – 17. Nine of these splice variants resulted in the loss of amino acids from the C-terminal of RASGRP1 due to lack of exon 16 and 17, caused by a premature translation termination codon. The resulting protein isoforms were unresponsive to diacylycerol (DAG), which resulted in reduced levels of the protein. These isoforms were found in patients with systemic lupus erythematosus (SLE), an autoimmune disease (Yasuda *et al*. 2007). SLE patients carrying an alternatively spliced isoform had diminished levels of functional RASGRP1. In one particular SLE patient studied, the patient was unable to produce significant amounts of IL-2 when activated. This suggests that alternative splicing of *RASGRP1* may result in lower expression levels of RASGRP1, and this alters T cell function. The SNPs found in this study did not result in amino acid changes or alternative splice isoforms of RASGPR1 such as reported in Yasuda *et al.* (2007) study. However, these variations may result in change in expression levels of *RASGPR1* or efficiency of DAG binding to the C-terminal domain.

Other roles of RASGRP1 in sheep have not been explored. In this study the *RASGRP1 A* variant was associated with increased occurrence of flystrike, but this variant was also present at the highest frequency, suggesting that it might have another function or functions in sheep. Consequently, the selection of sheep that had a greater immunity to flystrike (selection for *A*) may have other implications, immunological or otherwise for sheep. As there is a strong correlation between fleecerot and flystrike, and dags and internal parasites, it could be possible that RASGRP1 may be influencing these diseases as well as flystrike. This would suggest that further investigation of ovine RASGRP1 function in sheep is justified.

The immune system is activated in response to invading larvae once sheep have been struck. Therefore, future research should look at the gene expression of *RASGRP1* in the skin of sheep with active flystrike. To investigate if the different variations in *RASGRP1* identified here alter the expression of RASGRP1 and thus alter the T cell response to flystrike. This could be done in a similar way to the study of Smith *et al*. (2010) study, where microarray analysis was used to profile gene expression changes before, during and after fleecerot infection. Ideally, sheep carrying the different

variations of *RASGRP1* seen here would be investigated, and their gene expression measured before, during and after flystrike infestation.

Variation in the ovine *RASGRP1* could potentially be exploited as a gene-marker for use in breeding programmes to reduce the incidence of flystrike, thereby improving animal welfare and decreasing the on-farm use of chemicals. This study only looked at exon 16 of *RASGRP1*, as it is the largest exon of *RASGRP1* and encodes most of the C-terminal tail domain. However, the ovine *RASGRP1* gene is 17 exons long. Future research could look for variation in the whole RASGRP1 gene in association with flystrike resilience.

Chapter 7. Ovine *ABCC11* variation and its association with flystrike susceptibility

In the Trangie lines of susceptible and resistant sheep, Smith *et al*. (2010) identified the ABCC11 gene (*ABCC11*, or *MRP8* as it was formerly known) as one of three lipid metabolism genes that could play a role in fleecerot resistance (the other two genes were *FABP4* and *FADS1*). *ABCC11* has a key role in the intracellular transport of lipids, cholesterol and fatty-acids (Toyoda and Ishikawa, 2010; Yabuuchi *et al*. 2001) and it encodes an ATP-driven efflux pump for amphipathic anions (Ota *et al*. 2010).

The ABC transporters are one of the largest protein families encoded by the human genome and they play a key role as membrane transporters, or ion channel modulators (Higgins, 1992; Ishikawa *et al*. 2013; Klein *et al*. 1999). The ABC superfamily is well characterised, and members have the ability to transport various exogenous and endogenous substances across membranes, and against concentration gradients using ATP hydrolysis (Chen and Tiwari, 2011). As such, they have a wide range of biological functions, including the membrane transport of drugs, endogenous substance or ions, lipids, bile salts, toxic compounds, and peptides for antigen presentation (Ishikawa *et al*. 2013; Toyoda and Ishikawa, 2010).

The protein family was designated ABC, based on its conserved ATP-binding cassette (Choudhuri and Klaassen, 2006). The minimum structural requirement for a functional ABC transporter is two transmembrane domains (TMD) and two ATP-binding cassette (ABC or NBD) units (Tusnády *et al.* 2006). The ABCC sub-family can be further separated into the subclasses, 'short' and 'long' based on their domain arrangement and membrane topology (Tusnády *et al*. 2006).

The ABC subclass C11 gene (*ABCC11*) is 68 kb in size and in sheep it consists of 29 exons, in contrast to the 30 exons observed in humans (Yamada *et al*. 2014). The *ABCC11* and *ABCC12* gene in humans are found together, with both located on human chromosome 16q12.1 in a tail to head orientation, and separated by 20 kb (Yabuuchi *et al*. 2001). They are very similar genes, with *ABCC11* consisting of 30 exons, 4203 bp of coding sequence and 1385 aa, while *ABCC12* consists of 29 exons a coding sequence of a 7486 bp and a 1367 aa protein. Interestingly, mice and rats do not have an orthologue corresponding to human *ABCC11*. This suggests that *ABCC11* is not an orthologous gene, but is in fact a paralogous gene generated by gene duplication in the human genome (Shimizu *et al*. 2003).

A SNP in human *ABCC11* has been identified as the cause for some individuals producing dry earwax, rather than wet earwax (Toyoda and Ishikawa, 2010). The same SNP in *ABCC11* also has a strong association with axillary (arm-pit) odour in humans (Martin *et al*. 2010). Axillary odour is caused by apocrine sweat gland secretions and bacteria on the skin surface (Emter and Natsch, 2008; Harker *et al*. 2014). In axillary osmidrosis, apocrine sweat glands in the axillary region secrete a variety of odour

precursors that are transformed into volatile odoriferous substances by bacterial enzymes on the skin surface (Natsch *et al.* 2003; 2006). The main contributors to axillary odour are (i) unsaturated or hydroxylated branched fatty-acids, (ii) sulphonyl-alkanols, (Hasegawa *et al*. 2004; Natsch *et al*. 2004; Troccaz *et al*. 2004), and (iii) the odoriferous steroids, which exhibit a urine- or musk-like smell (Bird and Gower, 1981).

The precursors of the odorant acids have been shown to be glutamine conjugates that are cleaved specifically by a bacterial Nα-acyl-glutamine-aminoacylase from *Corynebacterium striatum A*x20. The sulphonyl-alkanols are secreted from apocrine sweat glands as cysteine–(S) or cysteine–glycine–(S) conjugates (Emter and Natsch, 2008; Starkenmann *et al*. 2005) and are set free by the sequential action of a bacterial dipeptidase, tpdA, and a cysteine β-lyase, which have been cloned from the same bacterial strain (Emter and Natsch, 2008). *Corynebacterium* is a common bacteria species known to produce strong odours in association with apocrine gland secretion (Leyden *et al*. 1981; Emter and Natsch, 2008). Interestingly, *Corynebacterium* species were found to be the most abundant species present in the fleece of the susceptible line of Trangie sheep (Dixon *et al*. 2007). Indicating that it could have a similar role in the production of odour from apocrine gland secretion in both humans and sheep.

In sheep, all wool follicles have a sebaceous gland, while the apocrine sweat glands are only found associated with the primary follicles (Lyne and Hollis, 1968). Given that *ABCC11* has an important role in the production of ear wax in humans, it may have a similar role in the production of the wool wax which is important for waterproofing the fleece produced by the sebaceous glands in the skin.

Loss of lipids, and therefore changes in wool wax quality, from the fleece are thought to contribute to the development of fleecerot (Norris *et al.* 2008). Thus *ABCC11* may play a role in the development of fleece rot which is a predisposing factor to flystrike. Furthermore, the bacterial infection that is associated with the fleecerot attracts flies to lay their eggs on the affected area and newly hatched larvae feed on protein in exudates associated with bacterial dermatitis (Sandeman *et al*. 1987). *ABCC11* may also have a role in creating odour, which may attract flies to sheep, especially the species *L. cuprina* (Ashworth and Wall, 1994). Collectively, the evidence suggests that variation in *ABCC11* may be associated with variation in susceptibility to flystrike.

Materials and Methods

Animals

Blood samples were collected from 890 sheep collected during five strike seasons, 2013 to 2017. Sheep were from commercial and stud farms and were of various breeds. Samples from 74 sheep selected from different breeds, including Merino, Corriedale, Romney, Dorper, Poll Dorset, Texel, Dorset Down, Perendale, Suffolk and Coopworth to determine the frequency of Region 1 of *ABCC11* variants, along with 270 sheep from the flystrike research samples.

Of the amplified samples from the flystrike, 490 samples from 12 different breeds along with 18 samples from Dorper sheep were used to determine the frequency of *ABCC11* variants in Region 2.

Blood sample collection

Blood samples were collected using methods previously described in Chapters 3.

A series of blood samples were analysed from the Lincoln University Gene-Marker lab, of various breeds, including Merino, Corriedale, Perendale, Texel, Coopworth, Texel, Poll Dorset, Dorset Down, Suffolk, Shropshire, and Dorper in order to determine the variation and frequency of variants in *ABCC11* gene.

Primer design for ovine ABCC11 gene

Two sets of primers were designed for two regions of the *ABCC11*, based on the ovine *ABCC11* predictor sequence XM 012127121.1. *ABCC11*–up primer sequence 5'-AACTGGGATAGTTGG GTGAC-3' and *ABCC11*-dn primer sequence 5'-GTGGAGTCCTAACTACTGAC-3', for exon 5 of the ABCC11 gene, which corresponds to the functionally important region of human ABCC11 that causes variation in body odour and ear wax type. ABCC11-up primer sequence 5'-TCAGATGGCAAGTCTGC-3' ABCC11-dn primer sequence 5'-GAGGACCAGGACTGA-3', corresponding to part of intron 25, exon 26 and intron 26. This region, in part makes up the second ATP-binding site of the protein.

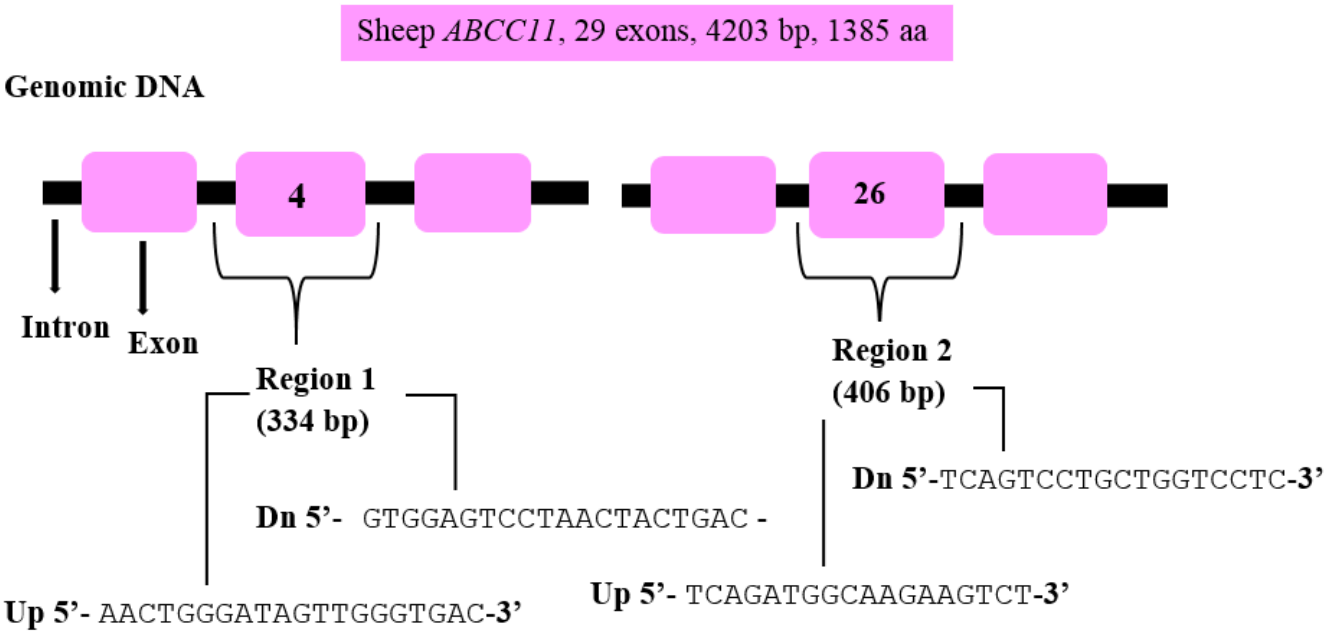


Figure 7.1 Primer location of *ABCC11* regions studied. Not drawn to scale; the ovine ABCC11 gene is 29 exons long.

Developing the PCR-SSCP protocols

DNA samples (n = 20) from several breeds were used to develop a PCR-SSCP protocol for both regions of ovine ABCC11. Different PCR protocols were tested, starting with annealing temperature of 58ºC, which was then optimised at 60ºC. Amplicons were visualized by electrophoresis in 1% agarose (Quantum Scientific, Queensland, Australia) gels, using 1 x TBE buffer (98mM Tris, 89mM boric acid, 2mM Na_2EDTA) and containing 200ng/ml of ethidium bromide. When the agarose gels produced a clear bright result, several samples were then run on 14% acrylamide gels at various temperatures to optimise clarity for identifying bands.

PCR-SSCP analysis and genotyping of ABCC11

PCR amplification was performed in a 15-μL reaction containing the genomic DNA on one 1.2-mm punch of FTA card, 0.25 μM of each primer, 150 μM dNTP's (Bioline, London, UK), 2.5 mM of Mg^{2+}, 0.5 U of Taq DNA polymerase (Qiagen, Hilden, Germany) and 1× the reaction buffer supplied with the enzyme. The thermal profile consisted of 2 min at 94 ºC, followed by 35 cycles of 30 s at 94 ºC, 30 s at 60 ºC and 30 s at 72 ºC, with a final extension of 5 min at 72 ºC. Amplification was carried out in S1000 thermal cyclers (Bio-Rad, Hercules, CA, USA).

A 0.7-μL aliquot of each amplicon was mixed with 7 μL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-col). After denaturation at 105 ºC for 5 min, samples were rapidly cooled on wet ice and then loaded on 16 cm × 18 cm, 14% acrylamide:bisacrylamide (37.5:1) (Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad), at 300 V for 19 h at 18 ºC in 0.5 × TBE buffer. Gels were silver-stained according to the method of (Byun *et al*. 2009a).

Sequencing of the ovine ABCC11 variant and sequence analysis

Homozygous sequence amplicons were identified for the two regions of ovine *ABCC11* by PCR-SSCP. Homozygous amplicons were then sequenced at the Lincoln University DNA Sequencing Facility. The computer program DNAMAN (version 5.2.10, Lynnon BioSoft, Canada) was used for sequence alignment, translations and comparisons. The Blast algorithm was used to search the NCBI GenBank database (http://blast.nci.nlm.nih.gov/) for homologous sequences.

Statistical analyses

All analyses were performed using SPSS version 24 (Chicago, IL, USA) as previously described in Chapter 3, Appendix A.3.

To determine which of these variables would be included in subsequent multivariate binary logistic regression models, univariate Pearson chi-square tests were performed to explore the association between the variables and the presence or absence of each of the gene region variants.

A Pearson chi-square test was undertaken to ascertain whether *ABCC11* Region 1 and Region 2 genotype was associated with the incidence of flystrike. No further analysis was performed for *ABCC11* Region 1 due to the lack of variation in this region.

For each gene variant, a Pearson chi-square test along with a binary logistic regression was performed to explore whether the presence or absence of the variant was associated with the presence or absence of flystrike and to determine the odds ratio, respectively. A multivariate binary logistic regression was then performed to determine the independent effects of gene variant on incidence of flystrike when year, breed, geographical location, age and gender were considered. If appropriate, an additional binary logistic regression model containing more than one of the gene variants was performed which included the variables from the previous models (year, breed, geographical location, and age_gender, along with any and gene variant from the previous univariate gene variant models with $P < 0.200$ (indicating a potential impact on the presence or absence of flystrike).

Results

Breed frequency for the two regions of *ABCC11* are shown in Table 7.1. The A_2 variant had the highest frequency, of 54.9%, while B_2 and D_2 had lower frequencies of 33.0% and 9.1% respectively. The C_2 and E_2 variants had very low frequencies, accounting for less than 3% of the total frequency.

Table 7.1 Breed frequency of ovine *ABCC11* exon 5.

		Region 1 Exon 4				**Region 2 Exon 26**				
Breed	***n***	***A_1A_1***	***A_1B_1***	***B_1B_1***	***n***	***A_2***	***B_2***	***C_2***	***D_2***	***E_2***
Merino	60	97.3	2.7	-	80	45.00	44.4	3.8	6.8	-
Corriedale	47	98.7	1.3	-	50	66.8	26.4	2.4	4.3	-
Coopworth	45	100	-	-	45	62.2	18.9	-	18.9	-
Romney	33	100	-	-	55	48.5	30.7	4.6	16.2	-
Perendale	2	100	-	-	47	55.2	24.0	5.2	15.6	-
Lincoln	7	100	-	-	7	50.0	28.6	-	21.4	-
Texel	13	100	-	-	13	88.5	11.5	-	-	-
South Suffolk	8	100	-	-	10	50.0	50.0	-	-	-
Suffolk	24	100	-	-	41	37.8	57.3	-	4.9	-
Shropshire	9	100	-	-	12	33.3	62.5	-	4.2	-
Crossbreed	49	98.3	1.7	-	100	45.10	40.05	4.05	10.75	0.05
Composite	20	100	-	-	20	57.5	27.5	-	15	-
Dorper	20	34.0	44.7	21.3	18	72.2	27.8	-	-	-
Total	*344*	*93.3*	*4.8*	*1.9*	*508*	*54.9*	*33.0*	*2.9*	*9.1*	*0.05*

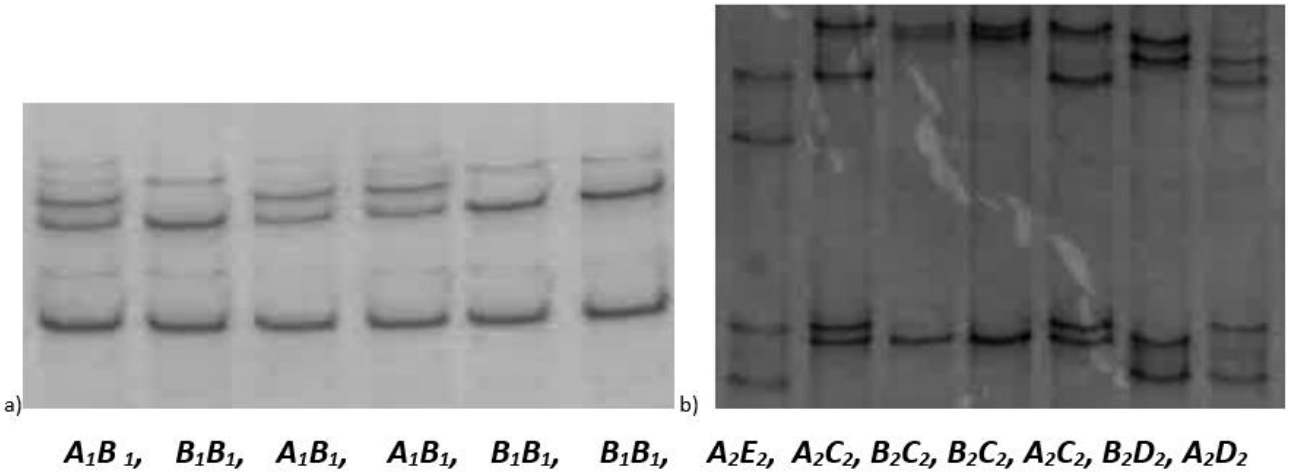


Figure 7.2 PCR-SSCP picture for ovine *ABCC11* (a) Region 1 corresponding to exon 5 and (b) Region 2 corresponding to exon 26.

