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Investigation of the molecular genetic basis of fly-strike resistance in NZ sheep: Analysis of the FABP4 gene.

A dissertation

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of the requirements for the Degree of

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by

Lucynda Emma Ruth Burrows

Lincoln University

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Abstract of a dissertation submitted in partial fulfilment of the requirements for the Degree of Bachelor of Science (Honours).

Investigation of the molecular genetic basis of fly-strike resistance in NZ sheep: Analysis of the FABP4 gene.

by

Lucynda Emma Ruth Burrows

Introduction: Fly-strike is a major economic and animal welfare issue in both the New Zealand and Australian sheep industries. There are several factors that predispose sheep to fly-strike, such as fleece-rot, urine staining of wool from the crutch and there is a strong genetic correlation (r=0.9) between fleece-rot and fly-strike. Previosuly, the fatty-acid binding protein gene FABP4 has been associated with variation in fleece-rot in sheep, so in this study susceptibility to fly-strike was investigated in the context of variation in the FABP4 gene.

Methods and materials: Blood samples were collected from sheep with and without fly-strike at shearing time and from different properties through out Canterbury. These samples were collected onto FTA cards for subsequent DNA typing. PCR-SSCP analysis was used to genotype a portion of the ovine *FABP4* gene.

Results: Four variants of *FABP4* were found (A_1, B_1, C_1 and D_1). There was a difference between sheep with and without fly-strike and the presence/absence of the A_1 and C_1 variant, (P=0.0073) and (P=0.0154) respectively. Sheep with the A_1 variant are less likely to get fly-strike than sheep with the C_1 variant. The overall Chi-squared test was insignificant, indicating that it cannot be determined from the genotype whether sheep will or will not get fly-strike.

Discussion: Based on these findings the development of a gene-marker test for selecting sheep that are genetically resistant to fly-strike is a possibility. However further studies need to be done, with a larger sample of sheep. The exact nature of why *FABP4* causes sheep to be resistant or susceptible to fly-strike also needs to be determined.

Keywords: Sheep: Fly-strike resistance: *FABP4*

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My oldest brother, John Burrows for the inspiration. At the start of the year I was worried that I would not be able to think of an idea for my Honours project. I had been working as a wool handler over the summer, and had noticed that there were a lot of sheep coming over the board with fly-strike. I was discussing this with John one day and he joked that his friend had tried to breed fly-strike resistant sheep, that wouldn't need dipping, however these sheep ended up dying of flystike. This conversation got me thinking, "why not breed sheep that are fly-strike resistant."

Thanks to my family for supporting my study. I realise I've missed a lot of family and farming time this year in order to work on my Honours project.

Thanks to my friends, who I've also some what negectled this year, thank you for understanding and supporting my studies.

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Table of Contents

List of Tables

List of Figures

Abbreviations

FABP4	Fatty acid binding protein 4
PCR-SSCP	Polymerase chain reaction-single strand conformation polymorphism
FBLN1	Fibulin
SNP	Single nucleotide polymorphism
kDa	kilodalton
ABCC11	ATP-binding cassette transporter sub family C member 11
FADS1	Fatty acid desaturase 1
Pten	Phosphatase and tension homology
PPARγ	Peroxisome proliferator-activated receptor gamma
ATP	Adenosine triphosphate
μL	micro litre
μM	micromolar
mM	millimolar
mm	millimetre
cm	centimetre
ng/mL	nanogram per millilitre

Chapter 1

Introduction

Cutaneous myiasis, commonly referred to as fly-strike, is a serious problem for both the New Zealand and Australian sheep industries. Sheep are commonly struck on the breech and body, (Figures 1.1 and 1.2). It is also an animal welfare issue, especially given that the practice of mulesing to prevent fly-strike, is coming under scrutiny from animal welfare organisations (Davidson *et al.* 2006). Organophosphorus insecticides have been used historically to control fly-strike, but this has led to the development of resistance to this class of insecticide (Board *et al.* 1994). With all these problems associated with fly-strike, the idea of breeding sheep that are naturally less susceptible to it is an attractive proposition for reducing its impact on the sheep industry.

Figure 1.1 Breech strike (Clark 2012).

Figure 1.2 Body strike. (Farming Ahead 2012, www.farmingahead.com.au).

In the early 1970's a program was established at Trangie near Dubbo, New South Wales (NSW), by the NSW Department of Primary Industries. It had the aim of examining the possibility of breeding sheep that are resistant to fly-strike. The Trangie sheep were scored for fleece-rot and fly-strike under both natural environmental conditions and after artificial wetting in sheds. Lines were bred for resistance and susceptibility using an index that included those scores for natural and induced fleece-rot and fly-strike. One of the important findings from this research was the observation of changes in wool attributes within the different selection lines. Sheep that were selected for resistance were found to have bright, white wool, with an even blocky tip that dried faster after wetting than susceptible sheep. They also had a higher level of antibodies to the bacteria *Pseudomonas aeruginosa (P. aeruginosa),* which is associated with the onset of fleece-rot (Colditz *et al*. 2006).

A recent study by Smith *et al.* (2010) of fleece-rot resistance, identified the fatty acid binding protein (FABP)4 and Fibulin (FBLN)1 genes as key factors associated with fleece-rot resistance in sheep. In the Trangie Merino's the susceptible line showed a significant association between two SNPs in *FABP4* and post- and pre-wetting fleece-rot scores. This could account for between 2.8% and 3.5% of the phenotypic variation. In the Trangie resistant line seven SNPs from the two genes (three for the *FABP4* gene and four for the *FBLN1* gene) were found to be associated with fleece-rot resistance scores.

FABPs are hydrophobic ligand-binding cytoplasmic proteins first discovered in 1971. They are involved in lipid metabolism through their intracellular binding and transport of long-chain fatty acids. Other studies implicate the FABP family of proteins in cell signalling, the inhibition of cell growth and cellular differentiation. FABP4 has also been suggested to have a role in sebaceous gland differentiation (Tsuda *et al.* 2009).

Lipids are vital to wool health as the loss of waxes and hydrophobicity is thought to be a major contributing factor to the development of fleece-rot (Norris *et al.* 2008). Lanolin is the lipid material in the fleece. It is sometimes referred to as wool fat and is secreted from the sebaceous glands of the follicle (Henderson 1965; Collins & Davidson 1997). Suint is the water-soluble material contained in the fleece that is produced by the sweat glands (Cottle 2010; Collins & Davidson 1997). Suint can act as a detergent for grease on wool and aid in its removal by rainfall. High levels of suint can also encourage retention of moisture within the fleece, causing susceptibility to fleece-rot, and fly-strike (Aitken *et al.* 1994).

FABP4 has previously been found to be associated with fleece-rot resistance and susceptibility in the Trangie lines (Smith *et al.* 2010). Due to fleece-rots high correlation with fly-strike (r=0.9), and as a consequence of the previous research that suggested variation in *FABP4* is associated with

fly-strike resistance in sheep, this study set out to determine whether there is an association between sheep with and without fly-strike and variation in the *FABP4* gene. The underlying hypothesis is that there will be a difference between sheep with fly-strike and sheep without fly-strike.

In order to do this, sheep with and without fly-strike were identified at shearing time throughout the Canterbury region, and blood samples were taken from both groups. Using sequence information published by Yan *et al.* (2012) two primers were designed to amplify a variable region of the *FABP4* gene containing part of exon 2 and intron 2. Five sequence variations (A_1-E_1) have been described within this region previously (Yan *et al.* 2012). Primers from this study will be used to genotype the blood samples collected from sheep with and without fly-strike, with the expectation that there will be a difference seen in the number of sheep with and without fly-strike correlating to specific variants of the FABP4 gene.

Chapter 2

Literature Review

Fly-strike, also known as cutaneous myiasis is a serious problem for both the New Zealand and Australian sheep industries. The disease is predominantly caused by the fly, *Lucilia cuprina* (*L. cuprina*), which is found in both countries (Raadsma *et al.* 1988). Fly-strike has an estimated prevalence of 3-5% in the New Zealand sheep flock (Heath & Bishop 1995) and is estimated to cost the industry around $50 million annually (Beef + Lamb New Zealand 1999). The cost is multifactorial in nature and includes production losses through reduction in wool and body growth, morbidity, and treatment and control costs, including the use of insecticides, prophylactic lamb shearing and ewe crutching (Pickering *et al*. 2012a). Furthermore the long-term use of organophosphorus insecticides as a method of control has led to the development of resistance to this class of insecticide (Board *et al.* 1994).

Fly-strike is not only a cost to the farming enterprise, but it is also considered a major animal welfare issue; especially with the practice of mulesing to prevent fly-strike coming under scrutiny from animal welfare organisations (Davidson *et al*. 2006).

With all these issues, the idea of breeding sheep that are less susceptible to flystrike is considered an attractive way to reduce its impact on the industry. Breeding for fly-strike resistance could be done with the use of selective breeding (Raadsma *et al.* 1989), and potentially gene-marker tests, once a suitable gene-marker has been discovered.

2.1 The species of fly that cause fly-strike

The larvae from *L. cuprina* and *Lucila sericata* (*L. sericata*) cause cutaneous myiasis in sheep, which is commonly referred to as fly-strike. In Australian around 90% of fly-strike is initiated by *L. cuprina*, but there are several other species involved as well. In New Zealand and Australia the main strike species are *L. cuprina, L. sericata, Calliphora stygia (C. stygia), and Chrysomya rufifacies (C. rufifacies)* (Levot 1995).

2.1.1 Lucilia cuprina

L. cuprina, also known as the “Austrilian Sheep blowfly”, is a small, shiny, green blowfly. In Victoria, Australia, it is found in 96% of all fly-strike cases. The maggots of *L. cuprina* are smooth and pale in colour. The young maggots cannot damage healthy tissue, but skin affected with

fleece-rot or soiled with urine or diarrhoea can weep fluid. This creates conditions that are ideal for maggots to establish in and inhabit the affected area (Department of Primary Industries 2010).

The introduction of *L. cuprina* into New Zealand was the single biggest influence on the epidemiology and natural history of fly-strike in New Zealand. *L. cuprina* was discovered in New Zealand in 1988, although it was likely introduced in the late 1970s. There was no evidence of *L.cuprina* in the South Island before 1989 (Tellam & Bowles 1997). Of the two subspecies of *L. cuprina* found in Australia, only one is found in New Zealand; *L. c. dorsalia*, and molecular genetic studies suggest that this species was introduced from Australia (Heath & Bishop 2006). Since its establishment in New Zealand *L. c. dorsalia* has surpassed *C. stygia, L. sericata* and *C. rufifacies* as the most common flies causing strike (Phillips 2009).

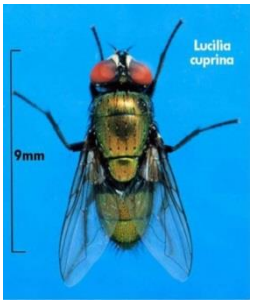

Figure 2.1 Image of *L. cuprina*. (Fly Biology http://www.flyboss.com.au/susceptibility/fly-biology.php#.Unk-zvkQ6So)

2.1.2 Lucila sericata

L. sericata is also known as the "Common Green Bottle Blowfly." This fly is an important ectoparasite of sheep and is found in New Zealand, Australia and most parts of northern Europe. Adult female *L. sericata* lay up to 200-250 eggs at a time (Cruickshank & Wall 2002; Smith & Wall 1998). In most regions of New Zealand *L. sericata* is the most common species found in fly traps (Gleeson & Heath 1997).

Figure 2.2 Image of *L. sericata*. (http://badufos.blogspot.co.nz/2012/11/ufos-infest-denver-according-to-fox.html).

2.1.3 Calliphora stygia

C. stygia is also known as the "Brown Blowfly" (Figure 2.3). This is found in both Australia and New Zealand. In New Zealand, prior to the introduction of *L. cuprina*, *C. stygia* was one of the major species involved in primary fly-strike and the earliest reports of *C. stygia* being involved in fly-strike were in 1841 (Phillips 2009).

Figure 2.3 Image of *C. stygia*. (*http://www.dpi.vic.gov.au/agriculture/pests-diseases-and-weeds/pest-insects/ag0081-sheep-flies-in-victoria*).