The SNP positions found in the two regions of ABCC11 are shown in Table 7.2 and Table 7.3.

Table 7.2 Sequence variation in exon 4 - intron 5 of ovine *ABCC11*, Appendix E.1.

Position	*Variant*	
	A1	***B1***
c.1080	C	T
c.1215+12	g	a

Table 7.3 Sequence variation in intron 25- exon 26 of ovine *ABCC11*, Appendix E.1.

Position	*Variant*				
	A2	***B2***	***C2***	***D2***	***E2***
c.4459+3522	c	a	c	a	c
c.4459+3556	g	g	g	a	g
c.4459+3594	t	t	a	t	t
c.4459+3694	g	g	a	g	g
c.4459+3728	c	c	t	c	c
c.4459+3735	t	t	c	t	t
c.4402	T	C	T	C	C

The frequencies of *ABCC11* variants for sheep with flystrike is shown in Table 7.4.

Table 7.4 Frequencies (expressed as a percentage) of variants in *ABCC11* and the occurrence of flystrike

ABCC11	*n*	Frequency	Fly-struck	% struck
***A*$_2$**	743	50.6	406	52.5
***B*$_2$**	546	37.2	276	35.7
***C*$_2$**	44	3.0	24	3.0
***D*$_2$**	130	8.9	68	8.8
***E*$_2$**	5	0.3	-	
Total	*1,468*	*100*	*774*	

Univariate analysis

The results of the univariate analyses exploring variable associations with the presence or absence of each *ABCC11* Region 2 variant and flystrike are shown in Table 7.5. *ABCC11* Region 2 genotype showed no association with flystrike (P = 0.477).

Table 7.5 The univariate association of the presence of each *ABCC11* Region 2 variant with the occurrence of flystrike.

Gene variant	Odds ratio[1]	95% Confidence Interval[1]		P value[2]
		Upper	Lower	
A$_2$	1.106	0.793	1.542	0.552
B$_2$	0.803	0.593	1.082	0.149
C$_2$	0.989	0.530	1.846	0.972
D$_2$	0.000	0.000		0.978
E$_2$	0.000	0.000		0.999

[1]Derived from a binary logistic regression with presence or absence of flystrike as the dependent variable and presence or absence of the gene variant as the independent variable.
[2]Derived from both the Pearson chi-square and the binary logistic regression.

Multivariate analysis

The multivariate analyses confirm that there is no association between *ABCC11* Region 2 variants and flystrike, Table 7.6.

Table 7.6 The odds[1] of flystrike occurrence given the presence of a particular *ABCC11* Region 2 variant.

Gene variant	Odds ratio	95% Confidence Interval		P value
		Upper	Lower	
***A*$_2$**	0.934	0.655	1.333	0.707
***B*$_2$**	0.887	0.644	1.220	0.460
***C*$_2$**	0.979	0.514	1.865	0.948
***D*$_2$**	0.000	0.000		0.996
***E*$_2$**	0.000	0.000		0.999

[1] The odds ratios and confidence intervals were derived from a multivariate binary logistic regression in which the presence or absence of flystrike was the dependent variable and the independent variable were presence or absence of the gene variant, year, breed, geographical region, and the combined variable of age_gender.

Discussion

The aim of this study was to investigate variation in ovine *ABCC11* and determine if variation in the gene, if it existed, was associated with the occurrence of flystrike. In the exon 4 region of *ABCC11* investigated, two nucleotide sequence variants were detected and these were named A_1 and B_1. These two variants were a result of two linked SNPs, one occurring in exon 4 and the other in intron 5. No

amino acid change resulted as a consequence of the exon 4 SNP. The ABCC11 protein is a full transporter and contains two conserved intracellular ATP-binding cassette domains and 12 putative transmembrane domains (Kruh *et al*. 2007; Yamada *et al*. 2014). Exon 4 is within the transmembrane domain. With the previous variation (538 G > A) located at a position where the change in residue would be expected to disturb the conformation of the proteins (Kruh *et al*. 2007). Although the SNP report in this study did not result in an amino acid change, they could still affect mRNA stability and secondary structure (Duan *et al*. 2003).

There was more variation seen in the second region of *ABCC11* investigated. This region displayed five variants (named A_2 – E_2), and these resulted from seven SNPs, six of which occurred in intron 25, with only one SNP being detected in exon 26. The SNP detected in exon 26 would give rise to an amino acid change, from arginine to cysteine, if expressed. This region of ABCC11 is part of the intracellular ATP-binding cassette domain (Ishikawa *et al*. 2013). While the non-synonymous SNP found in exon 26 of this study was not associated with flystrike, it may have a role in regulating the expression of *ABCC11*. A deletion of exon 28 of the human ABCC11 (that also makes up the second ATP-binding cassette domain) has the potential to reduce its expression (Yabuuchi *et al*. 2001).

Exon 4 was chosen for investigation because in humans the 538 G > A SNP results in an amino acid change glycine to arginine and influencing both ear wax type and odour (Martin *et al*. 2010; Toyoda and Ishikawa, 2010). There is 80% sequence similarity in exon 4 of *ABCC11* in humans and sheep. However the SNP in human exon 4 538 G>A was not found in sheep. All sheep investigated carried the corresponding G nucleotide at this location, which causes human carriers to have wet yellow earwax (Martin *et al*. 2010) and strong axillary odour (Toyoda and Ishikawa, 2010). If *ABCC11* has a similar biological function in sheep as it does in humans, this result could indicate that there is no difference in sheep odour and wax from *ABCC11*. Perhaps with further investigation sheep with a G>A change could be identified.

In this study, the Dorper breed had the highest frequency of the typically rarer B_1 variant, and the only B_1B_1 genotype found was in the Dorper sheep. This could indicate that *ABCC11* has a role in fleece production as Dorpers are shedding sheep and are considered to be a non-wool breed (Cloete *et al*. 2000), with high fibre diameter, and with a high proportion of kemp fibres in their fleece. As ABCC11 has been shown to be down-regulated in super fine wool sheep (Yue *et al*. 2015), perhaps it is up-regulated in strong wool breeds. However there is no study to show this, more research is required to determine if ABCC11 has any role in wool production in sheep.

Smith *et al*. (2010) selected ABCC11 for investigation in their study based off their DE results and because of its involvement in lipid metabolism and odour from apocrine gland secretion. Three SNPs in ovine *ABCC11*, (ABCIn0150 and ABCIn0270 in intron 26 and ABCex0667 in intron 11), but these SNPs did not have a significant association with fleecerot resistance. Similarily, neither region of *ABCC11* investigated had a significant association with flystrike susceptibility in the sheep studied.

There are 29 exons in the ovine ABCC11 gene, and only two were investigated in this study. It is possible that another region of *ABCC11* might give rise to an association with flystrike if the gene was more comprehensively studied. Further investigation of ovine *ABCC11* is required to determine if it has a similar function in sheep as it does in humans. If *ABCC11* effects body odour in sheep, it could be possible to test sheep on the variation in *ABCC11* and see if this gene is associated with flystrike susceptibility in sheep.

Chapter 8. Summary

In NZ, , flystrike is a growing problem for farmers, and especially with the spread of the Australian blowfly, *L. cuprina,* throughout the country. Existing management strategies for flystrike, which include the use of insecticides and mulesing, are often viewed negatively by consumers who want less chemical residue in wool (Levot, 1995), and better animal welfare standards.

Given the cost and issues around flystrike management, selecting sheep that are genetically less susceptible to being struck, may provide a more sustainable, and more socially acceptable method of managing this disease. As a consequence, the identification of genes that are associated with flystrike susceptibility, and that can be used as gene-markers for breeding, may allow more effective and better management of the problem.

There have been very few studies that have identified genes or gene activity that may contribute to the susceptibility of sheep to flystrike. This study investigated fives genes that were potentially associated with how sheep may respond or resist flystrike. The genes *FABP4* and *FBLN1* had been associated with fleecerot resistance in Merinos (Smith *et al*. 2010), and an association with susceptibility to flystrike was confirmed with the experiments conducted in this thesis.

This suggests both genes are worthy of further investigation. This might involve determining where and when these genes are being expressed in the epidermal tissues, and how their expression may affect skin, fleece, or wax/suint characteristics, in a way that contributes or alters flystrike susceptibility. Functional studies of this kind can be difficult to undertake, but they provide better evidence of the involvement of genes in determining phenotype.

Alternatively, and based on the knowledge derived from this thesis that particular variants of *FABP4* and *FBLN1* are associated with susceptibility to flystrike, one could set out to breed sheep that were carrying variants that were associated with either increased or decreased susceptibility. These sheep could be exposed to flystrike, and the outcome recorded. It might be expected that sheep carrying variants of the genes that are associated with decreased susceptibility, would have a lower flystrike incidence, providing evidence that improved sheep could be selectively breed for. This approach would take us closer to understanding whether a breeding solution to flystrike would be useful, but without giving us the improved understanding of how the FABP4 and FBLN1 genes are involved that the functional studies would enable. Ideally, both approaches would be used together.

Sheep have a complex immune response to flystrike and a combination of genes are likely to be involved in this process. It would seem more likely that the key genes contribute to pre-disposing conditions to flystrike, rather than directly to flystrike itself. This study looked at two genes involved in the immune system, *TLR4* and *RASGRP1*. No association between flystrike and *TLR4* variation was detected, but *RASGRP1* variation was found to be associated with flystrike susceptibility.

The majority of sheep in this study had breech strike, and there is a strong correlation between the occurrence of dags and breech strike (Leathwick and Atkinson, 1995), and high internal parasite burden and dag formation (Waghorn *et al*. 1999). Immune system genes may therefore be involved in response to internal parasites and have the secondary effect of affecting flystrike susceptibility. Further research is required to understand the roles *RASGRP1* has in the immune system of sheep and how sheep immune system genes are involved in responding to the disease. This may once again involve functional studies, and thus investigating *RASGRP1* expression in sheep with and without the disease. It might alos be useful to investigate whether *RASGRP1* variation has any association with variation in internal parasites resilience and dag score.

This study found evidence that gene variants *FABP4* A_1, *FBLN1* B_1 and *RASGRP1 C* have having a role in flystrike resilience. These findings could be used to develop a gene-marker test for breeding for less susceptibility to flystrike. However, before a gene-marker test could be developed, the associations identified in this study would need to be confirmed in a larger study or studies. This could be done by investigating several large research flocks around NZ, and following lambs from birth for several years to determine if they get flystrike throughout their lives, are struck just once or never struck. In such a study, every sheep would need to be genotyped for *FABP4* and *FBLN1* and the variant frequency measured against sheep with flystrike.

Furthermore, any genetic variation associated with flystrike would also need to be investigated as to its impact on other important sheep production traits. If the genes have various production effects such as have been described for *FABP4* (Yan *et al*, 2012; Bakhtiarizadeh *et al*. 2013), farmers will need to consider carefully what they are wanting to select for, as breeding for flystrike resilience in sheep could result in a fatter carcass, which might not suit current consumer demands.

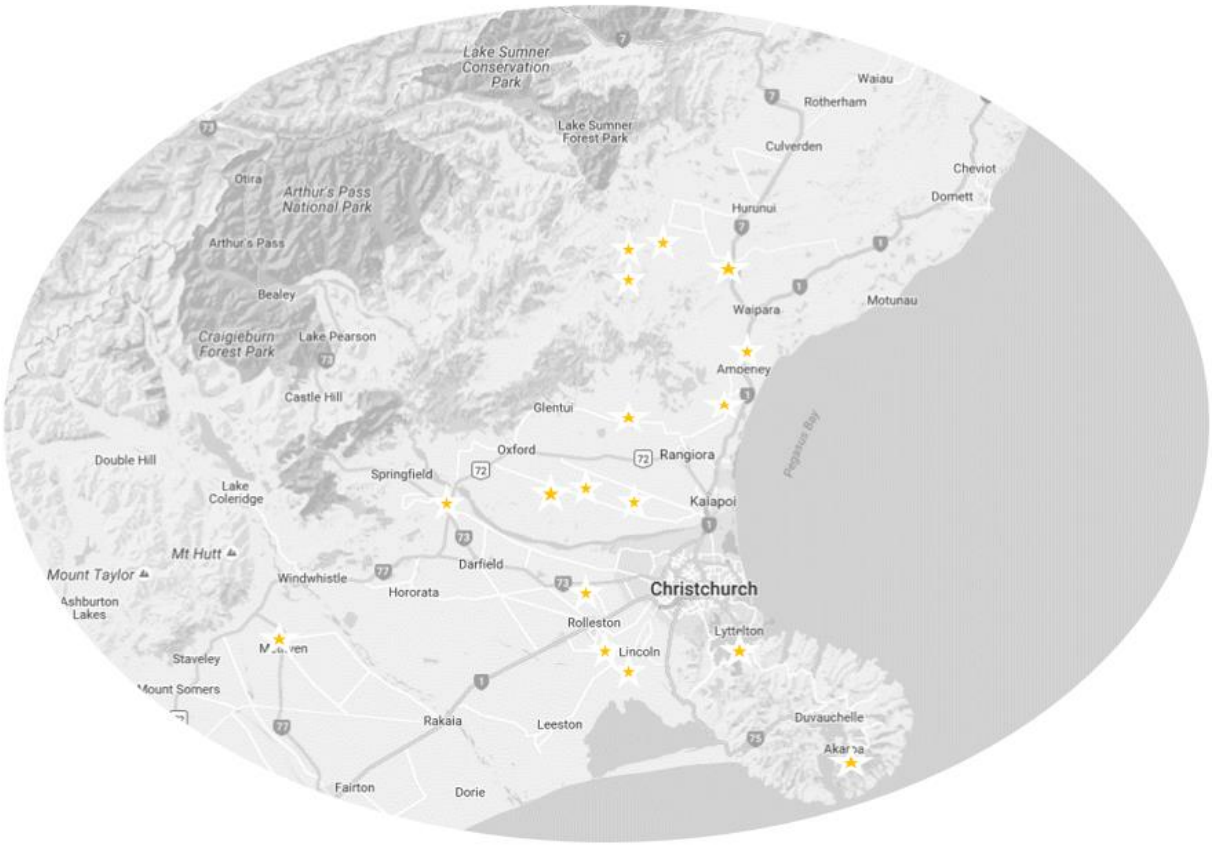
Another study that would be of great interest would be to genotype the Trangie resistant and susceptible line of sheep for the variants of *FABP4* and *FBLN1* described in this thesis, and to obverse the frequency of the gene variants in the two lines of sheep. This would require calibration with Trangie and the NSW Department of Primary Industries. This might be of benefit to both the NZ and Australian sheep industries, as a genetic approach to reduce the impact of flystrike should be achievable in both countries.

The occurrence of flystrike is environmentally dependant, and accordingly it is difficult to make genetic gain by direct selection under normal farming conditions. Accurately identifying sheep that are genetically resistant to flystrike, and selectively breeding for this trait, would be required. While a breeding approach to reduce flystrike susceptibility is undeniably desirable, getting accurate predictive measurements will require a great deal more research. Flystrike might never be completely eradicated, but the potential for a gene test, along with improved management practices, could reduce the prevalence of disease.

Appendix A

A.1 Map of the area where samples were taken from.

The farms in the Canterbury region where blood samples were collected.



A.2 Sequences of the *FABP4* fragments amplified

Exon 2:
GTGTGGGCTTTGCTACCAGGAAAGTGGCTGGCATGGCCAAACCCACTGTGATCATCAGTGTAAATGGG
GATGTGGTCAACATTAAATCAGAAAGCACCTTTAAAAATACTGAGATGTCCTTCAAATTGGGCCAGGA
ATTTGATGAAGTCACTCCAGATGACAGGAAAGTCAAG

Intron 2-3:
Gtgaggaataaagaactggagcagagtaaaagcctg[g/a]tttataaa[c/t][g/a]actgctgcc
tatatatagcaagccattttgtagaaggaggaaagccattccattataagccaaaaagctcagattgc
tagctctgaaccatgttactgttgatatttagttggtgaattgtctcccatttaataaaattgtcctt
attactttaaaaatgtttaacataataatttgtcataccatatatgtgtgtgtgtgtgtatatatata
tatatactatttaaagtaaattgaagtaacataacaatgttagagaacttttaaaagagtgggggaa
agaaaaaaaaaacacctatgatgctattccacataaatttattatgtatattctttcac[a/g]gtat
ttttcaaatgcatgtttgtataatattctgatcataatatacatgtaattttgcatattgtttttgg
cattcattgttttctttttcaacattttcttgtaatttagaattgctaagtacctcaaaataagcaaa
taaaagtgctctatttttttccccctccatgattgtaatcacttttaattatccccacag

Exon 3:
AGCATCATAAACTTAGATGAAGGTGCTCTGGTACAAGTACAAAACTGGGATGGGAAATCAACCACCAT
AAAGAGAAAACTTGTGGATGATAAGCTGGTGCTG

Intron 3-4:

Gtgagtatcttctgactacttcactctagattttagtgctaggtcatcccataatcgttatcctacct
agagaaatagacaatcgcccttgtagaatgaaaagttagtctattgggattatggtttcactctggca
attatccttctaagctctgtctatgtata[t/c]tgtgccccaggaagtattttcttatccctctcaa
tgtgaaccatattgtattgtgcatttctaattatgtttttcattcaccacatagattgtaagattcct
tgagggcaagacttgtatcttcttgatct[c/t]tgtgtctccctagtttattacaatatcaggtata
taagaagagccaagagtgaatat[c/t]tgattccttttgatgaacatttttccttctcagcattg
aaggagacaataaataaataaaccatgagttgtttagtcctgaggtttttaccaatattttgcctttg
tgcctag

Exon 4:
GAATGTGTCATGAACGGTGTCACTGCTACCAGAGTTTACGAGAGAGCATAA

Intron 4-5:
gccaagggatattgaaatggatgacgtttgcatcgaactccatgactttc.........

The red letters represent the Smith *et al*. (2010) primer coverage, the dark blue letters represent the primer coverage for Region 1 and Region 3, and the light blue letters represent the primer coverage for Region 3. The blue underlined letters represent the SNPs found in this study.

A.3 The prevalence of flystrike

	Flystrike (n)	Without flystrike (n)	Mob size	Prevalence
2013				
Charlie Croft	18	14	420	4.3%
Diamond Harbour	5	6	400	1.3%
Greg Lessen	6	6	360	1.7%
John McLaughlin	10	8	320	3.1%
John Thomas	18	16	550	3.3%
John Wiggly	19	18	450	4.2%
Kerry Prenter	11	12	650	1.7%
Malcom Menzie	5	10	150	3.3%
Okuku Pass	32	4	350	9.1%
Tom Burrows (Romney)	8	7	125	6.4%
Tom Burrows (Corriedale)	4	5	80	5.0%
Weka Pass	6	5	550	1.1%
John Jebson	1	1	Unknown	
Mike Smith	8	8	Unknown	
Rakahui	4	3	Unknown	
Rob James	12	15	Unknown	
Total	***168 -25 =143***	***138***	***4,405***	***3.2%***
2014				
Alistair Lawerance	12	12	200	6.0%
Diamond Harbour	1	3	450	0.2%
Angus Johnson	13	13	550	2.4%
Rowan Nesbit	6	5	150	4.0%
Kerry Prenter	12	6	750	1.6%
Greg Lessen	8	15	120	6.7%
Christen Beaten	5	2	40	1.3%
James Thomas	11	9	600	1.8%
Daniel Wheeler	2	2	50	4.0%
Eric Ross	1	1	100	1.0%
Tom Burrows (Shropshire)	4	12	90	4.4%
Fliss Gardiner	4	4	100	4.0%
Penny L	1	14	15	6.7%
Doc Sidey	7	7	Unknown	
Digger	1	1	Unknown	
Total	***88 -8 =80***	***106***	***3,245***	***2.5%***
2015				
Helen Heddell	7	7	125	5.6%
Fliss Gardiner	2	2	100	2.0%
Reuben Carter	9	9	80	11.25%
Tom Burrows (Dorset Down)	2	2	110	1.8%
Tom Burrows (Corriedale)	2	3	80	2.5%
Tom Burrows (Shropshire)	2	1	90	2.2%
Paul Gardiner	2	8	220	0.1%
Ashley Dene	8	8	300	2.7%
Bob Masefield	6	0	Unknown	
Lincoln University	35	22	Unknown	
Total	***75 -41 =34***	***56***	***1275***	***2.7%***
2016				
Dave Clarke	2	2	120	1.7%
Chris Hampton (Suffolk)	4	4	60	6.7%
Chris Hampton (South Suffolk)	4	4	80	4.8%
Tom Burrows (Shropshire)	3	3	90	3.3%
Tom Burrows (Corriedale)	7	6	80	7.5%
Tom Burrows (Dorset Down)	3	3	120	2.5%
Penni L	4	4	450	1%
Helen Heddell	24	21	125	18%
Neville Moorhead	18	14	165	11%
Roscoe Taggot	4	0	200	2%
Dunedin	9	4	200	1.6%
Ike Williams (Romney)	5	4	500	1.0%
Ike Williams (Lincoln)	3	6	95	3.2%
Doc Sidey	4	2	75	5.3%
Ashley Dene	5	4	320	1.6%
Total	***99***	***81***	***2,680***	***3.7%***
2017				
Chris Hampton (Suffolk)	4	4	60	6.5%
Helen Heddell	3	3	50	6.0%
Ike Williams (Romney)	9	9	500	1.8%
Hugh Taylor	2	2	450	0.4%
Tom Burrows (Shropshire)	5	4	95	5.0%
Tom Burrow (Corriedale)	6	6	80	7.5%
Tom Burrows (Perriedale)	6	6	120	3.0%
Penni Loffigan (Merino)	3	3	30	10.0%
Total	***40***	***39***	***1,385***	***2.9%***

Summary of the prevalence of flystrike in various breeds over the five-year study period.

Breed[a]	Number with flystrike	Total number investigated	Prevalence of flystrike
Merino	43	880	4.9%
Corriedale	110	2,240	4.9%
Perendale	54	1,150	4.7%
Romney	31	1,730	1.8%
Dorset Down	7	357	1.9%
Shropshire	14	365	3.8%
Suffolk	17	682	2.5%
Crossbred	72	3,078	2.3%
Crossbred (Black)	41	2,120	1.9%
Composite	9	209	4.3%
	398	12,811	3.2%

[a] Prevalence of the larger breed groups studied, excuse sheep where the mob size was unknown.

A.4 Re-coding of variables

Age_gender * age * gender cross tabulation

gender			age 1	2	3	4	Total
Ewe	Age_gender	Ewe2	0	157	0	0	157
		Ewe3	0	0	190	72	262
		Lamb	37	0	0	0	37
	Total		37	157	190	72	456
Ram	Age_gender	Lamb	11	0	0	0	11
		Ram2	0	32	0	0	32
		Ram3	0	0	21	8	29
	Total		11	32	21	8	72
Unknown	Age_gender	Lamb	268				268
	Total		268				268
Total	Age_gender	Ewe2	0	157	0	0	157
		Ewe3	0	0	190	72	262
		Lamb	316	0	0	0	316
		Ram2	0	32	0	0	32
		Ram3	0	0	21	8	29
	Total		316	189	211	80	796

Region * Region Cde cross tabulation

		Region_cde Mid Canterbury	North Canterbury	Otago	Total
Region	Banks Peninsula	0	20	0	20
	Mid Canterbury	295	0	0	295
	North Canterbury	0	183	0	183
	Northland	0	21	0	21
	Otago	0	0	17	17
	South Canterbury	0	0	308	308
Total		295	224	325	844

Breed Cde * Breed cross tabulation

		BreedNew Black_Face_Sheep	MerinoX	CrossBred	PureBred	Total
Breed Cde	Composite	0	0	21	0	21
	Coopworth	0	0	33	0	33
	Corriedale	0	224	0	0	224
	Corriedale X	0	0	33	0	33
	Crossbreed	0	0	79	0	79
	Crossbreed black	49	0	0	0	49
	Dorset Down	29	0	0	0	29
	Finn Texel	4	0	0	0	4
	Lincoln	0	0	0	7	7
	Merino	0	70	0	0	70
	Merino X	0	9	0	0	9
	Perendale	0	0	0	66	66
	Perendale X	0	0	16	0	16
	Poll Dorset	15	0	0	0	15
	Romney	0	0	0	59	59
	Romney X	0	0	54	0	54
	Shropshire	25	0	0	0	25
	South Down	1	0	0	0	1
	South Suffolk	7	0	0	0	7
	Suffolk	39	0	0	0	39
	Texel	4	0	0	0	4
Total		173	303	236	132	844

A.5 Pearson chi-squared analyses to determine variables for the Binary Logistic Regression Models

Region_cde * fly-strike

Crosstab

			fly-strike		
			0	1	Total
Region_cde	Mid Canterbury	Count	138	157	295
		% within Region_cde	46.8%	53.2%	100.0%
	North Canterbury	Count	114	110	224
		% within Region_cde	50.9%	49.1%	100.0%
	Otago	Count	151	174	325
		% within Region_cde	46.5%	53.5%	100.0%
Total		Count	403	441	844
		% within Region_cde	47.7%	52.3%	100.0%

Chi-square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson chi-square	1.214[a]	2	0.545
Likelihood Ratio	1.214	2	0.545
N of Valid Cases	844		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 106.96.

Breed New * fly-strike

Crosstab

			fly-strike		
			0	1	Total
Breed New	BF Sheep	Count	95	78	173
		% within Breed New	54.9%	45.1%	100.0%
	CrossBred	Count	110	145	255
		% within Breed New	43.1%	56.9%	100.0%
	Merino X	Count	142	142	284
		% within Breed New	50.0%	50.0%	100.0%
	PureBred	Count	56	76	132
		% within Breed New	42.4%	57.6%	100.0%
Total		Count	403	441	844
		% within Breed New	47.7%	52.3%	100.0%

Chi-square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson chi-square	7.810[a]	3	0.050
Likelihood Ratio	7.823	3	0.050
N of Valid Cases	844		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 63.03.

Age_Gender * fly-strike

Crosstab

			fly-strike		
			0	1	Total
Age_Gender	Ewe2	Count	66	94	160
		% within Age_Gender	41.3%	58.8%	100.0%
	Ewe3	Count	140	146	286
		% within Age_Gender	49.0%	51.0%	100.0%
	Lamb	Count	143	173	316
		% within Age_Gender	45.3%	54.7%	100.0%
	Ram2	Count	28	4	32
		% within Age_Gender	87.5%	12.5%	100.0%
	Ram3	Count	16	13	29
		% within Age_Gender	55.2%	44.8%	100.0%
Total		Count	393	430	823
		% within Age_Gender	47.8%	52.2%	100.0%

Chi-square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson chi-square	24.571[a]	4	0.000
Likelihood Ratio	26.799	4	0.000
N of Valid Cases	823		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 13.85.

Year * fly-strike

Crosstab

			fly-strike		
			0	1	Total
Year	2013	Count	125	146	271
		% within Year	46.1%	53.9%	100.0%
	2014	Count	105	83	188
		% within Year	55.9%	44.1%	100.0%

	2015	Count	56	74	130
		% within Year	43.1%	56.9%	100.0%
	2016	Count	80	99	179
		% within Year	44.7%	55.3%	100.0%
	2017	Count	37	39	76
		% within Year	48.7%	51.3%	100.0%
Total		Count	403	441	844
		% within Year	47.7%	52.3%	100.0%

Chi-square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson chi-square	7.067[a]	4	0.132
Likelihood Ratio	7.071	4	0.132
Linear-by-Linear Association	0.281	1	0.596
N of Valid Cases	844		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 36.29.

A.6 Pearson chi-square analyses for *FABP4* Region 1

***FABP4* * fly-strike**

Crosstab

			fly-strike 0	fly-strike 1	Total
FABP4	aa	Count	19	11	30
		% within FABP4	63.3%	36.7%	100.0%
	ab	Count	75	58	133
		% within FABP4	56.4%	43.6%	100.0%
	ac	Count	68	69	137
		% within FABP4	49.6%	50.4%	100.0%
	ad	Count	9	5	14
		% within FABP4	64.3%	35.7%	100.0%
	bb	Count	47	57	104
		% within FABP4	45.2%	54.8%	100.0%
	bc	Count	90	121	211
		% within FABP4	42.7%	57.3%	100.0%
	bd	Count	14	21	35
		% within FABP4	40.0%	60.0%	100.0%
	cc	Count	58	77	135
		% within FABP4	43.0%	57.0%	100.0%
	cd	Count	21	17	38
		% within FABP4	55.3%	44.7%	100.0%
	dd	Count	2	5	7
		% within FABP4	28.6%	71.4%	100.0%
Total		Count	403	441	844
		% within FABP4	47.7%	52.3%	100.0%

Chi-square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson chi-square	15.073[a]	9	0.089
Likelihood Ratio	15.170	9	0.086
N of Valid Cases	844		

a. 2 cells (10.0%) have expected count less than 5. The minimum expected count is 3.34.

FABP4 A1 * fly-strike

Crosstab

			fly-strike		
			0	1	Total
FABP4 A1	N	Count	232	298	530
		% within FABP4 A1	43.8%	56.2%	100.0%
	Y	Count	171	143	314
		% within FABP4 A1	54.5%	45.5%	100.0%
Total		Count	403	441	844
		% within FABP4 A1	47.7%	52.3%	100.0%

Chi-square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson chi-square	9.023[a]	1	0.003		
Continuity Correction[b]	8.600	1	0.003		
Likelihood Ratio	9.029	1	0.003		
Fisher's Exact Test				0.003	0.002
N of Valid Cases	844				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 149.93.

b. Computed only for a 2x2 table

FABP4 B1 * fly-strike

Crosstab

			fly-strike		
			0	1	Total
FABP4 B1	N	Count	177	184	361
		% within FABP4 B1	49.0%	51.0%	100.0%
	Y	Count	226	257	483
		% within FABP4 B1	46.8%	53.2%	100.0%
Total		Count	403	441	844
		% within FABP4 B1	47.7%	52.3%	100.0%

Chi-square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson chi-square	.415[a]	1	0.519		
Continuity Correction[b]	0.330	1	0.565		
Likelihood Ratio	0.415	1	0.519		
Fisher's Exact Test				0.531	0.283
N of Valid Cases	844				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 172.37.

b. Computed only for a 2x2 table

FABP4 C1 * fly-strike

Crosstab

			fly-strike		
			0	1	Total
FABP4 C1	N	Count	166	157	323
		% within FABP4 C1	51.4%	48.6%	100.0%
	Y	Count	237	284	521
		% within FABP4 C1	45.5%	54.5%	100.0%
Total		Count	403	441	844
		% within FABP4 C1	47.7%	52.3%	100.0%

Chi-square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-square	2.785[a]	1	0.095		
Continuity Correction[b]	2.554	1	0.110		
Likelihood Ratio	2.785	1	0.095		
Fisher's Exact Test				0.103	0.055
N of Valid Cases	844				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 154.23.

b. Computed only for a 2x2 table

FABP4 D1 * fly-strike

Crosstab

			fly-strike		
			0	1	Total
FABP4 D1	N	Count	357	393	750
		% within FABP4 D1	47.6%	52.4%	100.0%
	Y	Count	46	48	94
		% within FABP4 D1	48.9%	51.1%	100.0%
Total		Count	403	441	844
		% within FABP4 D1	47.7%	52.3%	100.0%

Chi-square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-square	.060[a]	1	0.807		
Continuity Correction[b]	0.018	1	0.893		
Likelihood Ratio	0.060	1	0.807		
Fisher's Exact Test				0.827	0.446
N of Valid Cases	844				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 44.88.

b. Computed only for a 2x2 table

A.7 Logistic Regression for *FABP4* Region 1

Case Processing Summary

Unweighted Cases[a]		N	Percent
Selected Cases	Included in Analysis	844	100.0

	Missing Cases	0	0.0
	Total	844	100.0
Unselected Cases		0	0.0
Total		844	100.0

a. If weight is in effect, see classification table for the total number of cases.

Dependent Variable Encoding

Original Value	Internal Value
0	0
1	1

Categorical Variables Coding

		Frequency	Parameter coding (1)
FABP4 A1	N	530	0.000
	Y	314	1.000

Block 1: Method = Enter

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	Upper
Step 1[a]	FABP4 A1(1)	-0.429	0.143	8.982	1	0.003	0.651	0.492	0.862
	Constant	0.250	0.088	8.176	1	0.004	1.284		

a. Variable(s) entered on step 1: FABP4 A1.

Logistic Regression

Case Processing Summary

Unweighted Cases[a]		N	Percent
Selected Cases	Included in Analysis	844	100.0
	Missing Cases	0	0.0
	Total	844	100.0
Unselected Cases		0	0.0
Total		844	100.0

a. If weight is in effect, see classification table for the total number of cases.

Dependent Variable Encoding

Original Value	Internal Value
0	0
1	1

Categorical Variables Coding

		Frequency	Parameter coding (1)
FABP4 B1	N	361	0.000
	Y	483	1.000

Block 1: Method = Enter

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	Upper
Step 1[a]	FABP4 B1(1)	0.090	0.139	0.415	1	0.519	1.094	0.833	1.437
	Constant	0.039	0.105	0.136	1	0.713	1.040		

a. Variable(s) entered on step 1: FABP4 B1.

Logistic Regression

Case Processing Summary

Unweighted Cases[a]		N	Percent
Selected Cases	Included in Analysis	844	100.0
	Missing Cases	0	0.0
	Total	844	100.0
Unselected Cases		0	0.0
Total		844	100.0

a. If weight is in effect, see classification table for the total number of cases.

Dependent Variable Encoding

Original Value	Internal Value
0	0
1	1

Categorical Variables Coding

		Frequency	Parameter coding (1)
FABP4 C1	N	323	0.000
	Y	521	1.000

Block 1: Method = Enter

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	Upper
Step 1[a]	FABP4 C1(1)	0.237	0.142	2.782	1	0.095	1.267	0.959	1.673
	Constant	-0.056	0.111	0.251	1	0.617	0.946		

a. Variable(s) entered on step 1: FABP4 C1.

Logistic Regression

Case Processing Summary

Unweighted Cases[a]		N	Percent
Selected Cases	Included in Analysis	844	100.0
	Missing Cases	0	0.0
	Total	844	100.0
Unselected Cases		0	0.0
Total		844	100.0

a. If weight is in effect, see classification table for the total number of cases.

Dependent Variable Encoding

Original Value	Internal Value
0	0
1	1

Categorical Variables Coding

		Frequency	Parameter coding (1)
FABP4 D1	N	750	0.000
	Y	94	1.000

Block 1: Method = Enter

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	Upper
Step 1[a]	FABP4 D1(1)	-0.054	0.219	0.060	1	0.807	0.948	0.617	1.456
	Constant	0.096	0.073	1.727	1	0.189	1.101		

a. Variable(s) entered on step 1: FABP4 D1.

Logistic Regression

Case Processing Summary

Unweighted Cases[a]		N	Percent
Selected Cases	Included in Analysis	823	97.5
	Missing Cases	21	2.5
	Total	844	100.0
Unselected Cases		0	0.0
Total		844	100.0

a. If weight is in effect, see classification table for the total number of cases.

Dependent Variable Encoding

Original Value	Internal Value
0	0
1	1

Categorical Variables Coding

			Parameter coding			
		Frequency	(1)	(2)	(3)	(4)
Age_Gender	Ewe2	160	1.000	0.000	0.000	0.000
	Ewe3	286	0.000	1.000	0.000	0.000
	Lamb	316	0.000	0.000	1.000	0.000
	Ram2	32	0.000	0.000	0.000	1.000
	Ram3	29	0.000	0.000	0.000	0.000
Year	2013	271	1.000	0.000	0.000	0.000
	2014	182	0.000	1.000	0.000	0.000
	2015	122	0.000	0.000	1.000	0.000
	2016	172	0.000	0.000	0.000	1.000
	2017	76	0.000	0.000	0.000	0.000

Breed New	BF Sheep	156	1.000	0.000	0.000
	Crossbred	255	0.000	1.000	0.000
	Merino X	284	0.000	0.000	1.000
	PureBred	128	0.000	0.000	0.000
Region_cde	Mid Cant	295	1.000	0.000	
	North Ca	214	0.000	1.000	
	Otago	314	0.000	0.000	
FABP4 A1	N	516	0.000		
	Y	307	1.000		

Block 1: Method = Enter

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	Upper
Step 1[a]	FABP4 A1(1)	-0.373	0.151	6.059	1	0.014	0.689	0.512	0.927
	Year			4.925	4	0.295			
	Year(1)	0.078	0.280	0.077	1	0.782	1.081	0.624	1.871
	Year(2)	0.012	0.303	0.001	1	0.970	1.012	0.559	1.830
	Year(3)	0.077	0.316	0.060	1	0.807	1.080	0.581	2.007
	Year(4)	0.473	0.296	2.562	1	0.109	1.605	0.899	2.865
	Breed New			4.728	3	0.193			
	Breed New(1)	-0.460	0.266	2.994	1	0.084	0.632	0.375	1.063
	Breed New(2)	-0.001	0.248	0.000	1	0.997	0.999	0.615	1.624
	Breed New(3)	-0.311	0.245	1.616	1	0.204	0.733	0.453	1.184
	Region_cde			1.425	2	0.490			
	Region_cde(1)	0.219	0.199	1.216	1	0.270	1.245	0.843	1.838
	Region_cde(2)	0.021	0.208	0.010	1	0.920	1.021	0.680	1.534
	Age_Gender			#####	4	0.005			
	Age_Gender(1)	0.263	0.441	0.357	1	0.550	1.301	0.548	3.088
	Age_Gender(2)	0.171	0.409	0.175	1	0.675	1.187	0.532	2.649
	Age_Gender(3)	0.162	0.423	0.147	1	0.701	1.176	0.514	2.693
	Age_Gender(4)	-1.950	0.671	8.445	1	0.004	0.142	0.038	0.530
	Constant	0.089	0.500	0.032	1	0.858	1.093		

a. Variable(s) entered on step 1: FABP4 A1, Year, Breed New, Region_cde, Age_Gender.

Logistic Regression

Case Processing Summary

Unweighted Cases[a]		N	Percent
Selected Cases	Included in Analysis	823	97.5
	Missing Cases	21	2.5
	Total	844	100.0
Unselected Cases		0	0.0
Total		844	100.0

a. If weight is in effect, see classification table for the total number of cases.