2.1.4 Chrysomya rufifacies

C. rufifacies is also referred to as the "Hairy Maggot Blowfly". It cannot initiate fly-strike, but once other species such as *L.cuprina* have initiated strike and damaged the skin, *C. rufifacies* can become involved in secondary strike. Once involved, the damage to the sheep increases within a matter of hours, because the *C. rufifacies* maggots are much larger and have more vigorous mouth parts than *L.cuprina. C. rufifacies* involvement can soon lead to the death of the sheep. *C. rufifacies* maggots often out compete the maggots of *L. cuprina* (Cottle 2010).

Figure 2.4 Image of *C. rufifacies*. (http://www.dpi.vic.gov.au/agriculture/pests-diseases-and-weeds/pest-insects/ag0081-sheep-flies-in-victoria).

2.2 Life cycle of *L. cuprina*

L. cuprina is the primary species involved in fly-strike and an adult fly can lay 200 eggs at one time (Figure 2.5). The presence of fly-strike on a sheep is a factor that attracts even more female flies

to lay their eggs on the sheep, further spreading the infection. Another factor that attract female flies is the presence of fleece-rot (Tellam & Bowles 1997).

Fly eggs hatch from eight hours to three days, depending on temperature, after they have been deposited on the skin of the sheep (Tellum & Bowles 1997; Australian Museum 2009). Once hatched, larvae require the presence of wet wool, the soiling of moist wool and wounded or inflamed skin to be able to initiate the "strike". The 1st stage larvae establish themselves on the dermis of the sheep skin by the excretion and regurgitation of digestive proteases. Once the larvae reach the 2nd and 3rd instars (within two to three days after hatching) they have rasping hook mouth parts, which along with the proteases allows the larvae to penetrate deep into the dermis, where they can feed on tissue fluids, dermal tissue and blood (Tellam & Bowles 1997).

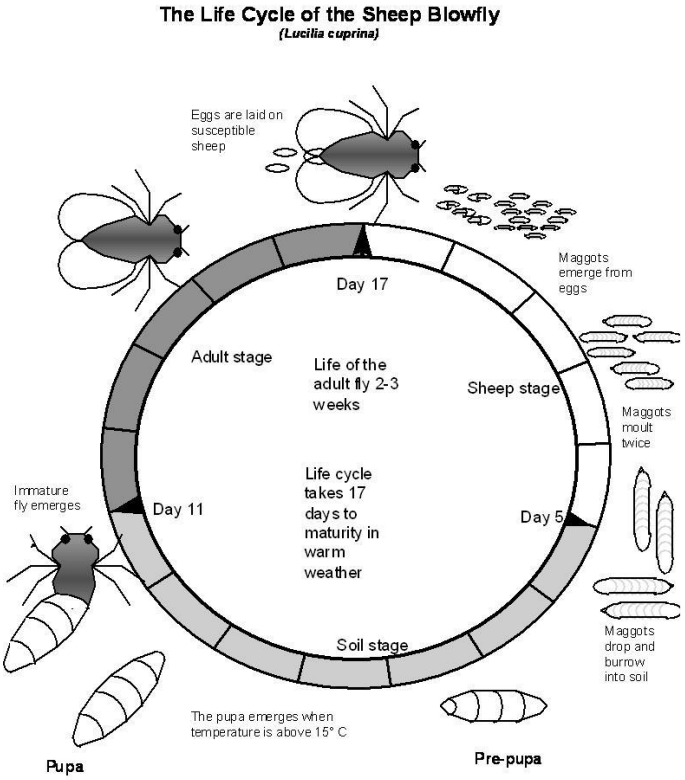

Figure 2.5 The life cycle of the fly. (Government of Western Australia. Wa.gov.au).

Flies spend a major part of their life cycle in the soil. Post-feeding the 3rd instar larvae burrow into the soil to pupate (Molyneux & Bedding 1984). This stage is dependent on soil temperature. At 30°C the pupal stage takes about six days, whereas at 15°C it takes 25 days (Foster *et al.* 1975). The adult fly then emerges. Adults feed on plant and animal material and the females require protein before they can develop and lay mature eggs (Tellam & Bowles 1997).

Much is known about the behaviour and activities of sheep flies, but less is known about the development and survival of the immature stages, especially after they leave the sheep to pupate in the soil (De Cat *et al*. 2012). In natural soils, larvae burrow down less than two inches. Larvae tend not to wander more than a few feet from where they fall. If they mature on hosts that have died, they are found in the soil below and around the carcass. It is likely that there are many mortality factors operating against prepupae and puparia in soil. For example, most puparia are likely to be sensitive to soil moisture content (Norris 1965).

Studies have found that there are several factors that affect the fly population. These factors include intra- and inter-specific competition and both the ambient temperature and microclimate (Fuller 1934, Nicholson 1948; Lane 1975). One study showed that nematodes feed on *L. cuprina* larvae, and this has been found to differ in relation to soil moisture and texture (Molyneux & Bedding 1984).

2.3 A history of fly-strike in Australasia

In the early 20th Century, fly-strike became a wide spread problem in Australia. It was uncommon until 1903, when it became a wide-spread problem in New South Wales and Victoria. This "emergence" of the disease coincided with introductions of Merinos from Vermont, in the United States of America. These Merinos had wool with a high grease content and pronounced skin wrinkles. It was soon realised that sheep's body conformation, body wrinkling and wool characteristics all influenced how susceptible a sheep is to fly-strike (Colditz *et al*. 2001).

2.3.1 The Trangie breeding programme

In the 1970s a breeding program at Trangie was established by the New South Wales Department of Primary Industries. It had the aim of examining the possibility of breeding sheep that are resistant to fleece-rot and fly-strike. Trangie developed experimental conditions for inducing fleece-rot and fly-strike, using artifical wetting by overhead sprinklers of sheep temporarily housed indoors (McGuirk *et al*. 1978; Colditz *et al*. 2001). These sheep were scored for fleece-rot and fly-strike susceptibility, in the weeks following the artificial wetting. With these susceptibility scores, lines were then bred for resistance and susceptibility using an index that included scores for natural and induced fleece-rot and fly-strike (Colditz *et al*. 2006).

2.3.2 History of fly-strike in New Zealand

In New Zealand, fly-strike has been reported since 1870, but it was not until the 1920s that it became a major concern to farmers (Tellam & Bowles 1997). A survey at the time found that the primary fly species involved were *L. sericata* and *C. stygia*. Up until the 1980's fly-strike in New

Zealand was a seasonal disease, however once the species *L. cuprina* was introduced into New Zealand in the late 1980s, the prevalence of fly-strike increased (Glesson & Heath 1997). As can be seen from Figure 2.6, fly-strike is more prevalent in the North Island, where weather conditions are typically more humid and wetter. This is more pronounced when compared to the lower half of the South Island, where fly-strike is less common (<0.5%).

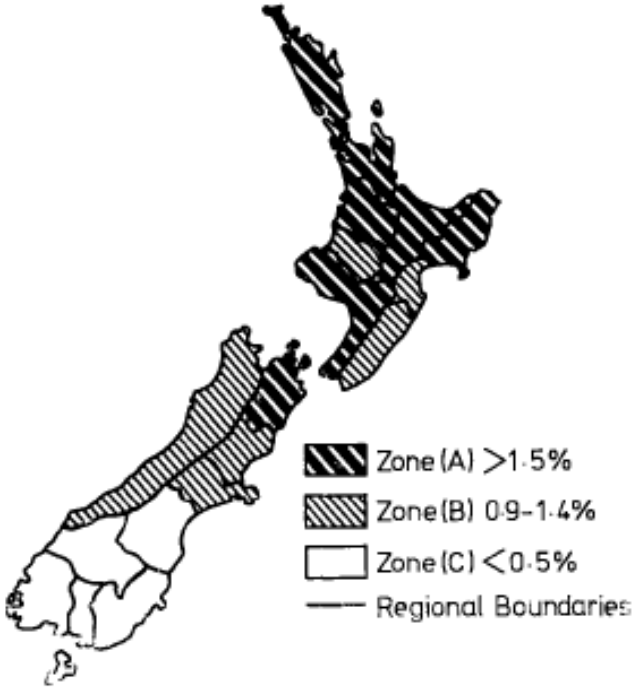

Figure 2.6 The percentage distribution of all sheep affected with fly-strike in New Zealand. (Tenquist & Wright 1976).

2.4 Climate conditions that promote fly-strike

The wetting of fleeces by rainfall can cause bacteria, primarily *P. aeruginosa,* to cause fleece-rot (Levot 1995). The bacterial growth discolours the fleece, resulting in a range of colours from green to yellow and brown (Chin & Watts 1992). This condition makes the sheep very attractive to flies such as *L. cuprina.* Fly-strike is more commonly seen with fleece-rot in warmer months because *L. cuprina* needs soil temperatures of above 15 ^{o}C to complete its life cycle and adult flies need warm conditions to be active. During spring and autumn fly waves occur throughout pastoral zones, and rainfall during this time creates ideal conditions for fly-strike. Sheep need to remain wet for several days before fly-strike occurs, so in Australia during summer when the sheep can dry within hours of being wet fly-strike is not as prevalent (Levot 1995; Hacker 2010).

Larval infestation from fly-strike results in reduced wool quality and quantity, it reduces ewe fertility and it can cause death with heavy infestations (Tellam & Bowles 1997). Fly-strike is a major animal welfare problem and it is thought that the presence of dags is a strong indication of fly-strike susceptibility (Pickering *et al* 2012b). In Australia, with the right conditions, as many as three million sheep per year can die from the effects of fly-strike (Tellam & Bowles 1997). The prevalence of fly-strike varies annually due to the climate conditions influencing the attractiveness of sheep for flies and variation in the activity and breeding of flies. In New Zealand, it is possible for fly-strike to occur every month of the year if the weather conditions are mild in winter, as well as being warm and moist during the rest of the year (Heath 1994).

2.5 When and where fly-strike occurs

In New Zealand fly-strike "challenges" vary both regionally and seasonally. Most fly-strike occurs from November to March, during warm and humid climatic conditions. In some years and regions, fly-strike can occur from October to May, if not longer. Fly-strike does not typically occur in cooler temperatures, such as those seen in winter. Typically, flies "over-winter" as pupae or adults. Once soil temperatures rise above 12^{o}C in the spring, the pupae hatch and the adult flies become active (West *et al.* 2009).

2.5.1 Where sheep get fly-strike

There are five main areas on the sheep where fly-strike can occur; the breech (crutch), poll, pizzle, tail and body (Figure 2.7).

Breech strike is the most common, due to this area being warm and moist (Tellam & Bowles 1997). 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 that usually occurs around the stump or sides of the tail (Phillips 2009).

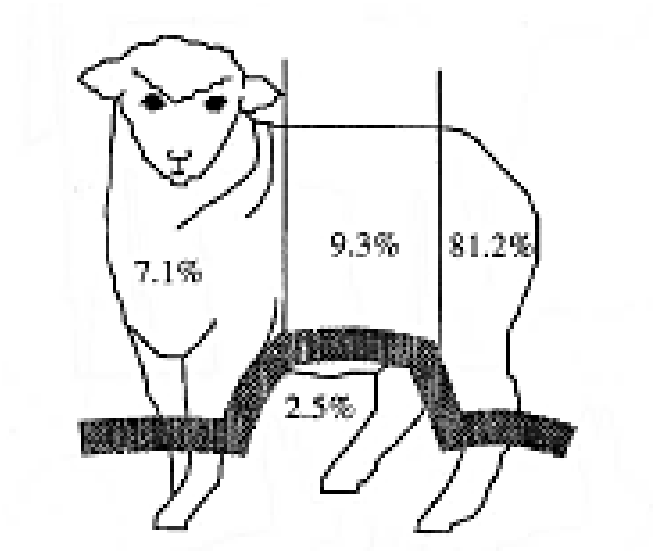

Figure 2.7 Areas of the sheep that are commonly struck by flies. he large black line cuts off the section of the sheep that has 2.5% prevalence for fly-strike from the rest of the sheep. (Fleming 2003).