Dependent Variable Encoding

Original Value	Internal Value
0	0
1	1

Categorical Variables Coding

			Parameter coding			
		Frequency	(1)	(2)	(3)	(4)
Age_Gender	Ewe2	160	1.000	0.000	0.000	0.000
	Ewe3	286	0.000	1.000	0.000	0.000
	Lamb	316	0.000	0.000	1.000	0.000
	Ram2	32	0.000	0.000	0.000	1.000
	Ram3	29	0.000	0.000	0.000	0.000
Year	2013	271	1.000	0.000	0.000	0.000
	2014	182	0.000	1.000	0.000	0.000
	2015	122	0.000	0.000	1.000	0.000
	2016	172	0.000	0.000	0.000	1.000
	2017	76	0.000	0.000	0.000	0.000
Breed New	BF Sheep	156	1.000	0.000	0.000	
	Crossbred	255	0.000	1.000	0.000	
	Merino X	284	0.000	0.000	1.000	
	PureBred	128	0.000	0.000	0.000	
Region_cde	Mid Cant	295	1.000	0.000		
	North Ca	214	0.000	1.000		
	Otago	314	0.000	0.000		
FABP4 B1	N	351	0.000			
	Y	472	1.000			

Block 1: Method = Enter

Variables in the Equation

								95% C.I.for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1[a]	FABP4 B1(1)	0.104	0.147	0.503	1	0.478	1.110	0.832	1.480
	Year			4.646	4	0.326			
	Year(1)	0.073	0.279	0.068	1	0.794	1.076	0.622	1.860
	Year(2)	0.006	0.302	0.000	1	0.985	1.006	0.556	1.819
	Year(3)	0.140	0.317	0.197	1	0.657	1.151	0.619	2.141
	Year(4)	0.457	0.295	2.404	1	0.121	1.580	0.886	2.816
	Breed New			5.114	3	0.164			
	Breed New(1)	-0.469	0.265	3.142	1	0.076	0.625	0.372	1.051
	Breed New(2)	-0.025	0.247	0.011	1	0.918	0.975	0.601	1.581
	Breed New(3)	-0.369	0.243	2.304	1	0.129	0.691	0.429	1.114
	Region_cde			1.497	2	0.473			
	Region_cde(1)	0.228	0.198	1.326	1	0.249	1.256	0.852	1.852
	Region_cde(2)	0.033	0.207	0.026	1	0.872	1.034	0.689	1.551
	Age_Gender			#####	4	0.003			
	Age_Gender(1)	0.320	0.439	0.532	1	0.466	1.377	0.583	3.254
	Age_Gender(2)	0.196	0.408	0.230	1	0.631	1.216	0.547	2.706
	Age_Gender(3)	0.206	0.421	0.240	1	0.624	1.229	0.538	2.805
	Age_Gender(4)	-1.966	0.671	8.589	1	0.003	0.140	0.038	0.521
	Constant	-0.126	0.503	0.063	1	0.802	0.881		

a. Variable(s) entered on step 1: FABP4 B1, Year, Breed New, Region_cde, Age_Gender.

Logistic Regression

Case Processing Summary

Unweighted Cases[a]		N	Percent
Selected Cases	Included in Analysis	823	97.5
	Missing Cases	21	2.5
	Total	844	100.0
Unselected Cases		0	0.0
Total		844	100.0

a. If weight is in effect, see classification table for the total number of cases.

Dependent Variable Encoding

Original Value	Internal Value
0	0
1	1

Categorical Variables Coding

			Parameter coding			
		Frequency	(1)	(2)	(3)	(4)
Age_Gender	Ewe2	160	1.000	0.000	0.000	0.000
	Ewe3	286	0.000	1.000	0.000	0.000
	Lamb	316	0.000	0.000	1.000	0.000
	Ram2	32	0.000	0.000	0.000	1.000
	Ram3	29	0.000	0.000	0.000	0.000
Year	2013	271	1.000	0.000	0.000	0.000
	2014	182	0.000	1.000	0.000	0.000
	2015	122	0.000	0.000	1.000	0.000
	2016	172	0.000	0.000	0.000	1.000
	2017	76	0.000	0.000	0.000	0.000
Breed New	BF Sheep	156	1.000	0.000	0.000	
	Crossbred	255	0.000	1.000	0.000	
	Merino X	284	0.000	0.000	1.000	
	PureBred	128	0.000	0.000	0.000	
Region_cde	Mid Cant	295	1.000	0.000		
	North Ca	214	0.000	1.000		
	Otago	314	0.000	0.000		
FABP4 C1	N	317	0.000			
	Y	506	1.000			

Block 1: Method = Enter

Variables in the Equation

								95% C.I.for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1[a]	FABP4 C1(1)	0.189	0.151	1.574	1	0.210	1.209	0.899	1.625
	Year			4.158	4	0.385			
	Year(1)	0.095	0.280	0.116	1	0.733	1.100	0.635	1.905
	Year(2)	-0.014	0.302	0.002	1	0.962	0.986	0.545	1.781
	Year(3)	0.096	0.316	0.092	1	0.762	1.100	0.593	2.042
	Year(4)	0.429	0.295	2.119	1	0.145	1.536	0.862	2.738
	Breed New			4.786	3	0.188			
	Breed New(1)	-0.459	0.265	2.995	1	0.084	0.632	0.376	1.063
	Breed New(2)	-0.010	0.247	0.002	1	0.968	0.990	0.610	1.607
	Breed New(3)	-0.332	0.245	1.845	1	0.174	0.717	0.444	1.159
	Region_cde			1.717	2	0.424			
	Region_cde(1)	0.234	0.198	1.395	1	0.238	1.264	0.857	1.865
	Region_cde(2)	0.006	0.207	0.001	1	0.976	1.006	0.670	1.511
	Age_Gender			#####	4	0.004			
	Age_Gender(1)	0.293	0.439	0.443	1	0.506	1.340	0.566	3.171
	Age_Gender(2)	0.175	0.409	0.184	1	0.668	1.191	0.535	2.654
	Age_Gender(3)	0.190	0.421	0.203	1	0.652	1.209	0.530	2.762
	Age_Gender(4)	-1.944	0.670	8.407	1	0.004	0.143	0.038	0.533
	Constant	-0.170	0.503	0.115	1	0.735	0.843		

a. Variable(s) entered on step 1: FABP4 C1, Year, Breed New, Region_cde, Age_Gender.

Logistic Regression

Case Processing Summary

Unweighted Cases[a]		N	Percent
Selected Cases	Included in Analysis	823	97.5
	Missing Cases	21	2.5
	Total	844	100.0
Unselected Cases		0	0.0
Total		844	100.0

a. If weight is in effect, see classification table for the total number of cases.

Dependent Variable Encoding

Original Value	Internal Value
0	0
1	1

Categorical Variables Coding

			Parameter coding			
		Frequency	(1)	(2)	(3)	(4)
Age_Gender	Ewe2	160	1.000	0.000	0.000	0.000
	Ewe3	286	0.000	1.000	0.000	0.000
	Lamb	316	0.000	0.000	1.000	0.000
	Ram2	32	0.000	0.000	0.000	1.000
	Ram3	29	0.000	0.000	0.000	0.000
Year	2013	271	1.000	0.000	0.000	0.000
	2014	182	0.000	1.000	0.000	0.000
	2015	122	0.000	0.000	1.000	0.000
	2016	172	0.000	0.000	0.000	1.000
	2017	76	0.000	0.000	0.000	0.000
Breed New	BF Sheep	156	1.000	0.000	0.000	
	Crossbred	255	0.000	1.000	0.000	
	Merino X	284	0.000	0.000	1.000	
	PureBred	128	0.000	0.000	0.000	
Region_cde	Mid Cant	295	1.000	0.000		
	North Ca	214	0.000	1.000		
	Otago	314	0.000	0.000		
FABP4 D1	N	731	0.000			
	Y	92	1.000			

Block 1: Method = Enter

Variables in the Equation

								95% C.I.for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1[a]	FABP4 D1(1)	0.001	0.230	0.000	1	0.996	1.001	0.638	1.570
	Year			4.526	4	0.340			
	Year(1)	0.074	0.279	0.071	1	0.790	1.077	0.623	1.862
	Year(2)	-0.010	0.302	0.001	1	0.974	0.990	0.548	1.788
	Year(3)	0.117	0.315	0.137	1	0.711	1.124	0.606	2.083
	Year(4)	0.445	0.294	2.281	1	0.131	1.560	0.876	2.777
	Breed New			5.088	3	0.165			
	Breed New(1)	-0.468	0.265	3.125	1	0.077	0.626	0.373	1.052
	Breed New(2)	-0.021	0.247	0.007	1	0.934	0.980	0.604	1.588
	Breed New(3)	-0.362	0.243	2.221	1	0.136	0.696	0.432	1.121

	Region_cde			1.499	2	0.473			
	Region_cde(1)	0.226	0.198	1.298	1	0.255	1.253	0.850	1.847
	Region_cde(2)	0.025	0.207	0.015	1	0.902	1.026	0.684	1.538
	Age_Gender			#####	4	0.003			
	Age_Gender(1)	0.320	0.439	0.533	1	0.465	1.378	0.583	3.254
	Age_Gender(2)	0.200	0.408	0.240	1	0.624	1.222	0.549	2.719
	Age_Gender(3)	0.211	0.421	0.252	1	0.616	1.236	0.541	2.820
	Age_Gender(4)	-1.959	0.671	8.537	1	0.003	0.141	0.038	0.525
	Constant	-0.063	0.496	0.016	1	0.900	0.939		

a. Variable(s) entered on step 1: FABP4 D1, Year, Breed New, Region_cde, Age_Gender

A.8 Copy number analysis for *FABP4 AA* region 1

			fly-strike		
			No	Yes	Total
ccA	0	Count	232	298	530
		% within ccA	43.8%	56.2%	100.0%
	1	Count	152	132	284
		% within ccA	53.5%	46.5%	100.0%
	2	Count	19	11	30
		% within ccA	63.3%	36.7%	100.0%
Total		Count	403	441	844
		% within ccA	47.7%	52.3%	100.0%

P = 0.002

Variables in the Equation

								95% C.I.for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1[a]	ccA	-0.394	0.125	9.955	1	0.002	0.674	0.528	0.861
	Constant	0.251	0.086	8.524	1	0.004	1.285		

Variables in the Equation

								95% C.I.for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1[a]	ccA	-0.363	0.132	7.589	1	0.006	0.695	0.537	0.900
	Year			4.952	4	0.292			
	Year(1)	0.079	0.280	0.079	1	0.779	1.082	0.624	1.874
	Year(2)	0.009	0.303	0.001	1	0.975	1.010	0.558	1.828
	Year(3)	0.077	0.316	0.060	1	0.807	1.080	0.581	2.008
	Year(4)	0.474	0.296	2.569	1	0.109	1.607	0.900	2.871
	Breed New			4.754	3	0.191			
	Breed New(1)	-0.465	0.266	3.064	1	0.080	0.628	0.373	1.057
	Breed New(2)	-0.001	0.248	0.000	1	0.998	0.999	0.615	1.624
	Breed New(3)	-0.303	0.245	1.532	1	0.216	0.738	0.457	1.194
	Region_cde			1.619	2	0.445			
	Region_cde(1)	0.231	0.199	1.348	1	0.246	1.260	0.853	1.861
	Region_cde(2)	0.015	0.208	0.005	1	0.944	1.015	0.675	1.525
	Age_Gender			14.803	4	0.005			
	Age_Gender(1)	0.251	0.442	0.321	1	0.571	1.285	0.540	3.055
	Age_Gender(2)	0.157	0.411	0.146	1	0.703	1.170	0.523	2.616

	Age_Gender(3)	0.149	0.424	0.124	1	0.724	1.161	0.506	2.665
	Age_Gender(4)	-1.951	0.672	8.423	1	0.004	0.142	0.038	0.531
	Constant	0.107	0.501	0.045	1	0.831	1.113		

A.9 Pearson chi-square for *FABP4* Region 2

Crosstabs

Region_cde * fly-strike

Crosstab

			fly-strike		
			0	1	Total
Region_cde	Mid Canterbury	Count	113	105	218
		% within Region_cde	51.8%	48.2%	100.0%
	North Canterbury	Count	76	66	142
		% within Region_cde	53.5%	46.5%	100.0%
	Otago	Count	102	120	222
		% within Region_cde	45.9%	54.1%	100.0%
Total		Count	291	291	582
		% within Region_cde	50.0%	50.0%	100.0%

Chi-square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-square	2.457[a]	2	0.293
Likelihood Ratio	2.460	2	0.292
N of Valid Cases	582		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 71.00.

Breed New * fly-strike

Crosstab

			fly-strike		
			0	1	Total
Breed New	BF Sheep	Count	72	51	123
		% within Breed New	58.5%	41.5%	100.0%
	CrossBred	Count	82	109	191
		% within Breed New	42.9%	57.1%	100.0%
	Merino X	Count	110	101	211
		% within Breed New	52.1%	47.9%	100.0%
	PureBred	Count	27	30	57
		% within Breed New	47.4%	52.6%	100.0%
Total		Count	291	291	582
		% within Breed New	50.0%	50.0%	100.0%

Chi-square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-square	7.944[a]	3	0.047
Likelihood Ratio	7.975	3	0.047
N of Valid Cases	582		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 28.50.

Age_Gender * fly-strike

Crosstab

			fly-strike		
			0	1	Total
Age_Gender	Ewe2	Count	52	58	110
		% within Age_Gender	47.3%	52.7%	100.0%
	Ewe3	Count	110	111	221
		% within Age_Gender	49.8%	50.2%	100.0%
	Lamb	Count	91	109	200
		% within Age_Gender	45.5%	54.5%	100.0%
	Ram2	Count	25	3	28
		% within Age_Gender	89.3%	10.7%	100.0%
	Ram3	Count	13	10	23
		% within Age_Gender	56.5%	43.5%	100.0%
Total		Count	291	291	582
		% within Age_Gender	50.0%	50.0%	100.0%

Chi-square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-square	19.629[a]	4	0.001
Likelihood Ratio	22.095	4	0.000
N of Valid Cases	582		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 11.50.

Year/date * fly-strike

Crosstab

			fly-strike		
			0	1	Total
Year/date	2013	Count	92	84	176
		% within Year/date	52.3%	47.7%	100.0%
	2014	Count	77	49	126
		% within Year/date	61.1%	38.9%	100.0%
	2015	Count	46	64	110
		% within Year/date	41.8%	58.2%	100.0%
	2016	Count	64	85	149
		% within Year/date	43.0%	57.0%	100.0%
	2017	Count	12	9	21
		% within Year/date	57.1%	42.9%	100.0%
Total		Count	291	291	582
		% within Year/date	50.0%	50.0%	100.0%

Chi-square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-square	12.920[a]	4	0.012
Likelihood Ratio	12.997	4	0.011
Linear-by-Linear Association	3.771	1	0.052
N of Valid Cases	582		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 10.50.

FABP4 2 * fly-strike

Crosstab

			fly-strike		
			0	1	Total
FABP4 2	AA	Count	151	137	288
		% within FABP4 2	52.4%	47.6%	100.0%
	AB	Count	85	102	187
		% within FABP4 2	45.5%	54.5%	100.0%

	AC	Count	7	7	14
		% within FABP4 2	50.0%	50.0%	100.0%
	BB	Count	45	43	88
		% within FABP4 2	51.1%	48.9%	100.0%
	BC	Count	1	2	3
		% within FABP4 2	33.3%	66.7%	100.0%
	CC	Count	2	0	2
		% within FABP4 2	100.0%	0.0%	100.0%
Total		Count	291	291	582
		% within FABP4 2	50.0%	50.0%	100.0%

Chi-square Tests

	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-square	4.605[a]	5	0.466
Likelihood Ratio	5.386	5	0.371
N of Valid Cases	582		

a. 4 cells (33.3%) have expected count less than 5. The minimum expected count is 1.00.

FABP4 A2 * fly-strike

Crosstab

			fly-strike 0	fly-strike 1	Total
FABP4 A2	N	Count	48	45	93
		% within FABP4 A2	51.6%	48.4%	100.0%
	Y	Count	243	246	489
		% within FABP4 A2	49.7%	50.3%	100.0%
Total		Count	291	291	582
		% within FABP4 A2	50.0%	50.0%	100.0%

Chi-square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-square	.115[a]	1	0.734		
Continuity Correction[b]	0.051	1	0.821		
Likelihood Ratio	0.115	1	0.734		
Fisher's Exact Test				0.821	0.411
N of Valid Cases	582				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 46.50.

b. Computed only for a 2x2 table

FABP4 B2 * fly-strike

Crosstab

			fly-strike 0	fly-strike 1	Total
FABP4 B2	N	Count	160	144	304
		% within FABP4 B2	52.6%	47.4%	100.0%
	Y	Count	131	147	278
		% within FABP4 B2	47.1%	52.9%	100.0%
Total		Count	291	291	582
		% within FABP4 B2	50.0%	50.0%	100.0%

Chi-square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-square	1.763[a]	1	0.184		
Continuity Correction[b]	1.549	1	0.213		
Likelihood Ratio	1.764	1	0.184		

Fisher's Exact Test				0.213	0.107
N of Valid Cases	582				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 139.00.

b. Computed only for a 2x2 table

FABP4 C2 * fly-strike

Crosstab

			fly-strike		
			0	1	Total
FABP4 C2	N	Count	281	282	563
		% within FABP4 C2	49.9%	50.1%	100.0%
	Y	Count	10	9	19
		% within FABP4 C2	52.6%	47.4%	100.0%
Total		Count	291	291	582
		% within FABP4 C2	50.0%	50.0%	100.0%

Chi-square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-square	.054[a]	1	0.816		
Continuity Correction[b]	0.000	1	1.000		
Likelihood Ratio	0.054	1	0.816		
Fisher's Exact Test				1.000	0.500
N of Valid Cases	582				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 9.50.

b. Computed only for a 2x2 table

A.10Logistic Regression for *FABP4* Region 2

Logistic Regression

Case Processing Summary

Unweighted Cases[a]		N	Percent
Selected Cases	Included in Analysis	582	100.0
	Missing Cases	0	0.0
	Total	582	100.0
Unselected Cases		0	0.0
Total		582	100.0

a. If weight is in effect, see classification table for the total number of cases.

Dependent Variable Encoding

Original Value	Internal Value
0	0
1	1

Categorical Variables Coding

		Frequency	Parameter coding (1)
FABP4 A2	N	93	0.000
	Y	489	1.000

Block 1: Method = Enter

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	Upper
Step 1[a]	FABP4 A2(1)	0.077	0.226	0.115	1	0.734	1.080	0.693	1.683
	Constant	-0.065	0.207	0.097	1	0.756	0.938		

a. Variable(s) entered on step 1: FABP4 A2.

Logistic Regression

Case Processing Summary

Unweighted Cases[a]		N	Percent
Selected Cases	Included in Analysis	582	100.0
	Missing Cases	0	0.0
	Total	582	100.0
Unselected Cases		0	0.0
Total		582	100.0

a. If weight is in effect, see classification table for the total number of cases.

Dependent Variable Encoding

Original Value	Internal Value
0	0
1	1

Categorical Variables Coding

		Frequency	Parameter coding (1)
FABP4 B2	N	304	0.000
	Y	278	1.000

Block 1: Method = Enter

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	Upper
Step 1[a]	FABP4 B2(1)	0.221	0.166	1.761	1	0.184	1.247	0.900	1.727
	Constant	-0.105	0.115	0.841	1	0.359	0.900		

a. Variable(s) entered on step 1: FABP4 B2.

Logistic Regression

Case Processing Summary

Unweighted Cases[a]	N	Percent

Selected Cases	Included in Analysis	582	100.0
	Missing Cases	0	0.0
	Total	582	100.0
Unselected Cases		0	0.0
Total		582	100.0

a. If weight is in effect, see classification table for the total number of cases.

Dependent Variable Encoding	
Original Value	Internal Value
0	0
1	1

Categorical Variables Coding

		Frequency	Parameter coding (1)
FABP4 C2	N	563	0.000
	Y	19	1.000

Block 1: Method = Enter

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	Upper
Step 1[a]	FABP4 C2(1)	-0.109	0.467	0.054	1	0.816	0.897	0.359	2.240
	Constant	0.004	0.084	0.002	1	0.966	1.004		

a. Variable(s) entered on step 1: FABP4 C2.

Logistic Regression

Case Processing Summary

Unweighted Cases[a]		N	Percent
Selected Cases	Included in Analysis	582	100.0
	Missing Cases	0	0.0
	Total	582	100.0
Unselected Cases		0	0.0
Total		582	100.0

a. If weight is in effect, see classification table for the total number of cases.