Both breech and body strike are typically preceded by the bacterial dermatitis that develops when the fleece and skin become wet after extended periods of rainfall, or when the breech becomes damp from continuous soiling. The normal defence barriers of the skin breakdown under bacterial challenge and this provides an area that is more attractive to the female fly to lay her eggs. The moist bacteria-laden skin is also an ideal environment for the larvae to survive in, and grow on, once hatched (Colditz, Mahony and Elkington 2006). The breech is a more favourable area to strike when weather conditions are dry, but during wet conditions body strike becomes significantly more likely, with the back, withers and shoulders being targeted. Other areas that are less commonly struck include the flank, poll, pizzle, belly, udder, foot and scrotum (Phillips 2009).

2.5.2 What attracts flies to sheep?

Conditions that are indicators for body strike susceptibility are the presence fleece-rot and mycotic dermatitis. For head strike the main indicator is the presence of horns or a deep wrinkle at the horn site (Karlsson & Greeff 2012). There are three host barriers that have been identified that are involved in the development of resistance to fly-strike. These are the wool, the skin, and the immune system (Smith *et al.* 2010).

Wool characteristics and body conformation that favour prolonged wetting of the fleece result in the bacterial dermatitis, known as fleece-rot, which predispose sheep to infestation (Norris *et al.* 2008). Fleece-rot and fly-strike have been reported to occur as a disease complex, with a strong interdependence when the strike occurs on the body of the sheep (i.e. the shoulders, back and

flanks) (Colditz & Tellam 2000). In Eastern Australia, fleece-rot is the most important factor predisposing sheep to body strike. Fleece-rot causes inflammation and ulceration of the skin which attracts flies to lay their eggs. Fleece-rot also provides moisture for the eggs to hatch along with soluble protein for the hatched larvae to feed on (Smith *et al.* 2010).

Urine and faecal stains on the wool, eye or skin damage caused by grass seeds, footrot affected hooves, wear around the horns on rams that have been fighting, mulesing wounds and lambing stain on ewes are also all attractive areas on the sheep for flies (Levot 1995).

2.5.3 The physiological effects of fly-strike on sheep

There are numerous effects of fly-strike on the sheep and as a result of the feeding activity of the maggots. These effects are both mechanical and chemical (Heath & Bishop 2006). The feeding of the larvae causes a reduced wool staple length due to the stress response of the sheep. This is indicated by elevated cortisol levels, elevated interleukin-6 levels, elevated serum amyloid A levels and elevated haptoglobin levels. Struck sheep often have a fever and reduced feed intake (Phillips 2009).

At the third larvae stage, the maggots release a toxin that elicits an acute-phase response in the host and can cause rapid progression of the disease. This can lead to mortalities within three days (Karlsson & Greeff 2012). Death due to fly-strike is usually a result of bacterial toxaemia and systemic toxaemia resulting from the large quantities of ammonia that are released by the larvae into the sheep (Tellam & Bowles 1997). One hundred *L. cuprina* larvae are capable of producing and excreting 80mg of ammonia nitrogen each day. *L. cuprina* larvae thrive in these alkaline environments, with the optimum pH for larval collagenase activity, 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 $\pm$ 0.3 and skin temperatures during myiasis can reach 53^{o}C (Guerrini 1988).

Sheep that are infected with the larvae of *L. cuprina* have been shown to mount an immune response, by producing anti-*L. cuprina* anibiodies (Skelly & Howells 1986). Bowles *et al.* (1992) described the cellular reaction of sheep struck by flies. They observed that within 48 hours the cellular infiltrate was comprised primarily of leukocytes, including a large number of CD45 + ve T cells. Neutrophils and eosinophils made up the major cell types found at the wound surface. The increased presence of neutrophils is a result of the physical damage done to the skin. Bowles *et al.* (1992) reported that during both primary and secondary fly infections there was an increase in eosinophil numbers in the skin.

Larval challenge resulted in a marked depression in wool production, not only during the challenge period, but also during a 30 day post-treatment period (Broadmeadow *et al.* 1984). While longitudinal growth did not change, the fibre diameter decreased. Fleece tenderness was also found, with the occurrence of brushed-ended fibres, indicating the fleece had begun to shed. This is due to the increased circulation of cortisol that is released as a result of stress associated with fly-strike (Broadmeadow *et al.* 1984).

O'Sullivan *et al.* (1984) showed an increase in rectal temperature and respiration rate of sheep infestated with larvae. They concluded that this was a systemic reaction to the larval challenge. When the number of larvae on the sheep decreased the sheep's rectal temperature and respiration rate declined. Metabolic challenges faced by the sheep infected with the larvae were reported to be due to the absorption of toxins by the sheep: either exogenous toxins produced by the larvae and/or an endogenous toxin that is a result of tissue damage (O'Sullivan *et al.* 1984).

Anorexia can occur soon after the larval challenge and continue progressively during the infestation, resulting in the liveweight loss. It is not until a larval challenge is over that sheep feed-intake increases along with an increase in liveweight (O'Sullivan *et al.* 1984).

Burrell (1990) showed that sheep that have been vaccinated against *P. aeruginosa* are more resistant to fleece-rot and fly-strike, but a commercial vaccine is not currently available (Colditz *et al.* 2006).

2.6 Methods to prevent fly-strike

There are a range of management strategies commonly used on farm to prevent and reduce the risk of fly-strike. These include shearing, crutching, mulesing and the application of various chemical dips.

2.6.1 Shearing and crutching

Shearing decreases the likelihood of fly-strike by reducing the time available for larval establishment. This is because short wool dries faster than long wool when wet. Shearing also improves the effectiveness of insecticides when they are applied to sheep, by allowing greater penetration of the chemical through the wool and onto the skin (Tellam & Bowles 1997).

Crutching is also widely used as a way of removing dags and urine stained wool from around the breech area, therefore reducing the attractiveness of the area to flies (Tellam & Bowles 1997). Crutching mid-way between shearing is a common preventive measure to prevent breech strike (Hacker 2010).

2.6.2 Mulesing

In order to provide a more permanent solution to breech strike, an operation known as 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, thus there is less wool available for contamination with either urine or faeces. As a result this area becomes less attractive to the female flies (Tellam & Bowles 1997; Plant & Coombes 1988). Mulesing reduces the occurrence of breech strike as well as increasing the survival rate of sheep that get breech strike (Plant & Coombes 1988).

While there are clear benefits of mulesing, there is also considerable producer and public opposition to this procedure, as it is a stressful procedure for sheep (Tellam & Bowles 1997; James 2006). Australia has seen 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 more intensive farming situations. There is currently no accepted alternative to mulesing, although there are several options under evaluation (Hacker 2010).

2.6.3 Dipping and chemical prophylaxis

Insecticides are heavily relied on in the wool industry as a method of controlling fly-strike. Not only are insecticides applied as a method to prevent fly-strike, they are also used as a dressing to already fly-struck areas on sheep. There has been a wide range of chemicals and insecticides used to control fly-strike. For example the earliest treatment for fly-strike used mixtures of arsenic trioxide, copper sulphate, sulphur and cresylic acid.

Introduced in late 1940's organochlorines were used extensively, but they were withdrawn from use in 1958 due to residue problems caused by the persistence of these insecticides in the adipose tissue of sheep. In this time fly larvae also developed resistance to this class of insecticide, particularly dieldrin. The organochlorines were replaced by a range of organophosphate insectides including: diazinon, fenthion, coumaphos, chlorfenvinphos, carbophenothion and malathion. These have been very successful as they can also be used to control lice on sheep as well as fly-strike. In the last 40 years organophosphorus insecticide use has become widespread, due to their relatively low cost of production, and the double benefit of controlling both lice and flies (Tellam & Bowles 1997).

There is now wide-spread resistance of *L. cuprina* to organophosphates, particularly diazinon. While organophosphate insecticides are still in use, their period of protection has changed from 12 weeks to 4-6 weeks (Tellam & Bowles 1997).

The most recent method of preventing fly-strike is with the use of insect growth regulators, such as triflumuron, dicyclanil and benzoyl-ureas diflubenzuron (Nottingham *et al.* 2001). These work by affecting the moulting process and development of larvae (West *et al*. 2009).

2.6.4 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 which kept quantitative historical records of fly numbers and strike incidence, indicated that fly-strike 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 traps them. The trapped flies die from starvation and dehydration (Tellam & Bowles 1997).

The development of a practical, effective and economical trapping system for the *L. cuprina* could make fly populations and strike incidence suppression a feasible method for controlling fly-strike. Such control methods could reduce the use of insecticides, which would also decrease the associated residues in sheep products (Urech *et al*. 2004).

Studies over the last 60 years have focussed on the type of volatile components emitted by natural sources that attract sheep flies. These studies have found that *L. cuprina* orientates towards bacterial strains that produce chemical attractants and volatile compounds from the myiatic lesions of sheep (Khoga *et al*. 2002; Urech *et al*. 2004). Therefore flytraps that contain such chemical attractants and volatile compounds can be used to reduce fly numbers and fly-strike. Liver and sodium sulphate has been found to be the best attractant for *L. cuprina* (Urech *et al.* 2002).

LuciTrap is a commercially available fly trap, which has been shown to reduce *L. cuprina* populations and the incidence of fly-strike when used at a recommended rate of 1 trap per 100 sheep (Urech *et al.* 2009).

2.6.5 Vaccination

Several studies have shown that sheep that have been vaccinated against *P. aeruginosa* are more resistant to fleece-rot and fly-strike (Burrell 1990), but commericial vaccines are not currently available.

In the past, fly vaccine development studies have shown that the sheep's immune system is able to recognise components of the maggots as foreign and are able to generate immune responses (Colditz *et al*. 2006). However another trial in which sheep were repeatedly immunised in such a way as to induce a hypersensitivity reaction to the maggots, revealed that despite exhibiting good levels of protection to the first larval challenge, the initial level of protection did not persist after repeated challenges (Bowles *et al*. 1987). The immune response of sheep that are repeatedly challenged with maggots declines over time, which contrasts the strong increase in immune response observed for other pathogens such as viruses and bacteria (Colditz *et al.* 2006). This is believed to occur because when vaccinated sheep are challenged with live fly maggots, the maggots secrete a molecule that inhibits a key pathway in the mobilisation of the immue system, thus enabling the establishment of fly-strike. This occurs in vaccinated sheep, because the vaccine contains individual components of the maggots, which is injected into the sheep, and recognised as foreign generating a specific immune response. The live maggots are able to modulate the sheep immune system, preventing it from recognising the maggots as foreign invaders (Colditz *et al*. 2006).

With the ongoing study of the host-immune response to myiasis and larval-host interactions it is possible that an affective vaccine against fly-strike will be designed in the future (Otranto 2001).

2.6.6 Resistance to fly-strike

Dr H.G. Belschner began the research into breeding sheep that were resistant to fly-strike at Trangie in the 1930s. The programmes primary objective was to develop sheep that are resistant to flystrike, while still maintaining other production attributes (McGuirk *et al.* 1980):

1. Measured characteristics such as fleece weight, fertility and resistance to fly-strike;
2. Developed measurement techniques and management procedures for the breeding flock;
3. Collected data from the lambs born;
4. Estimated genotypic and phenotypic parameters;
5. Formulated breeding plans and promoted these to the industry and breeders.

Fly-strike and fleece-rot occurrence has a high genetic correlation (r =0.9), and therefore can be selected together when breeding for fly-strike resistant sheep (Morris 2009). For Merino sheep the susceptibility to body-strike is heritable, and was estimated by Raadsma 1989 to be 0.35-0.4. It has also been suggested that a major gene may account for 20% of the phenotypic variation in fleece-rot and 15% of the variation in body strike for Merino sheep (Mortimer 2001).

2.6.7 Fleece characteristics and fly-strike resistance

The fleece is composed of the wool fibre, wax, suint and extraneous matter such as dirt, water, fungi and bacteria. Wool fibres are composed predominantly of fibrous proteins, keratin, and are coated by wax and suint. The sebaceous glands produce a lipid-rich wool wax or lanolin. The sebaceous glands are found in the dermis of various animals and the chemical composition of sebum is distinct in each species and even within members of the same species (Nikkari 1974: Cheng & Russell 2004). Wool wax is secreted by the sebaceous glands in order to protect the wool fibres from external damage. The wax is a mixture of cholesterol, lanosterol, fatty acids (e.g. palmitic and isostearic acid) and hydroxy fatty acids. Suint produced by the sweat glands contains diverse water soluble compounds, such as electrolytes (e.g. potassium carbonate and sulphate), fatty acids, organic acids, amino acids, urea and other nitrogenous compounds. These compounds provide nutrients for a variety of bacteria that survive in the fleece, including *P. aeruginosa, Bacillus subtilis, Enterobacter cloacae and Proteus mirabilis* (Figure 2.8) (Emmens & Murray 1982).

During periods of prolonged rainfall suint acts as a detergent that aids in the removal of wax from around the wool fibres. During this time the chemical composition of the wool wax is also altered. These conditions can give rise to both yellowing of wool and fleece-rot. Resistance to fleece-rot or yellowing have been described in sheep that have higher wax content than those which are more susceptible (Lipson *et al*. 1982; Evans and McGuirk 1983; Aitken *et al.* 1994).

The sebaceous wax in wool is an efficient barrier against water, and can reduce the ease with which the fleece of a sheep is wetted and penetrated. The penetration of the fleece by water, and the subsequent wetting of the skin is necessary for fleece-rot to develop. Resistance to fleece-rot is associated with the waterproofing effect of a high sebaceous wax content. The fatty acids in the wax lower the pH of the wool and skin surface which inhibits bacterial growth (Lambers *et al*. 2006). The components of sebum which are hypothesised to have the greatest antibacterial effects are oleic and palmitoleic acids. Oleic and palmitoleic acid are thought to inhibit fatty acid synthesis in bacteria (Smith & Thiboutot 2008).

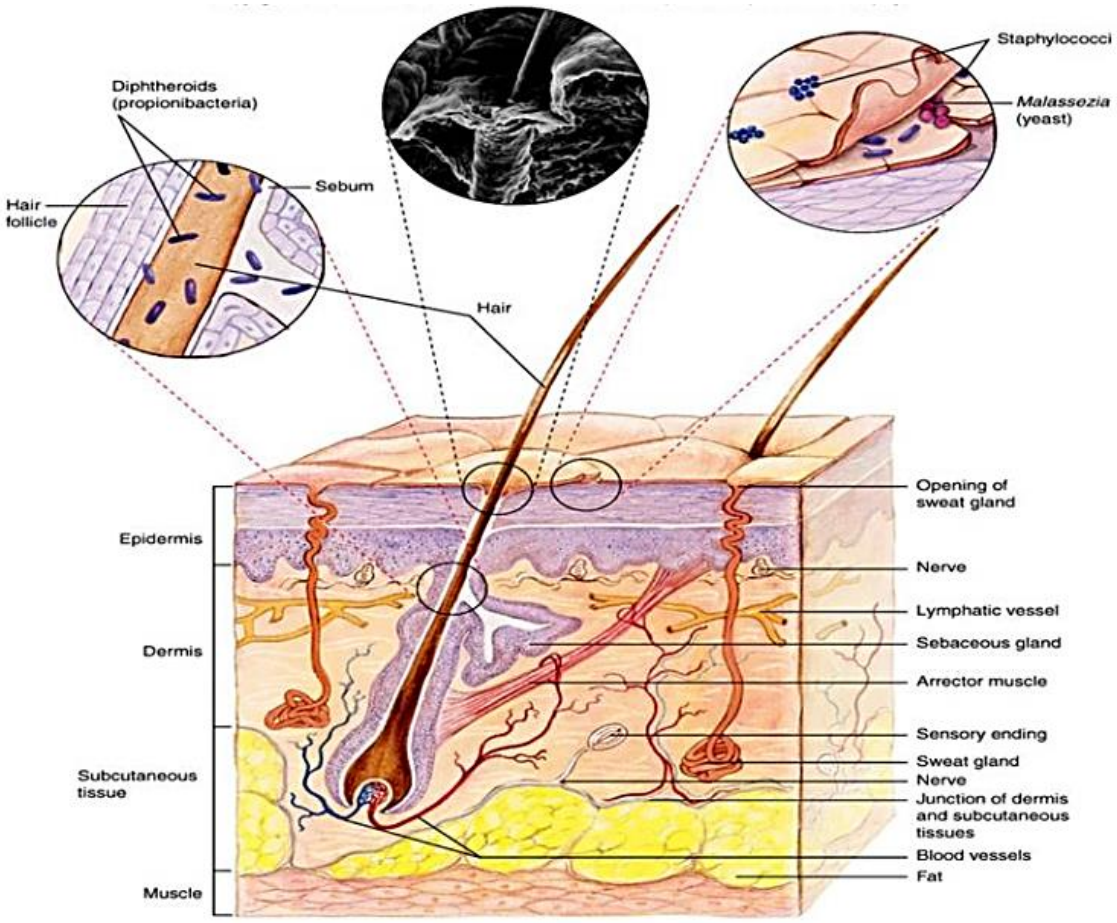

Figure 2.8 Image of human hair follicle, and the sebaceous gland releasing sebum (wool wax). (http://www.pc.maricopa.edu/Biology/rcotter/BIO%20205/LessonBuilders/Chapter%2014%20LB/Ch14LessonBuilder_print.html).

Reduction of the wax on the skin surface occurs due to wetting, and an increased amount of *Pseudomonas sp.* can be found in the fleece a day after wetting has occurred. This time period corresponds to the commencement of breakdown of the skin wax-layer and an increase in content of cholesterol and lanosterol in the skin wax (Merrit & Watts 1978; James *et al*. 1984). It has been theorised that the increase in cholesterol and lanosterol content of the wool during fleece-rot and dermatitis is due to bacterial action. Cholesterol is a powerful emulsifying agent in wool wax, and it seems likely that the physical breakdown in the skin-wax layer is caused by *Pseudomonas sp.* (Goodrich & Lipson 1978). The changes in wool wax seen in wetting experiments represent an increase in the hydrophilic character of the wax (Hay & Mills 1982; James *et al*. 1984).

Important predisposing factors for body strike are: the presence of fleece-rot and bacterial lesions of the skin fleece contaminated with *P. aeruginosa* (and particularly in high rainfall areas) and mycotic dermatitis, (which is an infection of the skin with the bacteria *Dermatophilus*

congolensis). These predisposing factors in sheep are present in combination with moisture, an abundance of soluble proteins and odours (Emmens & Murray 1982). These bacteria are not invasive but produce various extracellular enzymes and toxins which exacerbate the dermatitis, and attracts flies, thus increasing the risk of body strike (Burrell *et al*. 1982; Chin & Watts 1992) There is a high correlation between fleece-rot and body strike (0.7-0.9) and the resistances to both conditions has a moderate heritability (h2 = 0.35-0.4) (Raadsma *et al*. 1989).

Common fly-strike species display an orientation towards the wind in response to an olfactory stimulus, known as the anemotactic response. Various sulphur compounds, ammonia and carbon dioxide in elevated levels, along with volatile organic acids and indolic compounds are produced by bacteria (*P. aeruginosa, B. subtilis, P. mirabilis, E. cloacae*) in wounds or disintegrated animal tissue. These compounds can act as ovipositor stimuli to attract several species of fly including *L. cuprina,* and *L. sericata* (Emmens & Murray 1982; Khoga *et al.* 2002).

2.7 Protein and resistance to fleece-rot and fly-strike

The fatty acid-binding proteins (FABP) are small molecular-weight proteins which have a high binding affinity for long-chained fatty acids. These proteins transport fatty acids from the cell membrane to the sites of β-oxidation and triacylglycerol and phospholipid synthesis. FABPs come from a family of small cytoplasmic proteins that are around 14-15kDa in size, and are conserved through evolution from Drosophila to humans (Tuncmans *et al.* 2006). FABP's are found in almost all cell types.

There are about nine different types of FABPs that are recognised by the tissue in which they are found in. These included: (1) L-FABP or FABP1 which is found in the liver, (2) I-FABP or FABP2 which is found in the intestines, (3) H-FABP or FABP3 which is found in the heart, (4) A-FABP or FABP4 which is found in the adipocyte tissue. (5) E-FABP or FABP5 which is found in the epidermis, (6) IL-FABP or FABP6 which is found in the ileal tissue, (7) B-FABP or FABP7 which is found in the brain tissue, (8) M-FABP or FABP8 which is found in myelin, and finally (9) T-FABP or FABP9 which is located in the testis (Bai *et al.* 2013). *FABP4* has been described in humans, mice, pigs, chickens and cattle and it has been shown to have a conserved structure in all these species, with four exons interrupted by three introns (Yan *et al.* 2012).

The FABP are found in adipocytes and macrophages, and play an important role in the molecular pathway that integrates metabolic and inflammatory responses. FABPs show a tissue-specific expression pattern and they are often regulated by the metabolic demands of the cells in which they are found. FABPs have a role as cytoplasmic lipid chaperones and mediate other lipid signals

via their interaction with functional targets. Both adipocytes and macrophages express the same two FABP isoforms; FABP4 and FABP5 and both isoforms are expressed at similar levels in activated macrophages. They are regulated by various metabolic and inflammatory mediators (Tuncmans *et al.* 2006). *FABP4* is expressed in adipocytes and is regulated by peroxisome-proliferator-activated receptor-γ (PPARγ) agonists, insulin and fatty acids. The locus for the macrophage/adipocyte *FABP4* gene is vital in the regulation and dysregulation of metabolic and inflammatory responses in their relation to metabolic diseases. FABP4 acts to coordinate functional interactions between macrophages and adipocytes in the adipose tissue (Figure 2.9) (Furuhashi *et al.* 2007).

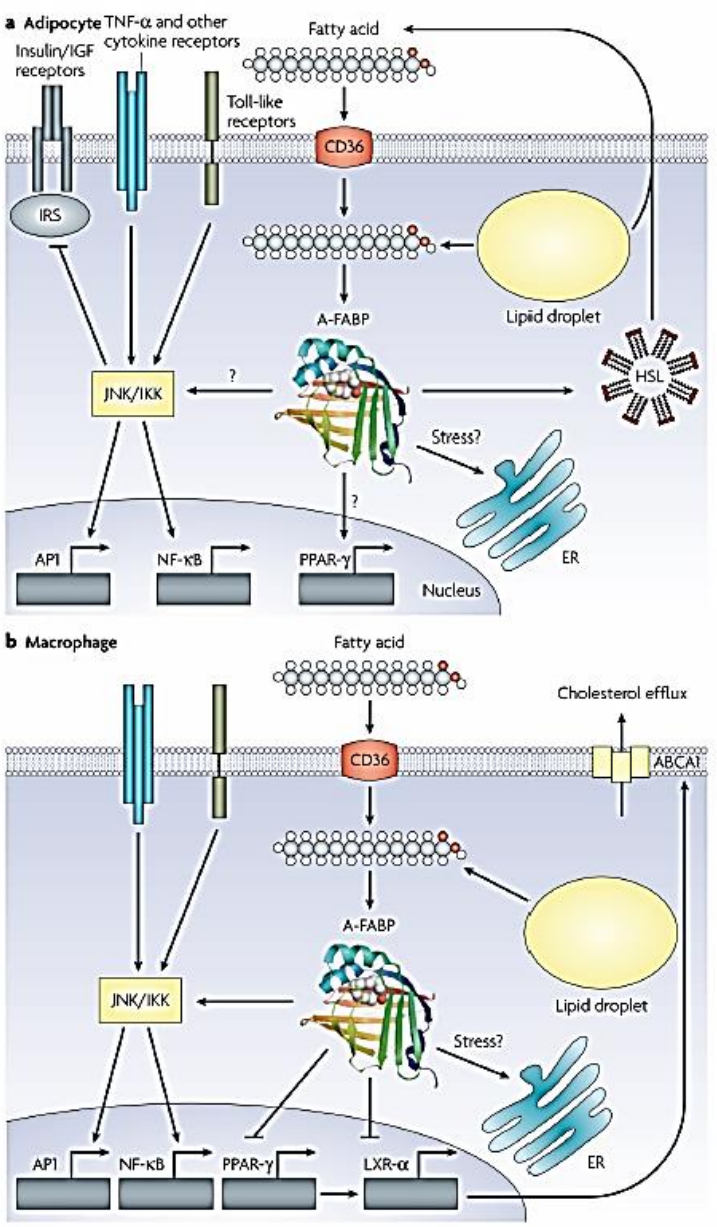

Figure 2.9 Function of FABP4 in the adipocyte and macrophage. (a) FABP4 interacts with hormone-sensitive lipase (HSL) to potentially modulate its catalytic activity and integrates several signalling networks that control inflammatory responses. In addition FABP4 is also important in controlling adipocyte lipid hormone production. (b) In the macrophages, FABP4 regulates inflammatory responses. In both macrophages and adipocytes, FABP4 has a critical role in integrating lipid signals to organelle responses, particularly in the endoplasmic reticulum (RE). In the diagram AP1 is adaptor receptor protein 1; IGF is insulin like growth factor; IRS is insulin receptor substrate and TNF is tumour necrosis factor. (Furuhashi & Hotamisligil 2008).