Dependent Variable Encoding

Original Value	Internal Value
0	0
1	1

Categorical Variables Coding

			Parameter coding			
		Frequency	(1)	(2)	(3)	(4)
Age_Gender	Ewe2	110	1.000	0.000	0.000	0.000
	Ewe3	221	0.000	1.000	0.000	0.000
	Lamb	200	0.000	0.000	1.000	0.000
	Ram2	28	0.000	0.000	0.000	1.000
	Ram3	23	0.000	0.000	0.000	0.000
Year/date	2013	176	1.000	0.000	0.000	0.000
	2014	126	0.000	1.000	0.000	0.000
	2015	110	0.000	0.000	1.000	0.000
	2016	149	0.000	0.000	0.000	1.000
	2017	21	0.000	0.000	0.000	0.000
Breed New	BF Sheep	123	1.000	0.000	0.000	
	Crossbred	191	0.000	1.000	0.000	
	Merino X	211	0.000	0.000	1.000	
	PureBred	57	0.000	0.000	0.000	
Region_cde	Mid Cant	218	1.000	0.000		
	North Ca	142	0.000	1.000		
	Otago	222	0.000	0.000		
FABP4 A2	N	93	0.000			
	Y	489	1.000			

Block 1: Method = Enter

Variables in the Equation

								95% C.I.for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1[a]	FABP4 A2(1)	0.096	0.240	0.158	1	0.691	1.100	0.687	1.762
	Year/date			9.865	4	0.043			
	Year/date(1)	-0.109	0.525	0.043	1	0.836	0.897	0.320	2.509
	Year/date(2)	-0.174	0.527	0.109	1	0.741	0.840	0.299	2.360

	Year/date(3)	0.291	0.542	0.288	1	0.592	1.337	0.462	3.869
	Year/date(4)	0.577	0.526	1.206	1	0.272	1.781	0.636	4.990
	Breed New			3.573	3	0.311			
	Breed New(1)	-0.338	0.378	0.798	1	0.372	0.713	0.340	1.497
	Breed New(2)	0.180	0.326	0.304	1	0.581	1.197	0.632	2.269
	Breed New(3)	-0.223	0.339	0.432	1	0.511	0.800	0.412	1.555
	Region_cde			0.629	2	0.730			
	Region_cde(1)	0.176	0.229	0.590	1	0.442	1.193	0.761	1.870
	Region_cde(2)	0.132	0.261	0.256	1	0.613	1.141	0.684	1.904
	Age_Gender			12.086	4	0.017			
	Age_Gender(1)	0.177	0.525	0.114	1	0.735	1.194	0.427	3.339
	Age_Gender(2)	0.239	0.474	0.254	1	0.614	1.270	0.502	3.212
	Age_Gender(3)	0.291	0.490	0.352	1	0.553	1.337	0.512	3.494
	Age_Gender(4)	-1.961	0.766	6.555	1	0.010	0.141	0.031	0.631
	Constant	-0.374	0.843	0.197	1	0.657	0.688		

a. Variable(s) entered on step 1: FABP4 A2, Year/date, Breed New, Region_cde, Age_Gender.

Logistic Regression

Case Processing Summary

Unweighted Cases[a]		N	Percent
Selected Cases	Included in Analysis	582	100.0
	Missing Cases	0	0.0
	Total	582	100.0
Unselected Cases		0	0.0
Total		582	100.0

a. If weight is in effect, see classification table for the total number of cases.

Dependent Variable Encoding

Original Value	Internal Value
0	0
1	1

Categorical Variables Coding

			Parameter coding			
		Frequency	(1)	(2)	(3)	(4)
Age_Gender	Ewe2	110	1.000	0.000	0.000	0.000
	Ewe3	221	0.000	1.000	0.000	0.000
	Lamb	200	0.000	0.000	1.000	0.000

	Ram2	28	0.000	0.000	0.000	1.000
	Ram3	23	0.000	0.000	0.000	0.000
Year/date	2013	176	1.000	0.000	0.000	0.000
	2014	126	0.000	1.000	0.000	0.000
	2015	110	0.000	0.000	1.000	0.000
	2016	149	0.000	0.000	0.000	1.000
	2017	21	0.000	0.000	0.000	0.000
Breed New	BF Sheep	123	1.000	0.000	0.000	
	Crossbred	191	0.000	1.000	0.000	
	Merino X	211	0.000	0.000	1.000	
	PureBred	57	0.000	0.000	0.000	
Region_cde	Mid Cant	218	1.000	0.000		
	North Ca	142	0.000	1.000		
	Otago	222	0.000	0.000		
FABP4 B2	N	304	0.000			
	Y	278	1.000			

Block 1: Method = Enter

Variables in the Equation

								95% C.I.for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1[a]	FABP4 B2(1)	0.149	0.181	0.681	1	0.409	1.161	0.814	1.655
	Year/date			8.720	4	0.068			
	Year/date(1)	-0.099	0.526	0.035	1	0.851	0.906	0.323	2.538
	Year/date(2)	-0.155	0.528	0.086	1	0.770	0.857	0.304	2.412
	Year/date(3)	0.265	0.542	0.240	1	0.625	1.304	0.451	3.774
	Year/date(4)	0.559	0.526	1.129	1	0.288	1.748	0.624	4.901
	Breed New			3.178	3	0.365			
	Breed New(1)	-0.344	0.378	0.828	1	0.363	0.709	0.338	1.487
	Breed New(2)	0.169	0.326	0.268	1	0.605	1.184	0.625	2.244
	Breed New(3)	-0.180	0.341	0.279	1	0.598	0.835	0.428	1.630
	Region_cde			0.439	2	0.803			
	Region_cde(1)	0.143	0.231	0.382	1	0.536	1.153	0.734	1.813
	Region_cde(2)	0.125	0.261	0.230	1	0.631	1.134	0.679	1.891
	Age_Gender			12.382	4	0.015			
	Age_Gender(1)	0.181	0.526	0.118	1	0.731	1.198	0.427	3.360
	Age_Gender(2)	0.219	0.476	0.212	1	0.645	1.245	0.490	3.166
	Age_Gender(3)	0.278	0.492	0.320	1	0.571	1.321	0.504	3.464
	Age_Gender(4)	-2.009	0.768	6.843	1	0.009	0.134	0.030	0.604
	Constant	-0.347	0.818	0.180	1	0.672	0.707		

a. Variable(s) entered on step 1: FABP4 B2, Year/date, Breed New, Region_cde, Age_Gender.

Logistic Regression

Case Processing Summary

Unweighted Cases[a]		N	Percent
Selected Cases	Included in Analysis	582	100.0
	Missing Cases	0	0.0
	Total	582	100.0
Unselected Cases		0	0.0
Total		582	100.0

a. If weight is in effect, see classification table for the total number of cases.

Dependent Variable Encoding

Original Value	Internal Value
0	0
1	1

Categorical Variables Coding

			Parameter coding			
		Frequency	(1)	(2)	(3)	(4)
Age_Gender	Ewe2	110	1.000	0.000	0.000	0.000
	Ewe3	221	0.000	1.000	0.000	0.000
	Lamb	200	0.000	0.000	1.000	0.000
	Ram2	28	0.000	0.000	0.000	1.000
	Ram3	23	0.000	0.000	0.000	0.000
Year/date	2013	176	1.000	0.000	0.000	0.000
	2014	126	0.000	1.000	0.000	0.000
	2015	110	0.000	0.000	1.000	0.000
	2016	149	0.000	0.000	0.000	1.000
	2017	21	0.000	0.000	0.000	0.000
Breed New	BF Sheep	123	1.000	0.000	0.000	
	Crossbred	191	0.000	1.000	0.000	
	Merino X	211	0.000	0.000	1.000	
	PureBred	57	0.000	0.000	0.000	
Region_cde	Mid Cant	218	1.000	0.000		
	North Ca	142	0.000	1.000		
	Otago	222	0.000	0.000		
FABP4 C2	N	563	0.000			

Y	19	1.000				

Block 1: Method = Enter

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B) Lower	Upper
Step 1[a]	FABP4 C2(1)	0.072	0.502	0.021	1	0.886	1.075	0.402	2.872
	Year/date			9.733	4	0.045			
	Year/date(1)	-0.097	0.531	0.033	1	0.855	0.907	0.320	2.570
	Year/date(2)	-0.166	0.532	0.098	1	0.755	0.847	0.298	2.404
	Year/date(3)	0.293	0.547	0.287	1	0.592	1.340	0.459	3.915
	Year/date(4)	0.580	0.531	1.193	1	0.275	1.786	0.631	5.055
	Breed New			3.561	3	0.313			
	Breed New(1)	-0.349	0.381	0.839	1	0.360	0.706	0.335	1.488
	Breed New(2)	0.180	0.326	0.304	1	0.581	1.197	0.632	2.269
	Breed New(3)	-0.218	0.339	0.415	1	0.520	0.804	0.414	1.562
	Region_cde			0.604	2	0.739			
	Region_cde(1)	0.172	0.229	0.560	1	0.454	1.187	0.757	1.862
	Region_cde(2)	0.133	0.261	0.261	1	0.610	1.143	0.685	1.906
	Age_Gender			12.193	4	0.016			
	Age_Gender(1)	0.182	0.525	0.120	1	0.729	1.199	0.428	3.359
	Age_Gender(2)	0.243	0.475	0.262	1	0.609	1.275	0.503	3.236
	Age_Gender(3)	0.290	0.491	0.349	1	0.555	1.336	0.511	3.496
	Age_Gender(4)	-1.970	0.767	6.595	1	0.010	0.140	0.031	0.627
	Constant	-0.303	0.822	0.136	1	0.712	0.739		

a. Variable(s) entered on step 1: FABP4 C2, Year/date, Breed New, Region_cde, Age_Gender.

Appendix B

B.1 Sequencing of exon 2 of the *FBLN1* gene

cctgggatgctgtgaggtggcgtccagatggtgcggccgggaggggggg[c/t]gcccttggcctcccga
gggaggctgcccatcccaggggccctgggggggtgtggtgagtcgcctgcc[a/g]cctgtcaccctgt
tctccttcctgcacag

Exon 2

[T/C]GGGCGCAGACGTCTCCATGGAGGCTTGCTGTGCAGATGGACATCGAATGGCCACGGAGCATAGG
GCCTGCTCACTGCCCTA[C/T]GCCTCGGAATCCAAGGAATGCAG

Intron 3

gtatgtctgccagcacttcaccagaagagcgttcctctagacaaaagcaggagaggaagggagagtgc

Intron 10

cttccttgccttccgttctgatcacgccccctcccacactaaagggttttcaatgtgtctttcttttc
tgcaag

Exon 10

ATGTGGACGAGTGCTCTCCCCCTTCTGAGCCCTGTGGGCCGGGGCACCTGTGTGTGAACTCCCCTGGA
AGTTTCCGCTGTGAGTGCAAGGCCGGGTACTACTTCGACGGCATCAGCAGGAC[A/G]TGTGT[A/G]
G

Gtatgtgt[g/t]ggtatccctgaggcgggtgctggccaggtgac[g/a]ccaaggagaaccagcctg
gggtgcacctggagtgaccgtggtctgctcccgacctctgacaatcctgagt

Exon 12

CTACCAGCTCAGCGACGTGGACGGGGTCACCTGCGAAG

Intron 12-13:

gtgagcgggagaggccgcccctctcctgtcgcccactccccgctcctcctggctggcgagtggccc[g
/a]ggtttcttccctctgagatgtgtttggatggactttgcccattgttgaaagtgaga[t/c]ttgt
aaaggaggaaacccactcccagattttcttcagtgacataaaatggaacggtggtggtggctatgggt
gccgcc[c/t]ggacctcaagcagggcgggggtgcgggaggcgggc[t/c]gaggagtcacaccctgg
ctcgaccctctgcctctggcctttcagaagcccctca[c/t]ggggcctgggccgccctc[t/c]tgt
gtaacatctgggttcatcttgcac

Red letters indicate the primers, blue letters represent the SNPs found.

B.2 Copy number analysis for FBLN1

ccB * fly-strike

			fly-strike		
			No	Yes	Total
ccB	0	Count	253	295	548
		% within ccB	46.2%	53.8%	100.0%
	1	Count	94	96	190
		% within ccB	49.5%	50.5%	100.0%
	2	Count	24	14	38
		% within ccB	63.2%	36.8%	100.0%
Total		Count	371	405	776
		% within ccB	47.8%	52.2%	100.0%

P = 0.109

ccC * fly-strike

			fly-strike		
			No	Yes	Total
ccC	0	Count	144	122	266
		% within ccC	54.1%	45.9%	100.0%
	1	Count	134	172	306
		% within ccC	43.8%	56.2%	100.0%
	2	Count	93	111	204
		% within ccC	45.6%	54.4%	100.0%
Total		Count	371	405	776
		% within ccC	47.8%	52.2%	100.0%

P= 0.093

Appendix C

C.1 Primer coverage for the *RASGRP1* gene

Intron 15:

aaagcctcaattgatactaatgtgtaaatttcgagtgcctgcttttctaagctaaagtaccaacatgt
cttcttcctacag

Exon 16:

CACCTGAGGAAGGACCTTTCACATTCCCTAACGGGGAGGCTGTGGAACACAGTGAGGAGAGTAAGGAT
CGGACCATCATGCTCATGGGGGTATCCTCACAGAAGATTTCTGTTCGGCTGAAGAGGAC[C/T]GTCA
CCCATAAGGCCACCCAGACTGAAGCACTGTCTTGGCCTGGCAGTGAGGGCCCTTCCAGTCACTTTGTA
CTGTCGTCCCCAAGGAGGACAGCCCAGGATACTCTGTATGTGCTGCC[C/T]AGCCCTACGTCTCCGT
GCCCCAG[C/T]CCAGTCTTGGTCAGGAAGCGGGCTTTCGTCAAGTGGGAGAACAAAGAATCCTTCAT
AAAATCAAAGGAGGAGCTCCGTCACCTCAAGCTCCCAACATACCAAGAGCTGGAACAG

Intron 16:

gtacctttttctttttttcagactctgagaaagtggggctaacaagggaagaacat

RASGRP1 Amino acid sequence

SEESKDRTIMLMGVSSQKISVRLKRTVTHKATQTEALSWPGSEGPSSHFVLSSPRRTAQDTLYVLPSP
TSPCPSPVLVRKRAFVKWENKESFIKSKEELRHLKLPTYQELEQ

C.2 Copy number analysis for RAGRP1

ccA * fly-strike

			fly-strike		
			No	Yes	Total
ccA	0	Count	93	82	175
		% within ccA	53.1%	46.9%	100.0%
	1	Count	160	204	364
		% within ccA	44.0%	56.0%	100.0%
	2	Count	109	129	238
		% within ccA	45.8%	54.2%	100.0%
Total		Count	362	415	777
		% within ccA	46.6%	53.4%	100.0%

P = 0.207

Appendix D

D.1 Sequence of *ABCC11*

Primer coverage for the ABCC11 gene:

Intron 4:
aactgggatagttgggtgacacaaccaaagagatttcatttgcctccatttccttcttttcaag

Exon 4:
GCTTCGTCGTCT[C/T]TGGGAAGAAGAAGTCTCAAGGCATGGAATTGACAAAGCTTCAGTGCTTCGA
GTGATGCTGCGATTCCAAAGAACAAGGCTTATTTTTGATACAATTATGAGCTGCTGCTTCTCCATCGC
AAGTGTTGTGGGGCCC

Intron 5:
gtaagtagcag[g/a]cttgatgaggtatccggtcttcaggctttggtgattagcatgtttagatcca
tgccaagaactggagatgtcttgagacttccctggtgggtcagtagttaggactccac

ABCC11 amino acid sequence
LRRLWEEEVS RHGIDKASVL RVMLRFQRTR LIFDTIMSCC FSIASVVGP

Exon 4 of human and sheep *ABCC11* (80.4% identity)

```
GCTTCACCGCCTTTGGGAAGAAGAAGTCTCAAGGCGAGGGATTGAAAAAGCTTCAGTGCTTC
|||||  || || ||||||||||||||||||||||  || ||||| ||||||||||||||||
GCTTCGTCGTCTCTGGGAAGAAGAAGTCTCAAGGCATGGAATTGACAAAGCTTCAGTGCTTC

TGGTGATGCTGAGGTTCCAGAGAACAAGGTTGATTTTCGATGCACTTCTGGGCATCTGCTTCT
  |||||||||  | ||||| |||||||||  | ||||| ||| || || || ||  ||||||||
GAGTGATGCTGCGATTCCAAAGAACAAGGCTTATTTTTGATACAATTATGAGCTGCTGCTTCT

GCATTGCCAGTGTACTCGGGCCA
 |||  ||  |||||   |  |||||
CCATCGCAAGTGTTGTGGGGCCC
```

Intron 25-26:
tcagatggcaagaagtctgcctgcaatgtgatacacctgggttcaatccctgggtccgaa[c/a]gat
cccccaaagatggaaatggcaacctactcc[a/g]gtattctagactggaaaattccatggacagagg
agcc[a/t]gtgggcttacaggccatggggtcccaaagagttggacacaactgagcaagtttcacttt
ctcaggcatcctcggtcacgtgctctgcggggtgcagac[g/a]acttgatgggcaagtgcgcttctt
gaacacgtc[c/t]ctttct[t/c]tcccg

Exon 26:
AGGCAGATTCAACCTGGACCCCTTTGAC[C/T]GCTGCACGGACGAGCAGATCTGGGATGCCTTGGAG
AGGACGTTCCTGAACAAGACG

Intron 26-27:
gtgagtaagtgcaaggctgaattctcagtcctgctggtcctc

References

Aitken, F. J., Cottle, D. J., Reid, T. C., Wilkinson, B. R. (1994). Mineral and amino acid composition of wool from New Zealand Merino sheep differing in susceptibility to yellowing. *Australian Journal of Agricultural Research* 45, 391-401.

Akira, S., Takeda, K., Kaisho, T. (2001). Toll-like receptors: critical proteins linking innate and acquired immunity. *Nature immunology* 2, 675-680.

Akira, S. (2003). Mammalian Toll-like receptors. *Current Opinion in Immunology* 15, 5-11.

Akira, S. Takeda, K. (2004). Toll-like receptor signalling. *Nature Reviews Immunology* 4, 499 – 511.

Anderson, J. M. E., McLeod, L. J., Shipp, E., Swan, A., Kennedy, J. P. (1990). Trapping sheep flies using bait-bins. *Australian Veterinary Journal* 67, 93-97.

Anon. (2005). Fly advice, a farmer's guide to blowfly control in sheep, Schering-Plough Coopers Upper Hutt, New Zealand. http://www.msd-animal-health.co.nz/binaries/FlyAdvice_r5_tcm51-36855.pdf. 17/5/2017.

Arbour, N. C., Lorenz, E., Schutte, B. C., Zabner, J., Kline, J. N., Jones, M., Frees, K., Watt, J. L., Schwartz, D.A. (2000). TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nature genetics* 25, 187-191.

Argraves, W. S., Dickerson, K., Burgess, W. H., Ruoslahti, E. (1989). Fibulin, a novel protein that interacts with the fibronectin receptor B subunit cytoplasmic domain. *Cell* 58, 623 - 629.

Argraves, W.S., Greene, L.M., Cooley, M.A., Gallagher, W.M. (2003). Fibulins: physiological and disease perspective. *EMBO Reports* 4, 1127-1131.

Ashworth, J. R., Wall, R. (1994). Responses of the sheep blowflies *Lucilia sericata* and *L. cuprina* to odour and the development of semiochemical baits. *Medical and Veterinary Entomology* 8, 303-309.

Aspberg, A., Adam, S., Kostka, G., Timpl, R., Heinegard, D. (1999). Fibulin-1 is a ligand for the C-type lectin domains of aggrecan and versican. *Journal of Biological Chemistry* 274, 114-122.