2.7.1 The structure of FABPs and FABP4

All FABPs bind long-chain fatty acids and they all have small structural differences between the isoforms that result in different ligand selectivity, binding affinity and binding mechanisms. FABPs

have a wide range of sequence diversity, from 15% to 70% sequence identity between different members. However all FABPs share an almost identical three-dimensional structure. Generally, one or two conserved basic amino-acid side chains are required to bind the carboxylate site of a fatty-acid ligand in the binding pocket of a FABP (Simpson *et al*. 1999).

All FABPs have a 10-stranded antiparallel β-barrel structure, which is formed by two orthogonal five-stranded β-sheets. The binding pocket is found inside the β-barrel, the opening of which is framed on one side by the N-terminal helix-loop-helix 'cap' domain, and fatty acids are bound to the interior cavity (Chmurzynska 2006; Furuhashi & Hotamisligil 2008).

All FABP have a conserved fingerprint which derives from three motifs. Motif 1 includes the G-x-W triplet, which forms part of the first β-strand (βA). Motif 2 spans the C terminus of strand 4 (βD) and includes stand 5 (βE). Motif 3 encodes strand 9 (βI) and 10 (βJ). In FABP4, potential functional domains include a nuclear localization signal (NLS) and its regulation sites, nuclear export signal (NES) and a hormone-sensitive ligase (HSL) binding site 17, 26, 100. Further detail can be found in the review by Furuhashi & Hotamisligil (Figure 2.10) (2008).

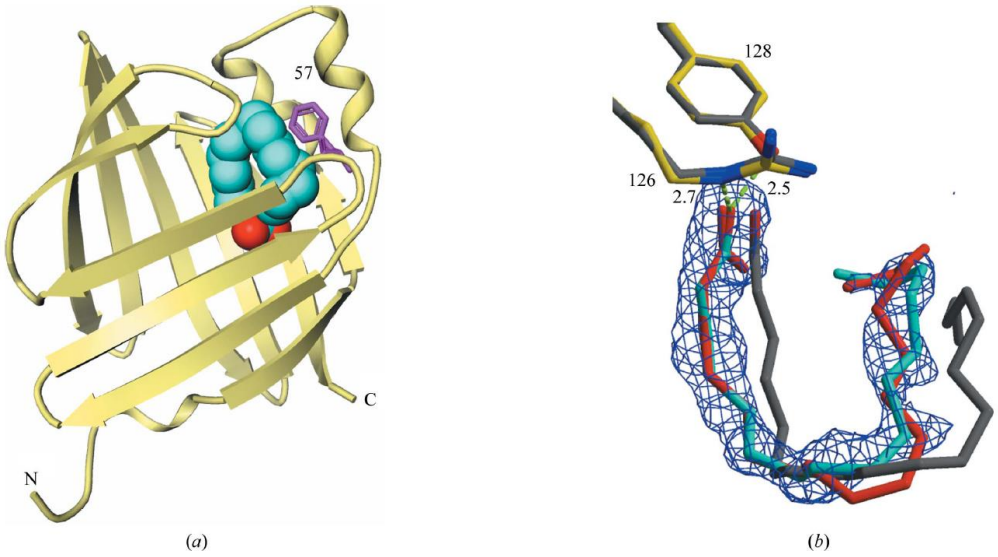

Figure 2.10 The structure of human FABP4 in complex with palmitic acid. (a) Ribbon diagram of FABP4. The bound palmitatic acid is shown as a cyan (the O atoms in red) CPK model. Phe75 is in magenta. The N- and C-termini are indicated as N and C. (b) the ligand-omitted electron-density map is contoured at the 2.5σ level. Key hydrogen bonds are depicted in broken lime green lines. The palmitate structure is shown in yellow and cyan. The structure overlay of a folded form of bound oleate acid into the observed electron density is shown in red. (Marr *et al*. 2006).

2.7.2 *FABP4* in sheep

In sheep *FABP4* has been shown to be involved in the regulation of macrophage endoplasmic reticulum (ER) stress and it functions as a cytosolic lipid chaperone in macrophages (Erbay *et al.* 2009). The adipose tissues house some of the most active lipid metabolism of any tissue. Adipocytes respond to insulin by the activation of glucose transport, the esterification of fatty acids into triacylglycerol and the synthesis of fatty acids (Baxa *et al.* 1989). FABP4 supplies long-chain fatty acids that are used as an energy source for muscle growth and maintenance. The long chain fatty acids tend to be used for fat storage within muscle fibres (Dervishi *et al.* 2011).

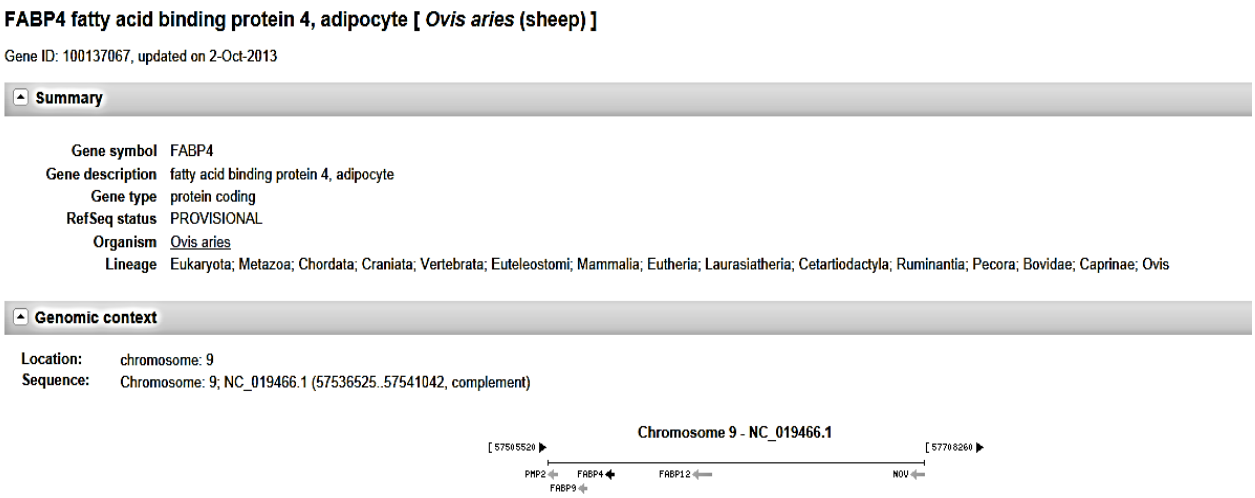

Figure 2.11 Overview of the *Ovis aries FABP4* gene (GenBank www.ncbi.nlm.nih.gov/gene/1.)

A study by Smith *et al.* (2010) looking at fleece-rot resistance identified the *FABP4* and *FBLN1* genes as key factors associated with fleece-rot resistance in sheep. In the Trangie Merino lines the susceptible line showed a significant association between the single nucleotide polymorphisms (SNPs) FABIn20237 and FABIn30360 and difference in post- and pre-wetting fleece-rot scores, ($P<0.05$) This accounted for between to 2.8% to 3.5% of the phenotypic variation. Significant results were also seen in the Trangie resistant line.

Table 2.1 The difference between post-wetting trails of three populations of sheep and SNP associated with fleece-rot resistance. (Adapted from Smith *et al*. 2010).

SNP	Armidale			Trangie Susceptible			Trangie Resistant		
	ß	R2	P	ß	R2	P	ß	R2	P
ABCIn0150	-0.01	0.00	0.973	0.14	0.30	0.528	-0.03	0.03	0.841
ABCIn0270	-0.28	2.04	0.235	-0.32	1.32	0.307	0.00	0.00	0.993
ABCex0667	0.05	0.04	0.829	-0.22	0.61	0.276	0.09	0.32	0.388
FABIn20115	-0.21	0.58	0.361	n/a	n/a	n/a	n/a	n/a	n/a
FABIn20237	0.15	0.54	0.368	**-0.46**	**2.78**	**0.041**	0.09	0.40	0.386
FABIn30227	0.21	0.66	0.328	-0.22	0.05	0.781	0.44	0.33	0.472
FABIn30360	-0.13	0.40	0.409	**0.54**	**3.50**	**0.012**	-0.10	0.36	0.362
FABIn30420	0.21	0.36	0.472	-0.71	0.65	0.276	0.10	0.12	0.614
FAD1g20645	0.17	0.37	0.470	-0.14	0.12	0.676	-0.33	1.14	0.169
FBLIn100090	0.25	0.90	0.478	-0.32	1.22	0.684	-0.17	0.98	0.63
FBLIn120135	-0.19	0.72	0.359	0.23	0.54	0.353	-0.09	0.29	0.493
FBLIn120280	0.03	0.01	0.886	-0.12	0.18	0.596	0.04	0.06	0.711
FBLIn120995	**0.52**	**5.71**	**0.005**	-0.09	0.07	0.774	-0.06	0.10	0.729
FBLs10075	0.02	0.01	0.896	0.09	0.10	0.730	-0.14	0.87	0.285
HMGIn40390	-0.05	0.05	0.772	-0.18	0.46	0.343	0.02	0.01	0.861

In the pre-wetting studies seven SNPs from two genes (three for the *FABP4* gene and four from the *FBLN1* gene) were found to be associated with fleece-rot resistance scores.

Yan *et al.* (2012) have studied the *FABP4* gene in sheep. In their study two regions of the ovine *FABP4* were analysed; in region 1 (exon 2-intron 2) they found five specific *FABP4* sequences (A_1-E_1). The sequence analysis showed that there were three nucleotide substitutions and one deletion in the intron. Four different patterns were detected in region 2 (exon 3-intron 3) with four nucleotide substitutions being revealed (A_2-D_2). The authors suggested that the variation seen in the FABP4 gene might underpin the differences seen in the fat and lean lines of sheep; with A_1 having the highest frequency of 51% in fat line and C_2 having the highest frequency of 59% in the lean line (Yan *et al.* 2012).

Bakhtiarizadeh *et al.* (2013) showed that the expression of *FABP4* in the fat-tail of Lori-Bakhtiari sheep was significantly higher than both the investigated tissues of the Zel sheep breed. Various other studies have reported the differential expressions of *FABP4* in different adipose tissues, and that the gene expression of *FABP4* is higher at the time of adipocyte differentiation (Bakhtiarizadeh *et al.* 2013).

2.7.3 Other roles for *FABP4*

Previous studies have suggested that *FAPB4* is directly related to fatness traits and in beef cattle may be a potential marker for tenderness and intramuscular fat content (Michal *et al*. 2006; Jurie *et al.* 2007; Hoashi *et al.* 2008; Barendse *et al.* 2009; Lee *et al*. 2010). *FAPB4* is expressed in the adipocytes, and therefore the relevance of *FABP4* in the muscle tissue is a relative measure of the amount of intramuscular adipose tissue or of the number of intramuscular adipocytes, which represents a proportion number of the total muscle volume (Jurie *et al.* 2007). This is in agreement with the findings of Avilés *et al.* (2013) who showed the *FABP4* marker (g7516G>C) was significantly associated with the percentage of intramuscular fat and the variation in the marker explained 0.22% of the variation in the trait.

Jurie *et al*. (2007) found that independent of genotype and muscle effect, the FABP4 protein content of muscle was significantly correlated with triacylglycerol content, strengthening the idea that *FABP4* expression is associated with intramuscular fat content. This study provided evidence that *FABP4* expression as measured by mRNA level and protein content is a good indicator of intramuscular adipocyte numbers, as well as oxidative enzyme activities and that FABP4's expression is associated with fatty acid catabolism, therefore fat turn-over, and this may be a major metabolic indicator of the ability of animals to deposit intramuscular fat according to breed and muscle type (Jurie *et al.* 2007).

2.7.4 *FABP4* association with wool wax:

Lipids are vital to barrier function as the loss of waxes, and hydrophobicity in general, is thought to be a major factor protecting skin and wool from the development of fleece-rot reviewed (Norris *et al*. 2008). Three genes: Adenosine triphosphate (ATP)-binding cassette transporter sub-family C member 11 (*ABCC11), FABP4* and fatty acid desaturase 1 (*FADS1*) were found to play an important role in lipid metabolism. Members of the ATP-binding transport protein superfamily, including *ABCC11*, are involved in the transport of sphingolipids, glycerophospholipids, cholesterol and fatty acids in epidermal lipid reorganization during keratinocyte terminal differentiation (Smith *et al.* 2010).

FABP4 has been shown to be induced in phoshatase and tensin (*Pten*)-null keratinocytes, suggesting a role in sebaceous gland differentiation (Tsuda *et al.* 2009). *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, that regulates genes involved in sebaceous differentiation (Michalik & Wahli 2007). Keratinocyte-specific *Pten*-null mice display distinct phenotypes, including wrinkled skin, ruffled shaggy and curly hair. Histological examination showed that these mice have acanthosis, sebaceous gland hyperplasia and accelerated hair follicle morphogenesis (Suzuki *et al.* 2003). Tsuda *et al.* (2009) study found the *FABP4* gene to be the strongest induced gene in the *Pten*-null keratinocytes; this suggests that *FABP4* plays a role in the development of the sebaceous gland hyperplasia seen in these mice (Michalik & Walter 2007; Tsuda *et al.* 2009).

FABP5 (another isotype of FABP) has been shown to be localised in sebaceous glands (Watanabe *et al.* 1996) and has been suggested to regulate sebaceous gland activity by modulating lipid signaling and/or lipid metabolism in sebocytes (Lin & Khnykin 2013).

Chapter 3

Methods

3.1 Blood sample collection

156 blood samples were taken from sheep with fly-strike and 130 from sheep without fly-strike and these samples were collected from 18 different farms throughout the greater Canterbury region. Samples were collected with farmer consent and according to normal farm management practices.

Sheep were identified as having fly-strike when maggots could be seen on the skin of the sheep as they were being shorn and the wool was discoloured and bad smelling. Sheep that had previously had fly-strike were also identified by having areas of pink skin with no wool growth, obvious scar tissue from maggot damage and flakey dry skin that had been damaged by maggots.

Sheep of various age and breed with fly-strike were identified at shearing, and blood samples were taken from them by cutting the lower part of the ear near the tip, where the prominent vein ends, Figure 3.1. Electrical side-cutters were used, to clip the ear, as these do not get contaminated by blood when the ear is clipped.

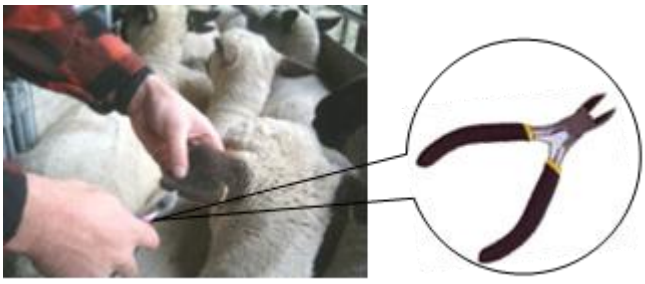

Figure 3.1 Use the electrical side cutters to make a cut in the bottom tip of the ear. (Instruction for Sheep Blood Collection on FTA Card).

Blood drops where collect onto FTA cards. The FTA cards were labelled according to farm and the presence or absence of fly-strike. The blood was allowed to dry and was stored in darkness at room temperature.

Figure 3.2 Collecting blood onto the FTA cards. (Instruction for Sheep Blood Collection on FTA Cards).

Control sheep were matched to the struck sheep by being from the same property as the sheep with fly-strike.

The blood samples were taken to the Lincoln University Gene Marker laboratory for testing.

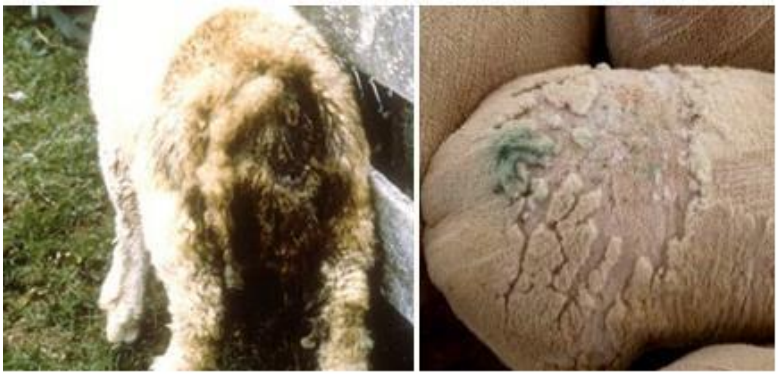

Figure 3.3 Active fly-strike and healed fly-strike (From http://www.liveexportshame.com/what_is_animal_welfare.htm and Curran 2006 respectively).

3.2 PCR-SSCP analysis and genotyping of ovine *FABP4* (undertaken by Dr H Zhou)

Two PCR primers, 3'-CAGGAATTTGATGAAGTCACT-5' and 3'-GTAACATGGTTCAGAGCTAG-5', were designed based on the published sequences JX290313-JX290317 (Yan *et al*., 2012; Appendices A and B) to amplify a variable region of ovine *FABP4* containing part of exon 2 and intron 2. The primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA).

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 ^{o}C, followed by 35 cycles

of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C, with a final extension of 5 min at 72 °C. Amplification was carried out in S1000 thermal cyclers (Bio-Rad, Hercules, CA, USA).

Amplicons were visualized 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 °C 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 250 V for 18 h at 11 °C in 0.5 × TBE buffer. Gels were silver-stained according to the method of Byun *et al.* (2009).

3.3 Statistical analysis

Of the 156 and 130 blood samples collected from sheep with flystrike and without flystrike respectively, only 92 sheep with fly-strike and 93 without fly-strike were genotyped in total. These were then tested using an odds ratio test to look at the presence/absence of each allele, and a Chi-square analysis to see if there was a significant difference between the presence and absence of various alleles and the presences and absence of fly-strike.

Chapter 4

Results

4.1 Variants of *FABP4*

The previously identified A_1, B_1, C_1 and D_1 variants of the exon-2-intron 2 region of the *FABP4* gene were all identified in the blood samples collected. Although there is an E_1 variant (Yan *et al*. 2012) of this region of the gene, it was not detected. The variant sequences have been previously deposited into GenBank with accession numbers JX290313-JX290317 (Yan *et al.* 2012). In the 185 sheep of various breeds studied, the B_1 variant was the most common, with very few D_1 variants found.

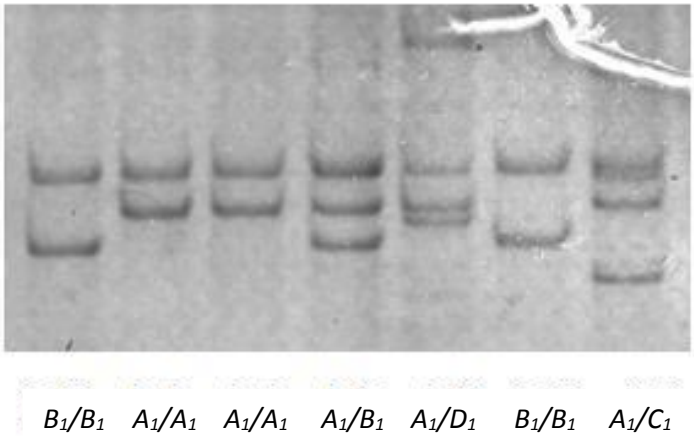

Figure 4.1 The PCR-SSCP banding patterns for the different combinations of variants of the *FABP4* gene.

The different banding patterns on the gel showed the variation in the *FABP4* gene. Figure 4.1 could be identified as two unique bands. B_1 also has a two bands pattern. but they are spaced further apart than the A_1 variant bands. The C_1 variant with A_1 as a heteropzygote has a single banding pattern further away from the A_1 bands, while the D_1 variant also found with A_1 in (Figure 4.1) has one band in close proximity to A_1 bands.

Table 4.1 Sequence variation in ovine FABP4. (Adapted from Yan *et al*. 2010).

Region	Position[a]	Nucleotide sequence[b]			
		A_1	B_1	C_1	D_1
Region 1	c.246 + 33	c	–	c	c
	c.246 + 37	g	a	a	a
	c.246 + 46	c	c	c	t
	c.246 + 47	g	g	g	g

Three nucleotide substitutions and a nucleotide insertion/deletion have previously been described by Yan *et al.* (2010). This shows the difference between the four variants of *FABP4* found in this study.

Table 4.2 Number of sheep with and without fly-strike observed with variant A_1, B_1, C_1 or D_1.

Variant	With fly-strike	Without fly-strike
A_1	25	43
B_1	62	68
C_1	53	33
D_1	13	6

4.2 Chi-squared

Table 4.3 Chi-squared table based on variant frequency, expressed as percentage of sheep with and without fly-strike carrying the respective variant.

Variant	With flystrike	Without flystrike
A_1	16	25
B_1	42	52
C_1	35	20
D_1	7	3

Chi-squared equals 21.746 with 3 degrees of freedom. The two-tailed P value is less than 0.0001. This demonstrated that there is a highly significant association between the variants of *FABP4* and resistance/susceptibility to fly-strike in sheep.

4.3 Odds ratio

Figure 4.2 Results of the odds ratio test

Variant	Odds ratio	P value
A_1	0.4677	0.0073
B_1	0.8216	0.3978
C_1	1.8791	0.0154
D_1	2.2286	0.1144

The odds ratio test (Table 4.4) gives a significant p value for the A_1 and C_1 variants, 0.0073 and 0.0154 respectively. These results suggest that sheep with the C_1 allele are more likely to have fly-strike than sheep with the A_1 variant. B_1 and D_1 do not show a significant difference, meaning if the sheep has the B_1 variant present it is just a likely to get fly-strike as it is to not get fly-strike. There was a very small number of sheep that expressed the D_1 variant, thus the insignificant result is not surprising, and it is possible that it might reach significance in a larger study.

Those sheep with the A_1 variant had 0.4677 the expected likelihood of getting flystrike while those with the C_1 variant had 1.8791 times the expected likelihood of getting flystrike.

Chapter 5

Discussion

The aim of this study was to determine if there is an association between fly-strike resistance in sheep and variations in the *FABP4* gene. Sheep with the A_1 variant of *FABP4* were less likely ($P<0.0073$) to get fly-strike than those without A_1. However it is still impossible to say absolutely if a sheep carrying the A_1 variant will, or will not get fly-strike.

There was also a difference seen with the presence of the C_1 variant compared to the absence of the C_1 variant, with C_1-carrying sheep being more likely to be struck by flies.

Sheep that carry the B_1 variant showed no significant difference in the presence and absence of fly-strike and very few sheep were found that carried the D_1 variant. No sheep were found that were homozygous for D_1 (Appendix C), therefore any affect is more likely to be the result of the other F*ABP4* allele they are carrying.

Although the results showed that sheep carrying the A_1 variant are less likely to get fly-strike than those that are not, and that sheep with the C_1 variant are more likely to get fly-strike than those without it, it is still impossible to predict which sheep will and will not get fly-strikes. The chi-square test performed on the results was highly significant ($P= 0.0001$) which suggest that genotype of the sheep can determine how likely a sheep is to get flystrike or not.

In this study, four variants of *FABP4* were detected (A_1-D_1). In contrast Yan *et al*. (2012) detected five specific sequences (A_1-E_1), E_1 was not found in these fly-strike and non-fly-strike sheep. The E_1 variant in Yan *et al*. (2012) was only found in a few sheep they studied, thus it is not that surprising that it was not found in this study due to the small number of sheep tested here. Larger studies may detect its presence in future.