Bakhtiarizadeh, M. R., Moradi-Shahrbabak, M., Ebranhimie, E. (2013). Underlying functional genomics of fat deposition in adipose tissue. *Gene* 521, 122-128.

Banchereau, J., Steinman, R. M., (1998). Dendritic cells and the control of immunity. *Nature* 392, 245-252.

Baron, R. W., Colwell, D. D. (1991). Mammalian immune response to myiasis. *Parasitology Today* 17, 353-355.

Bates, P. (2007). *Other ectoparasitic conditions. In Diseases of Sheep*, Fourth Edition. Edited by I. D. Aitken. Blackwell Publishing.

Batterham, P., Hill-Williams, A., Levot, G., Sales, N., McKenzie, J .A. (2006). The genetic bases of high-level resistance to diflubenzuron and low-level resistance to cyromazine in a field strain of the Australian sheep blowfly, *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae). *Australian Journal of Entomology* 45, 87 – 90.

Bell, J. K., Mullen, G. E., Leifer, C. A., Mazzoni, A., Davies, D. R., Segal, D. M. (2003). Leucine-rich repeats and pathogens recognition in Toll-like receptors. *Trends in immunology* 24, 528-533.

Bell, K. J. (2010). Sheep management. In *International sheep and wool handbook:* Cottle, D. J. Nottingham University Press. 407 – 411.

Belschner, H. G. (1937). Studies of the sheep blowfly problem. II. Observations on fleece rot and body strike in sheep, particularly in regards to their incidence, type of sheep susceptible, and economic importance. – *Science Bulletin, Department of Agriculture N.S.W.* 54, 61-95.

Beutler, B. (2000). TLR4: central component of the sole mammalian LPS sensor. *Current opinion in immunology* 12, 20-26.

Bird, S., Gower, D. B. (1981). The validation and use of a radioimmunoassay for 5α-androst -16-en-3-one in human axillary collections. *Journal of Steroid Biochemistry* 14, 213 -219.

Bishop, S. C., Morris, C. A. (2007). Genetics of disease resistance in sheep and goats. *Small Ruminant Research* 70, 48-59.

Bouchey, D., Argraves, W. S., Little, C. D. (1996). Fibulin-1, vitronectin, and fibronectin expression during avian cardiac valve and septa development. *Anatomical Record* 244, 540 – 551.

Bochud, P.-Y., Bochud, M., Telenti, A., Calandra, T. (2007). Innate immunogenetics: a tool for exploring new frontiers of host defence. *The Lancet infectious diseases* 7, 531-542.

Bowles, V. M., Carnegie, P. R., Sandeman, R. M. (1987). Immunization of sheep against infection with larvae of the fly *Lucilia cuprina. International Journal for Parasitology* 17, 753-758.

Bowles, V. M., Grey, S., Brandon, M. (1992). Cellular immune responses in the skin of sheep infected with larvae of *Lucilia cuprina*, the sheep blowfly. *Veterinary Parasitology* 44, 151-152.

Bowles, V. M., Meeusen, E. N. T., Chandler, K., Verhagen, A., Nash, A. D., Brandon, M. R. (1994). The immune response of sheep infected with larvae of the sheep blowfly *Lucilia cuprina* monitored via efferent lymph. *Veterinary Immunology and Immunopathology* 40, 341 – 352.

Brandsma, J. H., Blair, H. T. (1997). Heritability of resistance to flystrike in New Zealand Perendale sheep. *Proceedings of the New Zealand Society of Animal Production* 57, 41-42.

Broadmeadow, M., Butcher, G., O'Sullivan, B.M., Hopkins, P. S. (1984a). Effect of flystrike on wool production and economic returns. *Wool Technology and Sheep Breeding* 32, 9-11.

Broadmeadow, M., Gibson, J. E., Dimmock, C. K., Thomas, R. J., O'Sullivan, B.M. (1984b). The pathogenesis of fly-strike in sheep. *Wool Technology and Sheep Breeding* 32, 28 – 32.

Broughan, J. M. Wall, R. (2007). Fly abundance and climate as determinants of sheep blowfly strike incidence in southwest England. *Medical and Veterinary Entomology* 21, 231-238.

Burrell, D. H., Merritt, G. C., Watts, J. E., Walker, K. H. (1982). The role of *Pseudomonas aeruginosa* in the pathogenesis of fleecerot and the effect of immunization. *Australian Veterinary Journal* 58, 34-35.

Byun, S. O., Fang, Q., Zhou, H., Hickford, J. G. H. (2008). Rapid genotyping of the ovine ADRB3 gene by polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP). *Molecular and cellular probes*, 22, 69-70.

Byun, S. O., Fang, Q., Zhou, H., Hickford, J. G. H. (2009a). An effective method for silver-staining DNA in large numbers of polyacrylamide gels. *Analytical Biochemistry* 385, 174-175.

Byun, S. O., Zhou, H., Hickford, J. G. H. (2009b). Development of a simple typing method for the ovine Toll-like receptor 4 gene. *Veterinary Immunology and Immunopathology* 130, 272-274.

Byun, S. O., (2012). Genes associated with varioation in longevity and fecundity in sheep. A thesis submitted in fulfilment of the requirements for the Degree of Doctor of Philososphy, Lincoln University, New Zealand.

Chang, J.-S., Russell, G. C., Jann, O., Glass, E. J., Werling, D., Haig, D. M. (2009). Molecular cloning and characterization of Toll-like receptors 1-10 in sheep. *Veterinary immunology and immunopathology* 127, 94-105.

Chapman, R., Hollis, D., & Hemsley, J. (1984). How quickly does wetting affect the skin of Merino sheep. *Symposium conducted at the meeting of the Proceeding of Australian Society Animal Production* 15, 290 – 292.

Charles, C. A., Tomic-Canic, M., Vincek, V., Nassiri, M., Stojadinovic, O., Eaglstein, W. H., Kirsner, R. S. (2008). A gene signature of nonhealing venous ulcers: Potential diagnostic markers. *Journal of the American Academy of Dermatology* 59, 758-771.

Chemonges, S., Tung, J.-P., Fraser, J. F. (2014). Proteogenomics of selective susceptibility to endotoxin using circulating acute phase biomarkers and bioassay development in sheep: a review. *Proteome science* 12, 12.

Chen, Z. S., Tiwari, A., K. (2011). Multidrug resistance proteins (MRPs/ABCCs) in cancer chemotherapy and genetic diseases. *FEBS Journal* 278, 3226-3245.

Chin, J. C., Watts, J. E. (1991). Dermal and serological response against Pseudomonas aeruginsa in sheep bred for resistance and susceptibility to fleecerot. *Australian Veterinary Journal* 68; 28 – 31.

Chmurzyńska, A. (2006). The multigene family of fatty-acid-binding proteins (FABPs): Function, structure and polymorphism. *Journal of Applied Genetics* 47, 39-48.

Choudhuri, S., Klaassen, C. D. (2006). Structure, function, expression, genomic genomic organization, and single nucleotide polymorphisms of human ABCB1 (MDR1), ABCC (MRP), ABCG2 (BCRP) efflux transporters. *International journal of toxicology* 25, 213-259.

Chu, M. L., Tsuda, T. (2004). Fubulins in development and heritable disease. *Birth Defects Research Part C: Embryo Today: Reviews* 72, 25-36.

Cloete, S. W. P., Snyman, M. A., Heerselman, M. J. (2000). Productive performance of Dorper sheep. *Small Ruminant Research* 36, 119 - 135.

Colditz., I. G., Woolaston, R. R., Lax, J., Morimer, S. I. (1992). Plasma leakage in skin of sheep selected for resistance or susceptibility to fleece rot and fly strike. *Parasite Immunology* 14, 587-594.

Colditz, I. G., Lax, J., Mortimer, S. I., Clarke, R. A., Beh, K. J. (1994). Cellular inflammatory responses in skin of sheep selected for resistance or susceptibility to fleece rot and fly strike. *Parasite Immunology* 16, 289 - 296.

Colditz, I. G., Tellam, R. L., (2000). Host resistance to fleecerot and flystrike. Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction. *CAB International* 43, 437 - 448.

Colditz, I. G., Piper, L. R., Atkins, K. D. (2001). Breeding for resistance to fly-strike. *Proceedings of the FLICS Conference* 383 - 394.

Colditz, I. G., Walken-Brown, S. W., Baly, B. L., Crook, B. J. (2005). Some physiological responses associated with reduced wool growth during blowfly strike in Merino Sheep. *Australian Veterinary Journal* 83, 695 – 699.

Colditz, I. G., Mahony, T., Elkington, R., (2006). Using immunology and resistant sheep to beat the fly. Wool Meets Meat. Edited by P.B Conj'e & D. Maxwell. *Proceedings of the 2006 Australian Sheep Industry CRC Conference* 126 -130.

Collins, S., Davidson, R. S. (1997). Aspects of the photochemistry of wool yolk (wool wax and suint). *Review of Progress Coloration* 27, 42 - 58.

Cook, D. F. (1990). Trapping sheep blowflies using bait-bins – Review. *Medical and Veterinary Entomology* 8, 303 – 309.

Coughlin, J. J., Stang, S. L., Dower, N. A., Stone, J. C. (2005). RASGRP1 and RASGRP3 regulate B cell proliferation by facilitating B cell receptor-Ras signalling. *The Journal of Immunology* 175, 7179 - 7184.

Coughlin, J. J., Stang, S. L., Dower, N. A., Stone, J. C. (2006). The role of RASGRPs in regulation of lymphocyte proliferation. *Immunology letters* 105, 77 - 82.

Courtney, H. S., Li, Y., Twal, W. O., Argraves, W. S. (2009). Serum opacity factor is a Streptococcal receptor for the extracellular matrix protein Fibulin-1. *Journal of Biological Chemistry* 284, 12966 – 12971.

Cruickshank, I., Wall, R. (2002). Population dynamics of the sheep fly Lucilia sericata: seasonal patterns and implications for control. *Journal of Applied Ecology* 39, 493 - 501.

D'Arcy, J. B. (1990). *Sheep Management and Wool Technology*. UNSW Press, 314 - 317.

Dalrymple, B. P., Kirkness, E. F., Nefedov, M., McWilliam, S., Ratnakumar, A., Barris, W., O'Grady, M. Zhao, S., Shetty, J., Maddox, J. F., Nicholas, F., Crawford, A. M., Smith, T., de Jong, P. J., McEwan, J., Oddy, V. H., Cockett, N. E. (2007). Using comparative genomics to recorder the human genome sequence into a virtual sheep genome. *Genome biology* 8, R152.

Daly, R., Carter, H. (1956). Fleece growth of young Lincoln, Corriedale, Polwarth, and Fine Merino maiden ewes grazed on an unimproved Paspalum pasture. *Crop and Pasture Science* 7, 76-83.

Davenport, R., Heawood, C., Sessford, K., Baker, M., Baiker, K., Blacklaws, B., Kaler, J., Green, L., Tötemeyer, S. (2014). Differential expression of Toll-like receptors and inflammatory cytokines in ovine interdigital dermatitis and footrot. *Veterinary immunology and immunopathology* 161, 90-98.

De Vega, S., Iwamoto, T., & Yamada, Y. (2009). Fibulins: multiple roles in matrix structures and tissue functions. *Cellular and molecular life sciences* 66, 1890 -1902.

Dower, N. A., Stang, S. L., Bottorff, D. A., Ebinu, J. O., Dickie, P., Ostergaard, H. L., Stone, J. C. (2000). RASGRP is essential for mouse thymocyte differentiation and TCR signalling. *Nature Immunology* 1, 317 - 321.

Dixon, T., Mortimer, S., Norris, B. (2007). 16S rRNA gene microbial analysis of the skin of fleece rot resistant and susceptible sheep. *Crop and Pasture Science* 58, 739-474.

Duan, J., Wainwright, M. S., Comeron, J. M., Saitou, N., Sanders, A. R., Gelernter, J., Gejman, P. V. (2003). Synonymous mutations in the human dopamine receptor D2 (DRD2) affect mRNA stability and synthesis of the receptor. *Human Molecular Genetics* 12, 205-216.

e Sousa, C. R. (2001). Dendritic cells as sensors of infection. *Immunity* 14, 495 - 498.

Ebinu, J. O., Stang, S. L., Teixeira, C., Bottorff, D. A., Hooton, J., Blumberg, P. M., Barry, M., Bleakley, C. R., Ostergaard, H. L., Stone, J. C. (2000). RASGRP links T- cell receptor signaling to Ras. *Blood* 95, 3199-3203.

Eisemann, C., (1988). Upwind flight by gravid Australian sheep blowflies, *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae), in response to stimuli from sheep. *Bulletin of entomological research* 78, 273 – 279.

Eleanor Linscott, personal communication, August 30, 2016.

Elhay, J. M., Hanrahan, C. F., Bowles, V. M., Seow, V. H. F., Andres, E. a., Nash, A. D. (1994). Cytokine expression in skin in response to ectoparasite infection. *Parasite immunology* 16, 451 - 461.

Emmens, R. L., Murray, M.D. (1982). The role of bacterial odours in oviposition by *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae), the Australian sheep fly. *Bulletin of Entomological Research* 72, 367 - 375.

Emter, R., Natsch, A. (2008). The sequential action of a dipeptidase and a β-lyase is required for the release of the human body odorant 3-methyl-3-sulfanylhexan-1-ol from a Screated Cys-Gly-(S) Conjugated by Corynebacteria. *The Journal of Biological Chemistry* 283, 20645 -20652.

Evans, R., McGuirk, B. J. (1983). Estimating the association between wool and skin characters and fleecerot. *Australian Journal of Agricultural Research* 34, 47 - 52.

Fan, B., Du, Z.-Q., Gorbach, D. M., & Rothschild, M. F. (2010). Development and application of high-density SNP arrays in genomic studies of domestic animals. *Asian-Australasian Journal of Animal Sciences* 23, 833 - 847.

Ferwerda, B., McCall, M. B., Verheigen, K., Kullberg, B.-J., Van Der Ven, A. J., Van der Meer, J. W., Netea, M. G. (2008). Functional consequences of toll-like receptor 4 polymorphisms. *Molecular Medicine* 14, 356 - 352.

Fleming, P. H. (2003). *Farm Technical Manual*. Lincoln University. Canterbury. The Caxton Press, New Zealand. B - 21.

Flyboss. (2017). http://www.flyboss.com.au/treatment/choosing-the-right-chemical/insect-growth-regulators.php

Fogarty, N. M., Hopkins, D. L., van de Ven, R. (2000). Lamb production from diverse genotypes. 1. Lamb growth and survival and ewe performance. *Animal Science* 70, 135 – 145.

Fogarty, N., Safari, E., Taylor, P., Murry, W. (2003). Genetic parameters for meat quality and carcass traits and their correlation with wool traits in Australian Merino Sheep. *Crop and Pasture Science*, 54, 715 - 722.

Foster, G. G., Kitching, R. L., Vogt, W. G. Whitten, M. J. (1975). Sheep fly and its control in the pastoral ecosystem in Australia. *Proceedings of the Ecology Society of Australia* 9, 213 - 229.

French, N., Wall, R., Morgan, K. (1995). The seasonal pattern of sheep blowfly strike in England and Wales. *Medical and Veterinary Entomology* 9, 1 - 8.

Fuller, D. M., Zhu, M., Song, X., Ou-Yang, C.-W., Sullivan, S. A., Stone, J. C., Zhang, W. (2012). Regulation of RASGRP1 function in T cell development and activation by its unique tail domain. *PloS one* 7, e38796.

Furuhashi, M., Hotamisligil, G.S. (2008). Fatty-acid-binding proteins: role in metabolic disease and potential as drug targets. *Nature Reviews Drug Discovery* 7: 489. Doi:10.103/nrd2589.

GenBank www.ncbi.nlm.nih.gov/gene/1.

Gleeson, D. M., Heath, A. C. G., (1997). The population biology of the Australian sheep fly, *Lucilia cuprina*, in New Zealand. *New Zealand Journal of Agricultural Research* 40, 529-535.

Golglewski, R. P., Allerton, G. R., Rugg, D., Kawhia, D., Barrick, R. A., Eagleson, J. S. (1997). Demonstration of the sustained anthelmintic efficacy of a controlled-release capsule formulation of ivermectin in weaner lambs under field conditions in New Zealand. *New Zealand Veterinary Journal*, 45, 158-161.

Gopal, R., Birdsell, D., Monroy, F. P. (2008). Regulation of toll-like receptors in intestinal epithelial cells by stress and toxoplasma gondii infection. *Parasite Immunology* 30, 563 – 576.

Greeff, J. C., Safari, E., Fogarty, N., Hopkins, D., Brien, F., Atkins, K., Mortimer, S. I., Van Der Werf. J. (2008). Genetic parameters for carcass and meat quality traits and their relationships to liveweight and wool production in hogget Merino rams. *Journal of Animal Breeding and Genetics* 125, 205 - 215.

Greeff, J. C., Karlsson, L. J. E. (2009). Opportunities to breed for resistance to breech strike in Merino sheep in Mediterranean Environment. *Proceeding for the Association for the Advancement of Animal Breeding and Genetics* 18, 272 - 278.

Guerrini, V.H. (1988). Ammonia toxicity and alkalosis in sheep tnfested by *Lucilia cuprina* larvae. *International Journal for Parasitology* 18, 79-81.

Gupta, P., Roy, J., Prasad, M. (2001). Single nucleotide polymorphisms: a new paradigm for molecular marker technology and DNA polymorphism detection with emphasis on their use in plants. *Current Science* 80, 524-535.

Hacker, R. B. (2010). Extensive Grazing Systems. In *International sheep and wool handbook:* Cottle, D. J. Nottingham University Press. 523.

Harker, M., Carvell, A.-M., Marti, V.P., Riazanskaia, S., Kelso, H., Taylor, D., Grimshaw, S., Arnold, D. S., Zillmer, R., Shaw, J. Kirk, J. M., Alcasid, Z. M., Gonzales-Tanon, S., Chan, G. P., Rosing, E. A. E., Smith, A. M. (2014). Functional characterisation of a SNP in the ABCC11 allele –effects on axillary skin metabolism, odour generation and association behaviours. *Journal of dermatological science* 73, 23-30.

Harker, M., Carvell, A.-M., Marti, V.P., Riazanskaia, S., Kelso, H., Taylor, D., Grimshaw, S., Arnold, D. S., Zillmer, R., Shaw, J. Kirk, J. M., Alcasid, Z. M., Gonzales-Tanon, S., Chan, G. P., Rosing, E. A. E., Smith, A. M. (2014). Functional characterisation of a SNP in the ABCC11

allele –effects on axillary skin metabolism, odour generation and association behaviours. *Journal of dermatological science* 73, 23-30.

Hart, R. J., Cavey, W. A., Ryan, K. J., Strong, M. B., Moore, B., Thomas, P. L., Boray, J. C., von Orelli, M. CGA – 72662 – A new sheep blowfly insecticide. *Australian Veterinary Journal* 59, 104 – 109.

Hasegawa, Y., Yabuki, M., Matsukane, M. (2004). Identification of new odoriferous compounds in human axillary sweat. *Chemistry and Biodiversity* 1, 2042 -2050.

Hawn, T. R., Verbon, A., Janer, M., Zhao, L. P., Beutler, B., Aderem, A. (2005). Toll-like receptor 4 polymorphisms are associated with resistance to Legionnaires' disease. *Proceedings of the National Academy of Sciences of the United States of America* 102, 2487-2489.

Hay, J.B., Mills, S.C. (1982). Chemical changes in the wool wax of adult Merino sheep during prolonged wetting and prior to development of fleecerot. *Australian Journal of Agricultural Research* 33, 335-346.

Hayashi, K. (1999). Recent enhancements in SSCP. *Genetic Analysis: Biomolecular Engineering* 14, 193 – 196.