Yan *et al.* (2012) also found that some sequences were not observed in some of the breeds, and the variant frequencies between some breeds were quite different (Appendix B). They suggested that the variation seen in the *FABP4* gene might underpin the differences seen in fat and lean lines of sheep that they studied; with A_1 having the highest frequency 51% in the fat line and C_1 having the highest frequency 59% in the lean line.

There seems to be some correlation between the work of Yan *et al.* (2012) and the present study. While the variations in *FABP4* were in Hardy-Weinberg equilibrium in the sheep studied here,

Hardy-Weinberg equilibrium could not be analysed in the lean and fat lines as individual genotype data was not available. Given that the lean and fat lines are selection lines that have been inbred for many years (Yan *et al*. 2012) it would however seem unlikely that they are in Hardy-Weinberg equilibrium (Appendix C).

In the present study, sheep with the A_1 variant were the least likely to get fly-strike, and these sheep in Yan *et al.* (2012), were the sheep predominately 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 (Lambert *et al.* 2006). This would need further investigation to be confirmed.

The C_1 variant in Yan *et al*'s. (2012) study was more common in their lean line and fly-strike was also more prevalent with this variant of *FABP4* in this study. In contrast to the situation above, this may suggest that the likely mechanism behind this effect is that fewer lipids are present in the wool of the C_1 variant sheep, making it easier for bacteria to grow. If more bacteria grow and/or produce different odours in the right conditions, this might cause fleece-rot, which attracts flies. As concluded above, further research is also required to test this theory.

Smith *et al*. (2010) was the first study to report gene expression changes in the skin of sheep before, during and after the induction of fleece-rot challenge. At each time gene expression responses were compared between the Trangie resistant and susceptible populations of sheep and it was found that the *FABP4* and *FBLN1* genes had different expression patterns between these phenotypic extremes. Further gene association studies identified *FBLN1* and *FABP4* as key factors in the ability of sheep to resist fleece-rot. Validation of these markers in other populations could lead to vital tests for marker-assisted selection that would ultimately increase the resistance of sheep to fly-strike, thus reducing its prevalence. This study supported Smith *et al*.'s contention that *FABP4* is an important gene involved in the susceptibility and resistance of fleece-rot, and therefore fly-strike susceptibility and resistance, but as stated above further work needs to be done to define the role that *FABP4* plays in both fleece-rot and fly-strike susceptibility.

5.1 Other considerations in how *FABP4* variation may affect fly-strike susceptibility

From the literature there is evidence of *FABP4*'s role in adipose tissue metabolism. It could therefore be assumed that differences in fly-strike susceptibility and resistance in sheep carrying

different variants of the *FABP4* gene could also be associated with the differences in fat deposition, which can be further differentiated between breeds.

Bakhtiarizadeh *et al.* (2013) used Lori-Bakhtiari and Zel sheep to investigate the differences in gene expression in fat-tail and visceral adipose tissue. Their results showed that the expression of *FABP4* was significantly higher in the fat-tail of Lori-Bakhtiari sheep than that of both the fat-tail and visceral tissue of the Zel sheep. The higher expression of the *FABP4* gene in the fat-tail tissue of Lori-Bakhtiari sheep can be related to more fatty acid transportation into the fat-tail compared with the Zel's adipose tissue (fat-tail and visceral adipose tissue) (Bakhtiarizadeh *et al*. 2013).

If adipose fat is a factor that is causing fly-strike resistance in sheep then the higher expression of *FABP4*, leading to more fatty acid transport into various areas of the body could be a mechanism for this. In order to test this, the breech strike resistance of the Lori-Bakhtiari sheep could be tested compared to that of thin-tail breeds. Based on the raw comparison of Yan *et al.* (2012) and the present study (Appendix D) it is possible that the fat-tail sheep will have a higher frequency of the A_1 variant, and less fly-strike present, while the thin tail sheep will have a higher frequency of the C_1 variant and more fly-strike present.

5.2 Other evidence that FABP4 may affect sebaceous gland lipid production in animals

FABP4, along with *ABCC11* and *FADS1* have a role in lipid metabolism and have been found to be involved in the transport of sphingolipids, glycerophospholipids, cholesterol and fatty acids in epidermal lipid reorganization during keratinocyte terminal differentiation (Smith *et al*. 2010). Tsuda *et al.*'s (2009) study found the *FABP4* gene to be the strongest induced gene in the *Pten*-null keratinocytes; this suggests that *FABP4* plays a role in the development of the sebaceous gland hyperplasia seen in these mice. It has recently been suggested that *FABP4* selectively enhances PPARγ, a member of the nuclear hormone receptor family, which regulates genes involved in sebaceous differentiation (Michalik & Walter 2007; Tsuda *et al.* 2009). Therefore variations in *FABP4* could influence changes in sebaceous gland production, resulting in different levels of wool wax secretions, and even different lipids and long chain-fatty acids secretions into the wool.

The wool wax of sheep is considered to be an important barrier from infection. Older sheep are more resistant to *Dermatophilus congolensis* infection, because they can better maintain the integrity of the wool wax layer (Roberts 1963). In association with this, older sheep show greater resistances to fleece-rot and fly-strike than younger sheep (Belschner 1937). Warren *et al.* (1983)

found that mature sheep have a thicker wax layer than lambs, which could be why older sheep have a greater resistance to fleece-rot and fly-strike. Roberts (1863) found that the resistance to fleece-rot is associated with the waterproofing effect of high sebaceous wax content. In addition, he found that younger sheep have a relatively high incidence of fleece-rot, and suggested this could be due to the greater penetrability of their more open fleeces (Robert 1963). This would also make them more susceptible to fly-strike. In order to determine *FABP4*'s role in fleece-rot and fly-strike development the expression of *FABP4* could be compared between age groups. As younger sheep are more likely to get fleece-rot and fly-strike, it is possible that they have a lower expression of FABP4 than older sheep. However it could also be due to the acquisition of immunity with age.

5.3 Fly-strike resistance, animal odour and the role of lipids

Studies have focused on the orientation of *L. cuprina* towards fleece and chemical attractants (Eisemann 1995; Morris *et al*. 1998) and the identification of volatile components from bacterial strains isolated from myiatic lesions of sheep (Khoga *et al.,* 2002). These chemicals attractants from bacterial strains produce different odours which are likely to vary with the different variations of *FABP4* in sheep, and therefore affect their resistance or susceptibility to fly-strike. In order to test this a similar study to that done by Emmens & Murry (1982) could be done, where samples of wool from sheep that had been genotyped for FABP4 could be cultured with fleece-rot causing bacteria *in vitro* and exposed to flies to see if there is a difference in fly-strike occurring with the wool from sheep with different variations of *FABP4.*

5.4 The implications of FABP4 potentially affecting flystrike susceptibility in the context of the gene being associated with variation in other traits

As the *FABP4* gene appears to be associated with fat and lean lines of sheep (Yan *et al.* 2012), and various carcass traits in cattle (Michal *et al*. 2006; Jurie *et al*. 2007; Hoashi *et al.* 2008; Barendse *et al.* 2009; Lee *et al.* 2010) it is important to note that selecting sheep on their potential for fly-strike resistance may also affect their carcass trait characteristics. This could conflict with current consumer demands, especially as a major issue affecting the consumption of red meat is the need for improvement of lean meat yield, eating quality and human nutritional value. Consumers want meat with less fat, and this is most efficiently achieved by producing relatively leaner slaughter animals on farm (Pethick *et al*. 2011). Further study on the effects of *FABP4* on leanness and

fatness carcass traits of sheep and its association with fly-strike resistances and susceptibility are required, in order to demonstrate a clear correlation.

As mentioned above, there is a high correlation between fleece-rot and body strike (0.7-0.9), which has a moderate heritability of 0.35-0.4 (Raadsma *et al.* 1989). Therefore it is feasible to breed sheep that are resistant to fleece-rot, and in turn resistant to fly-strike. This idea has been the bases of the long standing breeding project at Trangie, NSW. However these sheep have been bred based on artificially induced fleece-rot and fly-strike in order to select sheep for resistance or susceptibility. This makes the idea of a commercial gene-test more compelling, as it means sheep do not need to be exposed to fleece-rot causing bacteria or fly-strike, which can cause wool production loses, as affected wool must be removed from the main line of fleeces (Henderson 1965). Sheep infected with fly-strike have a reduced feed-intake, high temperatures and show a reduction in wool production (Guerrine 1988). Exposing sheep to fleece-rot and fly-strike for research purposes would require ethical approval that looked at what is in the best interest of the animal.

Further studies would need to be done before a commercial gene test could be developed. Fly-strike might never be completely eradicated, however a gene test along with improved management practices could reduce the prevalence of fly-strike along with the use of chemical dips. Reducing fly-strike would save millions of dollars for the sheep industry. Management practices that might be incorporated along with gene-marker test selection include the use of fly-traps and crutching in-between shearing times.

5.5 Limitations in this study of *FABP4* and flystrike resistance

This was the first study to look at *FABP4*s association specifically with fly-strike. Therefore much more work needs to be done in this area. Although there is much research on *FABP4* function as an intracellular transporter, there is no current research about how variations in *FABP4* cause sheep to be more resistant or susceptible to fly-strike.

A major limitation to this study was the timing of year. The study began at the start of February, and collections of blood samples from sheep with fly-strike continued until May. It is unfortunate that the study did not begin early in the summer (December of the previous year) as this would allow for a greater sampling period, during peak fly-strike season. A further limitation is the time restraints of the study. Running the study over a period of two or three years would allow for more samples to be collected.

The sample size is another limitation, although it does relate to the timing of sampling. A greater sample size allows for a greater confidence in results.

Blood samples were collected based on the presence or absence of fly-strike, and the presence of fly-strike included sheep that had suffered from fly-strike, but had recovered. The two groups of fly-struck sheep were not separated. These sheep could show different genetic effects, but they would have to be separated in order to determine if this was the case.

Another limitation is that the sheep used were not recorded sheep, and there is no way of knowing if sheep from any given farm are related. Because the pedigree of these sheep is unknown it is impossible to know if there are any sire affects associated with their resistance/susceptibility. It is not known if there were half-siblings in this study, but there could be numerous other genes that could have been co-inherited through the sire-line and which could have various effects on traits that influence fly-strike susceptibility and resistance.

5.6 Suggestions for the future study of flystrike resistance with the possible role of *FABP4* in variations in susceptibility

Future research should focus on the effect of *FABP4* variations on the resistance and susceptibility sheep have to fly-strike. Although there were some significant results in this study much improvement could be made. In future studies the following should occur:

- The use of recorded sheep, therefore their pedigrees can be traced, and if need be their sire and dam can be genotyped.
- More animals should be used in the trial. If the effect of *FABP4* variations is small.
- Sheep should be separated into categories based on breed, gender, and age, as there is a difference seen between these traits and susceptibility to fly-strike. Female sheep are more likely to get fly-strike.
- The types of fly-strike occurring should also be recorded, such as breech, tail, pizzle, poll and body. The prevalence of the different types of fly-strike can vary at different times of year and between different classes of animals.
- In this trial sheep with fly-strike along with sheep that showed evidence of having had fly-strike were put into the same group. In future studies these sheep should be separated, as it could be a genetic effect causing the sheep to recover from the fly-strike, through innate or acquired immunity.

- Details of the region where sample collection took place should also be recorded. In the present study samples were taken from sheep throughout the Canterbury region. Areas within this region can vary considerably, and recording factors such as humidity, altitude, temperature and rainfall can affect the prevalence of fly-strike.
- The time of year should also be recorded, because as mentioned above the type of fly-strike occurring can vary with the time of year.
- The breeding program at Trangie (NSW, Australia) has selectively breed sheep to be susceptible or resistant to fly-strike, and therefore would be ideal sheep to genotype for the FABP4 gene in future studies.
- The breed of fly causing the fly-strike could also be recorded in future studies. This would require the collection of maggots to identify the species of fly. The fly-strike would have to be active in order to do this.