Hayman, R. (1953). Studies in fleecerot of sheep. *Crop and Pasture Science* 4, 430-463.

Heath, A.C.G. (1994). Ectoparasites of livestock in New Zealand. *New Zealand Journal of Zoology* 21, 23-38.

Heath, A.C.G., Bishop, D.M. (1995). Fly-strike in New Zealand. *Surveillance (Wellington)* 22, 11-13.

Heath, A.C.G., Bishop, D.M., (2006). Fly-strike in New Zealand: An overview based on a 16-year study, following the introduction and dispersal of the Australian sheep fly, *Lucilia cuprina* Wiedemann (Diptera: Calliphoridae). *Veterinary Parasitology* 137, 333-344.

Helmby, H., Grenise, R. K. (2003). Essential role for TLR4 and MyD88 in the development of chronic intestinal nematode infection. *European Journal of Immunology* 33, 2974 – 2979.

Henderson, A.E. (1965). *Relationship of wool follicle and wool fibre dimensions*: Lincoln College.

Higgins, C. F. (1992). ABC transporters: from microorganisms to man. *Annual review of cell biology* 8, 67-113.

Holloway, B. A. (1991). Identifcation of third-instar larvae of fly-strike and carrion-associated blowflies in New Zealand (Diptera: Calliphoridae). *New Zealand Entomology* 14, 24-128.

Horton, B. J.., Best, D. J., Butler, L. G., Gregory, G. G. (1997). Organophosphorus residues in wool grease resulting from specified on-farm lice and flystrike control treatments. *Australian Veterinary Journal* 75, 500-503.

Horton, J.D., Champion, S.C. (2001). Wool producer's knowledge of flystrike control. *Symposium conducted at the meeting of the Proceedings of the FLICS Conference*. Launceston, Tasmania, Australia. 433-442.

https://digitalinsectcollection.wikispaces.com/Common+Green+Bottle+Fly

http://agriculture.vic.gov.au/__data/assets/image/0010/226666/AG0081b.jpg

http://agriculture.vic.gov.au/agriculture/pests-diseases-and-weeds/pest-insects-and-mites/sheep-blowflies-in-victoria

http://www.terrain.net.nz/friends-of-te-henui-group/local-flies/fly-green-bottle-lucilia-sericata.html 5/6/2017.

http://www.terrain.net.nz/friends-of-te-henui-group/local-flies/fly-hairy-maggot-blow-fly-chrysomya-rufifacies.html 5/6/2017.

http://www.thefarmpage.com/wordpress/wp-content/uploads/2011/06/blowfly-2-300x205.jpg

https://www.agric.wa.gov.au/livestock-parasites/managing-non-mulesed-sheep?page=0 %2C2 24/8/2017.

https://www.agric.wa.gov.au/livestock-parasites/managing-non-mulesed-sheep?page=0 %2C2 24/8/2017.

https://www.agric.wa.gov.au/livestock-parasites/managing-non-mulesed-sheep?page=0 %2C2 24/8/2017

Hughes, P. B., Levot, G. W. (1987). Simulation of fly-waves to assess the ability of diflubenzuron to protect sheep against flystrike by *Lucilia cuprina. Veterinary Parasitology* 24, 275-284.

Ishikawa, T., Toyoda, Y., Yoshiura, K.-I., Niikawa, N. (2013). Pharmocogenetics of human ABC transporter ABCC11: new insights into apocrine gland growth and metabolite secretion. *Frontiers in genetics* 3, 306.

James, P.I., Warren, G.H., Neville, A. (1984). The effect of some fleece characters on the skin wax layer and fleecerot development in Merino sheep following wetting. *Australian Journal of Agricultural Research* 35, 413-422.

James, P. J., Ponzoni, R. W. (1992). Fibre diameter variability in South Australian Merinos-phenotypic and genetic relationships with wool quality parameters and fleece rot resistance. *Wool technology and sheep breeding* 40, 25-26.

James, P.I. (2006). Genetic alternative to mulesing and tail docking in sheep: a review. *Australian Journal of Experimental Agriculture* 64, 1-18.

Jiang, Y., Xie, M., Chen, W., Talbot, R., Maddox, J. F., Faraut, T., Wu, C., Muzny, D. M., Li, Y., Zhang, W., Stanton, J., Brauning, R., *et al*. (2014). The sheep genome illuminates biology of the rumen and lipid metabolism. *Science* 344, 1168-1173.

Karlsson, L.J.E., Greeff, J.C. (2012). Genetic aspects of sheep parasitic diseases. *Veterinary Parasitology* 189, 104-112.

Kawai, T., Akira, S. (2006). Innate immune recognition of viral infections. *Nature Immunology* 7, 131 – 137.

Kawai, T., Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature Immunology* 11, 373- 384.

Kawai, T., Akira, S. (2011). Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 34, 637- 350.

Kerlin, R. L., East, I. J., (1992). Potent immunosuppression by secretory/excretory products of larvae from the sheep blowfly *Lucilia cuprina. Parasite Immunology* 14, 595 -604.

Khoga, J. M., Tóth, E., Márialigeta, K., Borossay, J. (2002). Fly-attracting volatiles produced by Rhodococcus fascians and Mycobacterium aurum isolated from myiatic lesions of sheep. *Journal of Microbiology Methods* 48, 281-287.

Klein, I., Sarkadi, B., Vàradi, A. (1999). An inventory of the human ABC proteins. *Biochimica et Biophysica Acta (BBA)-Biomembranes* 1461, 237-262.

KosiK-Bogacka, D. I., Wojtkowiak-Giera, A., Kolasa, A., Salamatin, R., Jagodzinski, P. P., Wandurska-Nowak, E. (2012). Hymenolepis diminuta: Analysis of the expression of Toll-like receptor genes (TLR2 and TLR4) in the small and large intestines of rats. *Experimental Parasitology* 130, 261 – 266.

KosiK-Bogacka, D. I., Wojtkowiak-Giera, A., Kolasa, A., Czernomysy-Furowicz, D., Lanocha, N., Wandurska-Nowak, E., Salamatin, R., Jagodzinski, P. P. (2013). Hymenolepis diminuta: Analysis of the expression of Toll-like receptor genes (TLR2 and TLR4) in the small and large intestines of rats. Part II. *Experimental Parasitology* 135, 437 – 445.

Kostka, G., Giltay, R., Bloch, W., Addicks, K., Timpl, R., Fässler, R., Chu, M.-L. (2001). Perinatal lethality and endothelial cell abnormalities in several vessel compartments of fibulin-1-deficient mice. *Molecular and cellular biology* 21, 7025-7034.

Kruh, G. D., Guo, Y., Hopper-Borge, E., Belinsky, M. G., Chen, Z.-S. (2007). Abcc10, abcc11, and abcc12. *Pflügers Archiv-European Journal of Physiology* 453, 675-684.

Lambers, H., Piessen, S., Bloem, A., Pronk, H., Finkel, P (2006). Natural skin surface pH is on average below 5, which is beneficial for its resident flora. *Internalional Journal of Cosmetic Science* 28, 359 – 370.

Lang, M., Allen, G., Horton, B. (2001). The utilisation of carcasses by *Lucilia cuprina* (Weidemann) as a breeding site in Tasmania. *Symposium conducted at the meeting of the Proceedings of the FLICS Conference* 71 – 79.

Leathwick, D., Atkinson, D. (1995). Dagginess and flystrike in lambs grazed on Lotus corniculatus or ryegrass. *Symposium conducted at the meeting of the Proceedings-New Zealand Society of Animal Production* 55, 196 - 198.

Le Hir, H., Nott, A., Moore, M. J., (2003). How introns influence and enhance eukaryotic gene expression. *Trends in Biochemical Sciences* 28, 215 - 220.

Lee, C., Fisher, A. D. (2007). Welfare consequences of mulesing of sheep. *Australian Veterinary Journal* 85, 89 - 93.

Lee, S. H., Yun, S., Lee, J., Kim, M. J., Piao, Z-H., Jeong, M., Chung, J. W., Kim, T-D., Yoon, S. R., Greenberg, P. D., Choi, I. (2009). RASGRP1 is required for human NK cell function. *The Journal of Immunology* 183, 7931 – 7938.

Levot, G. W. (1993). Insecticide resistance: new developments and future options for fly and lice control on sheep. *Wool Technology and Sheep Breeding* 42, 108 – 119.

Levot, G. W., (1995). Resistance and the control of sheep ectoparsites. *International Journal for Parasitology* 25, 1355 - 1362.

Levot, G. W., Sales, N. (1997). Insecticide residues in wool from sheep jetted by hand and via automatic jetting races. *Australian Journal of Experimental Agriculture* 37, 737 - 742.

Levot, G. W., Sales, N., Barchia, I. (1999). *In vitro* larvicidal efficacy of flystrike dressings against the Australian sheep blowfly. *Australian Journal of Experimental Agriculture* 39, 541 - 547.

Levot, G. W., Sales, N. (2002). New high level resistance to Diflubenzuron detected in the Australian Sheep Blowfly, *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae). *General applied entomology* 31, 43 - 46.

Levot, G. W. (2016). Sheep blowflies. NSW Department of Primary Industries/PrimeFact, 485, 2-3. https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0005/.../Sheep-blowflies.pdf. 8/12/2017.

Leyden, J. J., McGinley, K. J., Hölzle, E., John, M. D., Labow, J. N., Mligman, A. M. (1981). The microbiology of the human axilla and its relationship to axillary odor. *Journal of Investigative Dermatology* 77, 413 - 416.

Lim K. H., Staudt, L. M. (2013). Toll-Like Receptor Signaling. Cold Spring Harbour Perspectives in Biology. http://cshperspectives.cshlp.org/content/5/1/a011247.full.pdf+html.

Lin, M. H., Khnykin, D. (2014). Fatty-acid transporters in skin development, function and disease. *Biochimica et Biophysica Acta* 1-7.

Lin, Y.-S., Zhou, H., Forrest, R. H. J., Frampton, C. M. A., Burrows, L. E. R., Hickford, J. G. H. (2016). Association between variation in faecal egg count for a natural mixed field-challenge of nematode parasites and TLR4 variation. *Veterinary parasitology* 218, 5-9.

Lipson, M., Hilton, R. A., Watts, J. E., Merritt, G. C. (1982). Factors influencing fleece rot in sheep. *Australian Journal of Experimental Animal Husbandry* 22, 168 – 172.

Liu, Y., Zhu, M., Nishida, K., Hirano, T., Zhang, W. (2007). An essential role for RASGRP1 in mast cell function and IgE-mediated allergic response. *The Journal of experimental medicine* 204, 93 -103.

Lucas, P., Horton, B. (2013). Comparative costs, chemical treatments and fly-strike rates in mulesed and unmulesed sheep flocks as predicted by a weather-driven model. *Animal Production Science* 53, 342 - 351.

Ludemann, C., Amer, P., Sise, J. (2010). Value propositions and trait prioritisation for the New Zealand sheep industry, AbacusBio Limted, Dunedin, New Zealand.

Lyne, A. G., Hollis, D. E. (1968). The skin of the sheep: A comparison of Body Regions. *Australian Journal of Biological Sciences* 21, 499 - 527.

MacDiarmid, J., Clarke, R., McClure, S., Bowen, F., Burrell, D. (1995). Use of a monoclonal antibody to ovine IgE for fly strike studies in sheep. *International Journal for Parasitology* 25, 1505 - 1507.

Mackerras, M. J. Fuller, M. E., Austin, K., Lefroy, E. (1936). Sheep Blowfly investigations. The effect of trapping on the incidence of strike in sheep. *Journal of the Council for Scientific and Industrial Research, Australia,* 9, 153 - 162.

Makowski, L., Brittingham, K. C, Reynolds, J. M., Suttles, J., Hotamisligil. G. S. (2005). The fatty acid-binding protein, aP2, corrdinates macrophages cholestrerol trafficking and inflammatory activity. *The Journal of Biological Chemiostry* 280, 12888 – 12895.

Mantovani, A., Allavena, P., Sica, A., Balkwill, F. (2008). Cancer-related inflammation. *Nature* 454, 436 - 444.

Martin, A., Saathoff, M., Kuhn, F., Max, H., Terstegen, L., Natsch, A. (2010). A functional ABCC11 allele is essential in the biochemical formation of human axillary odor. *Journal of Investigative Dermatology* 130, 529 - 540.

McGuirk, B. J., Atkins, D.A., Kowal, E., Thornberry, K. (1978). Breeding for resistance to fleecerot and body strike- the Trangie programme. *Wool Technology and Sheep Breeding* 26, 17 - 24.

Merritt, G. C., Watts, J. E. (1978). The changes in protein concentration and bacteria of fleece and skin during the development of fleecerot and body strike in sheep. *Australian Veterinary Journal* 54, 517 – 520.

Michalik, L., Wahli, W. (2007). Peroxisome proliferator-activated receptors (PPARs) in skin health, repair and disease. *Biochimica et Biophysica Acta* 1771, 991 - 998.

Miura, K., Yoshiura, K.-I., Miura, S., Shimada, T., Yamasaki, K., Yoshida, A., Yoshida, A., Nakayama, D., Shibata, Y., Niikawa, N. (2007). A strong association between human earwax-type and aprocrine colostrum secretion from the mammary gland. *Human Genetics* 121, 631 - 633.

Mockenhaupt, F. P., Cramer, J. P., Hamann, L., Stegemann, M. S., Eckert, J., Oh, N.-R., Otchwemah, R. N., Dietz, E., Ehrhardt, S., Schröder, N. W. J., Bienzle, U., Schumann, R. R. (2006). Toll-like receptor (TLR) polymporphisms in African children: common TLR-4 variants presidpose to severe malaria. *Proceedings of the National Academy of Sciences of the United States of America* 103, 177 - 182.

Molyneux, A. S., Bedding, R.A. (1984). Influence of soil texture and moisture on the infectivity of *Heterorhabditis Sp*. D1 and *Steinernema glaseri* for larvae of the sheep fly, *Lucilia cuprina*. *Nematogoica* 30, 358 - 365.

Morley, M., Donald, A., Donnelly, J., Axelsen, A., Waller, P. (1976). Blowfly strike in the breech region of sheep in relation to helminth infection. *Australian Veterinary Journal* 52, 325 - 329.

Morris, M. C., Woolhouse, A.D., Rabel, B., Joyce, M.A. (1998). Orientation stimulants from substances attractive to *Lucilia cuprina* (Diptera, Calliphoridae). *Australian Journal of Experimental Agriculture* 38, 461 - 468.

Morris, C. A. (2009). Review of genetic parameters for disease resistance in sheep in New Zealand and Australia. *Proceedings for the Association for the Advancement of Animal Breeding and Genetics* 18, 263 – 271.

Mortimer, S., Henshall, J., Tier, B. (2001). Major gene effects on resistance to body strike and fleece rot in Merino sheep revisited. *Proceedings for the association for the Advancement of Animal Breeding and Genetics* 14, 171 - 174.

Mucha, R., Bhide, M., Chakurkar, E., Novak, M., Mikula, I. (2009). Toll-like receptors TLR1, TLR2 and TLR4 gene mutations and natural resistance to *Mycobacterium avium subsp*. *Paratuberculosis* infection in cattle. *Veterinary Immunology and Immunopathology* 128, 381-388.

Nakano, N., Mica, N., Hirano, A., Yoshiura, K., Niikawa, N. (2009). A strong association of axillary osmidrosis with the wet earwax determined by genotyping of the ABCC11 gene. *BMC Genetics* 10.

Natsch, A., Gfeller, H., Gygax, P., Schmid, J., Acuna, G. (2003). A specific bacterial aminoacylase cleaves odorant precursors secreted in the human axilla. *The Journal of Biological Chemistry* 278, 5718 - 5727.

Natsch, A., Schmid, J., Flachsmann, F. (2004). Identification of odoriferous sulfanylalkanols in human axilla secretions and their formation through cleavage of cysteine precursors by a C_S Lyase isolated from axilla bacteria. *Chemistry and Biodiversity* 1, 1058 – 1072.

Natsch, A., Derrer, S., Flachsmann, F., Schmid, J. (2006). A broad diversity of volatile carboxylic acids, released by a bacterial, determination of human-body odor type. *Chemistry and Biodiversity* 3, 1 -20.

Newton, K., Dixit, V. M. (2012). Signaling in innate immunity and inflammation. *Cold Spring Harbour Perspectives in Biology.* http://cshperspectives.cshlp.org/content/4/3/a006049.full.pdf+html.

Noreen, M., Shah, M. A. A., Mall, S. M., Choudhary, S., Hussain, T., Ahmed, I., Jalil, S. F., Raza, M. I. (2012). TLR4 polymorphisms and disease susceptibility. *Inflammation Research* 61, 177 - 188.

Norment, A. M.., Bogatzki, L. Y., Klinger, M., Ojala, E. W., Bevan, M. J., Kay, R. J. (2003). Transgenic expression of RasGRP1 induces the maturation of double-negative thymocytes and enhances the production of CD8 single-positive thymocytes. *Journal of Immunology* 170, 1141 -1149.

Norris, B.J., Colditz, I.G., Dixon, T.J., (2008). Fleecerot and dermatophilosis in sheep. *Veterinary Microbiology* 128, 217 - 230.

Norris, K.R. (1965). The bionomics of blow flies. *Annual Review of Entomology* 10, 47 - 68.

Nottingham, R.M., Hosking, B.C., Schmid, H.R., Strehlau, G., Junquera, P. (2001). Prevention of blowfly strike on coarse and fine woolled sheep with the insect growth regulator dicyclanil. *Australian Veterinary Journal* 79, 51 - 57.

O'Donnell, I., Green, P., Connell, J., Hopkins, P. (1980). Immunoglobulin G antibodies to the antigens of *Lucilia cuprina* in the sear of fly-struck sheep. *Australian journal of Biological Sciences* 33, 27 - 34.

O'Meara, T. J., Nesa, M., Raadsma, H. W., Saville, D. G., Sandeman, R. M. (1992). Variation in skin inflammatory responses between sheep bred for resistance or susceptibility to fleece rot and blowfly strike. *Research in Veterinary Science* 52, 205 - 210.

O'Meara, T., Nesa, M., Seaton, D. S., Sandeman, R. M. (1995). A comparison of inflammatory exudates released from myiasis wounds on sheep bred for resistance or susceptibility to *Lucilia cuprina*. *Veterinary Parasitology* 56, 207 – 223.

O'Meara, T. J., Nesa, M., Sandeman, R. (1997). Antibody responses to *Lucilia cuprina* in sheep selected for resistance or susceptibility to *L. cuprina*. *Parasite Immunology* 19, 535 - 543.

O'Sullivan, B.M., Hopkins, P.S., Connell, J.A., (1984). The pathogenesis of fly-strike in sheep. *Animal Production in Australia* 15, 171 - 181.

Oddy, H., Dalrymple, B., McEwan, J., Kijas, J., Haye, B., van der Werf, J., Emery, D., Hynd, P., Longhurst, T., Fischer, T., Ferguson, D., Forage, R., Cockett, N., Nicholas, F. (2007). Sheep gemonics and the international sheep genomics consortium. *Symposium conducted at the meeting of the Proceedings of the Association for the Advancement of Animal Breeding and Genetics* 17, 411 – 417.

Ohara, T., Morishita, T., Suzuki, H., Hibi, T. (2006). Heterozygous Thr 135 Ala polymorphism at leucine-rich repeat (LRR) in genomic DNA of toll-like receptor 4 in patients with poorly-differentiated gastric adenocarcinomas. *International Journal of Molecular Medicine* 18, 59-64.

Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., Sekiya, T. (1989). Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proceedings of the National Academy of Science* 86, 2766 – 2770.

Ota, I., Sakurai, A., Toyoda, Y., Morita, S., Sasaki, T., Chishima, T., Yamakado, M., Kawai, Y., Ishidao, T., Lezhava, A., Yoshiura, K- I., Togo, S., Hayashizaki, Y., Ishikawa, T., Ishikawa, T., Endo, I., Shimada, H. (2010). Association between breast cancer risk and the wild-type allele of human ABC transporter ABCC11. *Anticancer research* 30, 5189 - 5194.

Otranto, D. (2001). The immunology of myiasis: parasite survival and host defence strategies. *Trends in Parasitology* 17, 176 - 182.

Pan, T.-C., Kostka, G., Zhang, R.-Z., Timpl, R., Chu, M.-L. (1999). Complete exon-intron organization of the mouse fibulins-1 gene and its comparison with the human fibulin-1 gene. *FEBS Letters* 444, 38 - 42.

Pethick, D. W., Ball, A.J., Banks, R.G., Hocquette, J.F. (2011). Current and future issues facing red meat quality in a competitive market and how to manage continuous improvement. *Animal Production Science* 51, 31 - 18.

Phillips, C. J. (2009). A review of mulesing and other methods to control flystrike (cutaneous myiasis) in sheep. *Animal Welfare* 18, 113 - 121.

Pickering, N. K., Dodds, K.G., Blair, H. T., Hickson, R.E., Johnson, P. L., McEwan, J. C., (2012). Estimated of genetic parameters for fly-strike in New Zealand Romney and Romney cross sheep. *Proceedings of the New Zealand Society of Animal Production* 72, 189 -191.

Pickering, N. K. (2013). Genetics of flystrike, dagginess and associated traits in New Zealand dual-purpose sheep: a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Animal Science at Massey University, Palmerston North, New Zealand.

Pickering, N. K., Blair, H. T., Hickson, R. E., Johnson, P. L., Dodds, K., McEwan, J. C. (2015). Estimates of genetic parameters for breech strike and potential indirect indicators in sheep. *New Zealand Veterinary Journal*, 63, 98 - 103.

Pitts, K., Wall, R. (2005). Winter survival of larvae and pupae of the blowfly, *Lucilia sericata* (Diptera: Calliphoridae). *Bulletin of entomological research* 95, 179 - 186.

Plant, J. W., Coombes, D., (1988). Sheep fly-strike and mulesing. *Parasitology Today* 4, 253-254.

Plant, J. W., Horton, B. J., Armstrong, R. T. F., Campbell, N. J. (1999). Modelling pesticide residues on greasy wool: using organophosphate and synthetic pyrethroid survey data. *Australian Journal of Experimental Agriculture* 39, 9 – 19.

Priatel, J.J., X Chen, L, A. Zenewicz., H. Shen., K.W., Harder., M.S. Horwitz., H.-S. Teh. (2007). Chronic immunodeficiency in mice lacking RASGRP1 results in CD4 T cell immune activation and exhaustion. *Journal of Immunology* 179, 2143 - 2152.

Priatel, J. J., Teh, S.-J., Dower, N. A., Stone, J. C., Teh, H.-S. (2002). RASGRP1 transduces low-grade TCR signals which are critical for T cell development, homeostasis, and differentiation. *Immunity* 17, 617 - 627.

Raadsma, H.W. (1987). Flystrike control: an overview of management and breeding options. *Wool Technology and Sheep Breeding* 35, 174 - 185.

Raadsma, H. W., Gilmour, A. R., and Paxton, W. J. (1989). Fleecerot and body strike in Merino sheep. II. Phenotypic and genetic variation in liability to fleecerot following experimental induction. *Australian Journal of Agricultural Research* 40, 207 - 20.

Raadsma, H. W. (1991). Fleece rot and body strike in Merino sheep. V. Heritability of liability to body strike in weaner sheep under flywave condition. *Crop and Pasture Science* 42, 279 - 293.

Raadsma, H. W. (1993). Fleece rot and body strike in Merino sheep. VI. Experimental evaluation of some physical fleece and body characteristics as indirect selection criteria for fleece rot. *Crop and Pasture Science* 44, 915-931.

Raadsma, H. W. (2000). Genetic aspects of resistance to ovine cutaneous myiasis. In Axford, R. E. F., Owen, J. B. Breeding for disease resistance in Farm Animals, 171- 194.

Rallabhandi, P., Bell, J., Boukhvalova, M. S., Medvedev, A., Lorenz, E., Arditi, M., Hemming, V. G., Blanoc, J. C. G., Segal, D. M., Vogel, S. N. (2006). Analysis of TLR4 polymorphic variants: new insights into TLR4/MD-2/CD14 stoichiometry, structure, and signaling. *The Journal of Immunity* 177, 322-332.

Rammell, C.G., Bentley, G.R. (1988). Organophosphate residues in the wool of sheep dipped for flystrike control. *New Zealand Journal of Agricultural Research* 31, 151-154.

Roark, E. F., Keene, D. R., Haudenschild, C. C. Godyna, S., Little, C. D., Argraves, W. S. (1995). The association of human fibulin-1 with elastic fibers: an immunohistological, ultrastructural, and RNA study. *Journal of Histochemistry and Cytochemistry* 43, 401 – 411.

Roberts, D.S. (1963). Barriers to *Dermatophilus dermatonomus* infection on the skin of sheep. *Australia Journal of Agricultural Research* 14, 492-508.

Rodriguez, S., Steer, C. D., Farrow, A., Golding, J., Day, I. N. (2013). Dependence of deodorant usage on ABCC11 genotype: scope for personalized genetics in personal hygiene. *Journal of Investigative Dermatology* 133, 1760-1767.

Roose, J. P., Mollenauer, M., Gupta, V. A., Stone, J., Weiss, A. (2005). A diacylglycerol-protein kinase C-RasGRP1 pathway directs Ras activation upon antigen receptor stimulation of T cells. *Molecular and Cellular Biology* 25, 4426 – 4441.

Rothwell, J. (2005). Modern chemical treatments for sheep infested with external parasites. *Ectoparasiticide use in contemporary Australian Livestock Production* 33.

Russell, I. (1994). Pesticides in wool: downstream consequences. *Wool Technology and Sheep Breeding* 42, 344 – 349.

Sandeman, R. M., Bowles, V. M., Stacey, I. N., Carnegie, P. R. (1986). Acquired resistance in sheep to infection with larvae of the blowfly, *Lucilia cuprina*. *International Journal of Parasitology* 16, 69-75.

Sandeman, R.M., Collins, B.J., Carnegie, P.R. (1987). A scanning electron microscope study of *L. cuprina* larvae and the development of blowfly strike in sheep. *International Journal for Parasitology* 17, 759-765.

Sandeman, R. M., Bowles, V. M., Colwell, D. D. (2014). The immunology of myiasis infections – whatever happened to vaccination? *Parasite Immunology* 36, 605 -615

Scholtz, A.J., Cloete, S.W.P., van Wyk, J.B., Misztal, I., du Toit, E., de K. van der Linde, T.C. (2010). Genetic (co)variances between wrinkle score and absence of breech strike in mulesed and unmulesed Merino sheep, using a threshold model. *Animal Production Science* 50, 210-218.

Scobie, D., O'Connell, D., Bray, A., Cunningham, P. (2002). Breech strike can be reduced by increased area of naturally bare skin around the perineum of lambs. *Symposium conducted at the meeting of the Proceedings the Australian Society of Animal Production* 24, 201 – 204.

Scobie, D., O'Connell, D., Morris, C., Hickery, S. (2007). A preliminary genetics analysis of breech and tail traits with the aim of improving the welfare of sheep. *Crop and Pasture Science* 58, 161-167.

Scobie, D., O'Connell, D. (2010). Breech bareness reduces flystrike in New Zealand crossbred sheep. *Animal Production Science* 50, 599-602.

Seaton, D. S., O'Meara, T. J., Chandler, R. A., Sandeman, R. M. (1992). The sheep antibody response to repeated infection with *Lucilia cuprina. International Journal for Parasitology* 22, 1169 – 1174.

Seddon, H. R., Belschner, H. G., Mulhearn, C. R. (1931) Studies on cutaneous myiasis of sheep (sheep blowfly attack). *Department of NSW Agriultural Science Bullentin. No 37*.

Segade, F. (2010). Molecular evolution of the fibulins: implications on the functionality of the elastic fibulins. *Gene* 464, 17-31.

Shimizu, H., Taniguchi, H., Hippo, Y., Hayashizaki, Y., Aburatani, H., Ishikawa, T. (2003). Characterization of the mouse *Abcc12* gene and its transcript encoding an ATP-binding cassette transporter, an orthologue of human ABCC12. *Gene* 310, 17 -28.

Smirnova, I., Hamblin, M. T., McBride, C., Beutler, B., Di Rienzo, A. (2001). Excess of rare amino acid polymorphism at the toll-like receptor 4 in humans. *Genetics* 158, 1657-1664.

Smith, J. L., Brewer, H. G., Dyall, T. (2009). Heritability and phenotypic correlations for breech strike and breech strike resistance indicators in Merinos. *Proceeding for the Association for the Advancement of Animal Breeding and Genetics* 18, 334-337.

Smith, K.E., Wall, R. (1998). Estimates of population density and dispersal in the fly *Lucilia sericata* (Diptera: Calliphoridae). *Bulletin of Entomological Research* 88, 65-73.

Smith, W.J.M., Li Y., Ingham, A., Collis, E., McWilliam, S.M., Dixon, T.J., Norris, B.J., Mortimer, S.I., Moore, R.J., Reverter, A. (2010). A genomics-informed, SNP associated study reveals FBLN1 and FABP4 as contributing to resistance to fleecerot in Australian Merino sheep. *BMC Veterinary Research* 6, 27.

Sneddon, J., Rollin, B. (2010). Mulesing and animal ethics. *Journal of Agricultural and Environmental Ethics* 23, 371-386.

Starkenmann, C., Niclass, Y., Troccaz, M., Clark, A. J. (2005). Identification of the precursor of (S)-3-methyl-3-sulfanylhexan-1-ol, the sulfury malodour of human axilla sweat. *Chemistry and Biodiversity* 2, 705 -716.

Stevens, J. R., Wall, R. (1996). Species, sub-species and hybrid populations of the blowflies *Lucilia cuprina* and *Lucilia sericata* (Diptera: Calliphoridae). *Proceedings of the Royal Society of London B: Biological Sciences* 263, 1335-1341.

Stevens, J.R. (2003). The evolution of myiasis in blowflies (Calliphoridae). *International Journal for Parasitology* 33, 1105-1113.

Stone, J. C. (2011). Regulation and function of the RASGRP family of Ras activators in blood cells. *Genes and Cancer* 2, 320-334.

Supek, F., Miñana, B., Valcárcel, J., Galbaldón, T., Lehner, B. (2014). Synonymous mutations frequently act as driver mutations in human cancers. *Cell* 156, 1324-1335.

Sultzer, B. M. (1968). Genetic control of leucocyte responses to endotoxin. *Nature* 219, 1253-1254.

Suzuki, A., Itamia, S., Ohishi, M., Hamada, K., Inoue, T., Komazawa, N., Senoo, H., Sasaki, T., Takeda, J., Manabe, M., Mak, T.W., Nakano. (2003). Keratinocyte-specific Pten deficiency results in epidermal hyperplasia, accelerated hair follicle morphogenesis and tumor formation. *Cancer Research* 63: 674-681.

Takeda, K., Kaisho, T., Akira, S. (2003). Toll-like receptors. *Annual review of Immunology* 21, 335-376.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., Akira, S. (1999). Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 11, 443 – 451.

Taneera, J., Lang, S., Sharma, A., Fadista, J., Zhou, Y., Ahlqvist, E., Jonsson, A., Lyssenko, V., Vikman, P., Hansson, O., Parikh, H. (2012). A systems genetics approach identifies genes and pathways for type 2 diabetes in human islets. *Cell Metabolism* 16, 122 – 134.

Taylor, D. L., Zhong, L., Begg, D. J., de Silva, K., Whittington, R. J. (2008). Toll-like receptor genes are differentially expressed at the sites of infection during the progression of Johne's disease in outbred sheep. *Veterinary Immunology and Immunopathology* 124, 132-151.

Tellam, R.L., Bowles, V.M. (1997). Control of fly-strike in sheep: Current strategies and future prospects. *International Journal for Parasitology* 27, 261-273.

Toyoda, Y., Ishikawa, T. (2010). Pharmacogenomics of human ABC transporter ABCC11 (MRP8): potential risk of breast cancer and chemotherapy failure. *Anti-Cancer Agents in Medicinal Chemistry* 10, 617-6254.

Troccaz, M., Starkenmann, C., Niclass, Y., van de Wall, M., Clark, A. J. (2004). 3-Methyl-3-sulfanylhexan-1-ol as a major descriptor for the human axilla-sweat odour profile. *Chemistry and Biodiversity* 1, 1022 -1035.

Tsuda, M., Inoue-Nartia, T., Suzuki, A., Itami, S., Blumenberg, M., Manabe, M. (2009). Induction of gene encoding FABP4 in Pten-null keratinocytes. *FEBS Letters* 583, 1319-1322.

Tuncman, G., Erbay, E., Hom, X., De Vivo, I., Campos, H., Rimm, E.B., Hotamisligil, G.S. (2006). A genetic variant at the fatty-acid-binding protein aP2 locus reduces the risk for hypertriglyceridemia type 2 diabetes, and cardiovascular disease. *PNAS* 103, 6970-6975.

Tusnády, G. E., Sarkadi, B., Simon, I., Váradi, A. (2006). Membrane topology of human ABC proteins. *FEBS letters* 580, 1017-1022.

Urech, R., Green, P.E., Rice, M.J., Brown, G.W., Duncalfe, F., Webb, P. (2004). Compostion of chemical attractants affects trap catches of the Australian sheep fly, *Lucilia cuprina,* and other flies. *Journal of Chemical Ecology* 30, 851-866.

Urech, R., Green, P.E., Rice, M.J., Brown, G.W., Webb, P., Jordan, D., Wingett, M., Mayer, D.G., Butler, L., Joshua, E., Evans, I., Toohey, L., Dadour, I.R. (2009). Suppression of populations of Australian sheep fly, *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae), with a novel fly trap. *Australian Journal of Entomology* 48, 182-188.

Valachová. I., Bohová, J., Kozánek, M., Takáč, T., Majtán. J. (2013). *Lucilia sericata* medicinal maggots: a new source of antimicrobial compounds. *Microbial pathogens and strategies for combating them: science, technology and education* (A. Méndez-Vilas, Ed.) 1745 – 1753.

Vignal, A., Milan, D., SanCristobal, M., Eggen, A. (2002). A review on SNP and other types of molecular markers and their use in animal genetics. *Genetics Selection Evolution* 34, 275-306.

Volk, M. G. (2007). An examination of the evidence supporting the association of dietary cholesterol and saturated fats with serum cholesterol and development of coronary heart disease. *Alternative Medicine Review* 12, 228-245.

Waghorn, G.C., Gregory, N.G., Todd, S.E., Wesselink, R. (1999). Dags in sheep: a look at faeces and reasons for day formation. *Proceedings of the New Zealand Grassland Association* 61, 43-49.

Wall, R., French, N., Morgan, K. (1992). Effects of temperature on the development and abundance of the sheep blowfly *Lucilia sericata* (Diptera: Calliphoridae). *Bulletin of Entomological Research* 82, 125-131.

Wardhaugh, K., Morton, R. (1990). The incidence of flystrike in sheep in relation to weather conditions, sheep husbandry, and the abundance of the Australian sheep blowfly, *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae). *Australian Journal of Agricultural Research* 41, 1155 – 1167.

Wardhaugh, K., Mahon, R., Bedon, D. (2001). Factors affecting the incidence of flystrike in sheep – a description and analysis of data from three separate areas in eastern Australia. FLICS: *Flystrike and Lice IPM Control Strategies* 80-87.

Watanabe, R., Fujiib, H., Yamamotoa, A., Hashimotoa, T., Kamedac, K., Itoa, M., Onob, T. (1997). Immunohistochemical distribution of cutaneous fatty-acid-binding protein in human skin. *Journal of Dermatological Science* 16, 17 – 22.

Watson, J., Riblet, R., Taylor, B. A., (1977). The response of recombinant inbred stains of mice to bacterial lipopolysaccharides. *The Journal Immunology* 118, 2088-2093.

Watts, J.E., Murry, M.D., Grahma, N.P.H. (1979). The blowfly strike problem of sheep in New South Wales. *Australian Veterinary Journal* 55, 325-334.

Weiss, D. S., Raupach, B., Takeda, K., Akira, S., Zychlinsky, A. (2004). Toll-like receptors are temporally involved in host defense. *The Journal of Immunology* 172, 4463-4469.

Werling D., Coffey, T. J. (2007). Pattern recognition receptors in companion and farm animals – The key to unlocking the door to animal disease? *The Veterinary Journal* 174, 240 -251.

West, D.M., Brue're, A.N., Rider, A.L., (2009). The Sheep, Health, Disease and Production. 3rd Edition. Published by VetLearn Foundation.

White, S. N., Taylor, K. H., Abbey, C. A., Gill, C. A., Womack, J. E. (2003). Haplotype variation in bovine Toll-like receptor 4 and computational prediction of a positively selected ligand-binding domain. *Proceedings of the National Academy of Sciences* 100, 10364-10369.

Yabuuchi, H., Shimizu, H., Takayanagi, S.-I., Ishikawa, T. (2001). Multiple splicing variants of two new human ATP-binding cassette transporters, ABCC11 and ABCC12. *Biochemical and Biophysical Research Communications* 288, 933-939.

Yamada, A., Takabe, K., Terracina, K. P., Ishikawa, T., Endo, I. (2014). Gene section. http://AtlasGeneticsOncology.org. 540.

Yan, W., Zhou, H., Luo, Y., Hu, J., Hickford, J. G. H., (2012). Allelic variation in ovine fatty-acid binding protein (FABP4) gene. *Molecular Biology Reports* 39, 10621-10625.

Yan, W., Zhou, H., Hu, J., Luo, Y., Hickford, J. G. H., (2018, Unpublished Manuscript). Sequence variation in the FABP4 gene and its association with growth and carcass traits in sheep.

Yasuda, S., Stevens, R. L., Terada, T., Takeda, M., Hashimoto, T., Fukae, J., Horita, T., Kataoka, H., Atsumi T., Koike, T. (2007). Defective expression of Ras guanyl nucleotide-releasing protein 1 in a subset of patients with systemic lupus erythematosus. *The Journal of Immunology* 179, 4809-4900.

Yue, Y., Guo, T., Liu, J., Guo, J., Yuan, C., Feng, R., Niu, C., Sun, X., Yang, B. (2015). Exploring differently expressed genes and natural antisense transcripts in sheep (Ovis aries) skin with different wool fiber diameters by digital gene expression profiling. *PLOS one* 10, 1-18.

Zhou, H., Hickford, J. G. H., Fang, Q. (2006). A two-step procedure for extracting genomic DNA from dried blood spots on filter paper for polymerase chain reaction amplification. *Analytical Biochemistry* 354, 159-161.

Zhou, H., Hickford, J. G. H., Fang, Q., Lin, Y-S. (2007). Allelic variation of the ovine Tool-like receptor 4 gene. *Development and Comparative Immunology* 31, 105-108.

Zhou, H., Hickford, J. G. H. (2008). Clonal polymerase chain reaction – single-strand conformational polymorphism analysis: An effective approach for identifying cloned sequences. *Analytical Bochemistry* 378, 111-112.

Zhou, X-J. Nath, S. K., Qi, Y-Y., Sun, C., Hou, P., Zhang, Y-M., Lv, J-C., Shi, S-F., Liu, L-J., Chen, R., Yang, W., He, K., Li, Y., Zhang, H. (2016). Novel identified associations of RGS1 and RASGRP1 variants in IgA Nephropathy. *Scientific Reports Nature* 1-7.