The method of identifying sheep with fly-strike at shearing time and taking blood samples accordingly worked well in the present study, and would work well in future studies. Shearing time is an ideal time to collect blood sample from sheep with and without fly-strike, as it is easy to identify sheep that have been fly-struck as they are being shorn. It is also an easy time to collect the blood from the sheep as they are being held by the shearer. In some cases the shearer will unintentionally cut the sheep, allowing for blood collecting without having to additionally cut the sheep. Many farmers shear, or crutch during the summer months. This is when fly-strike is most prevalent, and it is an ideal way to get many blood samples, without having to bring the sheep in again.

Chapter 6

Conclusion

It is generally accepted that breeding for resistance to myiasis could be a good long-term solution against this significant issue. There are several notable traits found that increase susceptibility to breech strike. The main indicator traits that have been identified for myiasis in the breech area are: accumulated faecal matter, urine stains, wrinkles, breech wool coverage and other wool traits. The *FABP4* gene has previously been associated with fleece-rot resistance (Smith *et al*. 2010), making it an ideal candidate gene to investigate for fly-strike resistance in sheep. In this study sheep with the A_1 variant of *FABP4* had significantly less fly-strike than sheep without the A_1 variant and sheep with the C_1 variant had significantly more fly-strike than those without it. This suggests that the A_1 and C_1 variants could be markers for the selection of fly-strike resistance into breeding programs. With further study it might be possible to develop a gene-marker test, which will allow farmers to selectively breed sheep that are resistant to fly-strike.

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Appendix A

Published sequences of FABP4

The gene bank accession numbers of *FABP4*: JX290313-JX290317.

A.1 *Ovis aries* fatty acid binding protein 4 (*FABP4*) gene, *FABP4-A* variant, exon 2 and partial cds

ORIGIN

1 tgtgggcttt gctaccagga aagtggctgg catggccaaa cccactgtga tcatcagtgt
61 aaatggggat gtggtcaaca ttaaatcaga aagcaccttt aaaaatactg agatgtcctt
121 caaattgggc caggaatttg atgaagtcac tccagatgac aggaaagtca aggtgaggaa
181 taaagaactg gagcagagta aaagcctggt ttataaacga ctgctgccta tatatagcaa
241 gccattttgt agaaggagga aagccattcc attataagcc aaaaagctca gattgctagc
301 tctgaaccat gttactgttg atatttagtt ggtgaattgt ctcccattta

A.2 *Ovis aries* fatty acid binding protein 4 (*FABP4*) gene, *FABP4-B* variant, exon 2 and partial cds

ORIGIN

1 tgtgggcttt gctaccagga aagtggctgg catggccaaa cccactgtga tcatcagtgt
61 aaatggggat gtggtcaaca ttaaatcaga aagcaccttt aaaaatactg agatgtcctt
121 caaattgggc caggaatttg atgaagtcac tccagatgac aggaaagtca aggtgaggaa
181 taaagaactg gagcagagta aaagctgatt tataaacgac tgctgcctat atatagcaag
241 ccattttgta gaaggaggaa agccattcca ttataagcca aaaagctcag attgctagct
301 ctgaaccatg ttactgttga tatttagttg gtgaattgtc tcccattta

A.3 *Ovis aries* fatty acid binding protein 4 (*FABP4*) gene, *FABP4-C* variant, exon 2 and partial cds

ORIGIN

1 tgtgggcttt gctaccagga aagtggctgg catggccaaa cccactgtga tcatcagtgt
61 aaatggggat gtggtcaaca ttaaatcaga aagcaccttt aaaaatactg agatgtcctt
121 caaattgggc caggaatttg atgaagtcac tccagatgac aggaaagtca aggtgaggaa
181 taaagaactg gagcagagta aaagcctgat ttataaacga ctgctgccta tatatagcaa

241 gccattttgt agaaggagga aagccattcc attataagcc aaaaagctca gattgctagc

301 tctgaaccat gttactgttg atatttagtt ggtgaattgt ctcccattta

A.4 *Ovis aries* fatty acid binding protein 4 (*FABP4*) gene, *FABP4-D* variant, exon 2 and partial cds

ORIGIN

1 tgtgggcttt gctaccagga aagtggctgg catggccaaa cccactgtga tcatcagtgt

61 aaatggggat gtggtcaaca ttaaatcaga aagcaccttt aaaaatactg agatgtcctt

121 caaattgggc caggaatttg atgaagtcac tccagatgac aggaaagtca aggtgaggaa

181 taaagaactg gagcagagta aaagcctgat ttataaatga ctgctgccta tatatagcaa

241 gccattttgt agaaggagga aagccattcc attataagcc aaaaagctca gattgctagc

301 tctgaaccat gttactgttg atatttagtt ggtgaattgt ctcccattta

A.5 *Ovis aries* fatty acid binding protein 4 (*FABP4*) gene, *FABP4-E* variant, exon 2 and partial cds

ORIGIN

1 tgtgggcttt gctaccagga aagtggctgg catggccaaa cccactgtga tcatcagtgt

61 aaatggggat gtggtcaaca ttaaatcaga aagcaccttt aaaaatactg agatgtcctt

121 caaattgggc caggaatttg atgaagtcac tccagatgac aggaaagtca aggtgaggaa

181 taaagaactg gagcagagta aaagcctgat ttataaacaa ctgctgccta tatatagcaa

241 gccattttgt agaaggagga aagccattcc attataagcc aaaaagctca gattgctagc

301 tctgaaccat gttactgttg atatttagtt ggtgaattgt ctcccattta

Appendix B

Raw data from FABP4 gene analysis

LAB ID	Farm	Fly-strike	FABP4
JT 23	JT	N	aa
OP 17	OP	Y	aa
RJ 19	RJ	N	aa
TB 7	TB	Y	aa
TB'5	TB	N	aa
CC 21	CC	N	ab
CC 26	CC	N	ab
GL4	GL	Y	ab
JT 28	JT	N	ab
JT 32	JT	N	ab
R 7	R	N	ab
RJ 18	RJ	N	ab
RJ 24	RJ	N	ab
RJ 25	RJ	N	ab
RJ 26	RJ	N	ab
RJ 27	RJ	N	ab
RJ 4	RJ	Y	ab
TB 1	TB	Y	ab
TB 10	TB	N	ab
TB 2	TB	Y	ab
WP 9	WP	N	ab
BT4	BT	Y	ab
DH4	DH	Y	ab
JW11	JW	Y	ab
JW12	JW	Y	ab
JW21	JW	N	ab
JW23	JW	N	ab
JW27	JW	N	ab
JW5	JW	Y	ab
TB'6	TB	N	ab
TB'7	TB	N	ab
CC 32	CC	N	ac
CC 6	CC	Y	ac
BT6	BT	N	ac
JW24	JW	N	ac
CC 11	CC	Y	ad
RJ 22	RJ	N	aa
CC 22	CC	N	bb
CC 30	CC	N	bb
GL7	GL	N	bb
JT 22	JT	N	bb

JT 4	JT	Y	bb
KP 2	KP	Y	bb
MM 13	MM	N	bb
MM 2	MM	Y	bb
MM 4	MM	N	bb
MS 10	MS	N	bb
MS 11	MS	N	bb
MS 12	MS	N	bb
MS 5	MS	Y	bb
R 5	R	N	bb
RJ 13	RJ	N	bb
RJ 14	RJ	N	bb
RJ 15	RJ	N	bb
RJ 17	RJ	N	bb
RJ 20	RJ	N	bb
RJ 21	RJ	N	bb
RJ 3	RJ	Y	bb
RJ 5	RJ	Y	bb
RJ 7	RJ	Y	bb
TB 13	TB	N	bb
TB 14	TB	N	bb
TB 3	TB	Y	bb
WP 4	WP	Y	bb
DH11	DH	N	bb
DH5	DH	Y	bb
JW16	JW	N	bb
JW17	JW	N	bb
JW18	JW	N	bb
JW19	JW	N	bb
JW20	JW	N	bb
JW26	JW	N	bb
JW30	JW	N	bb
TB'1	TB	Y	bb
TB'3	TB	Y	bb
CC 15	CC	Y	bc
CC 9	CC	Y	bc
JT 11	JT	Y	bc
JT 12	JT	Y	bc
JT 24	JT	N	bc
JT 25	JT	N	bc
JT 29	JT	N	bc
JT 6	JT	Y	bc
TB 15	TB	N	bc
WP 7	WP	N	bc
BT3	BT	Y	bc
JW2	JW	Y	bc
JW25	JW	N	bc

JW28	JW	N	bc
TB'4	TB	Y	bc
TB'8	TB	N	bc
CC 10	CC	Y	bd
CC 13	CC	Y	bd
CC 33	CC	N	bd
GL10	GL	N	bd
JM 1	JM	Y	bd
JM 3	JM	Y	bd
MM 14	MM	N	bd
OP 36	OP	Y	bd
TB 12	TB	N	bd
TB 6	TB	Y	bd
OP 21	OP	Y	bb
RJ 10	RJ	Y	bb
RJ 12	RJ	Y	bb
RJ 16	RJ	N	bb
RJ 11	RJ	Y	cc
WP 2	WP	Y	cc
RJ 1	RJ	Y	cd
JT 10	JT	Y	cc
KP 3	KP	Y	cc
KP 5	KP	Y	cc
JW3	JW	Y	cc
JT 15	JT	Y	ac
JT 21	JT	N	ac
KP 13	KP	N	ac
KP 20	KP	N	ac
KP 23	KP	N	ac
MM 3	MM	N	ac
MM 6	MM	N	ac
MS 1	MS	Y	ac
GL3	GL	Y	cc
JM 15	JM	N	cc
JM 16	JM	N	cc
JM 17	JM	N	cc
KP 1	KP	Y	cc
MM 12	MM	Y	cc
OP 19	OP	Y	cc
OP 24	OP	Y	cc
R 6	R	N	cc
TB 4	TB	Y	cc
JW6	JW	Y	ab
JW9	JW	Y	ab
GL11	GL	N	ab
JT 30	JT	N	ab
JT 31	JT	N	ab

JT 8	JT	Y	ab
KP 17	KP	N	ab
KP 18	KP	N	ab
WP 8	WP	N	ab
BT2	BT	Y	ac
BT5	BT	N	ac
DH1	DH	Y	ac
DH10	DH	N	ac
DH8	DH	N	ac
GL9	GL	N	ac
JT 26	JT	N	ac
JW15	JW	Y	ac
MM 10	MM	Y	ac
MM 9	MM	N	ac
OP 18	OP	Y	ac
TB 8	TB	Y	ac
GL6	GL	Y	ad
JM 18	JM	N	ad
DH7	DH	N	bb
BT1	BT	Y	bb
JW1	JW	Y	bb
JW10	JW	Y	bc
JW13	JW	Y	bc
CC 28	CC	N	bc
DH2	DH	Y	bc
DH3	DH	Y	bc
GL 1	GL	Y	bc
JT 13	JT	Y	bc
JT 7	JT	Y	bc
JW4	JW	Y	bc
KP 12	KP	N	bc
KP 6	KP	Y	bc
KP 7	KP	Y	bc
KP 9	KP	Y	bc
OP 1	OP	Y	bc
OP 10	OP	Y	bc
OP 16	OP	Y	bc
OP 20	OP	Y	bc
OP 26	OP	N	bc
OP 5	OP	Y	bc
R 3	R	Y	bc
RJ 9	RJ	Y	bc
TB 11	TB	N	bc
TB 9	TB	N	bc
TB'2	TB	Y	bc
JM 11	JM	N	cd
JM 6	JM	Y	cd

JT 9	JT	Y	cd
R 2	R	Y	cd
TB 5	TB	Y	cd

Raw data:

Alleles	With flystrike	Without flystrike
aa	3	3
ab	12	23
ac	9	15
ad	2	1
bb	17	28
bc	27	13
bd	6	4
cc	12	4
cd	5	1
	93	92

Allele frequencies of raw data:

Allele	With flystrike	Without flystrike
A_1	29	45
B_1	79	96
C_1	65	37
D_1	13	6
	186	184

Allele frequency as a percentage:

Allele	With flystrike	Without flystrike
A_1	16	25
B_1	42	52
C_1	35	20
D_1	7	3

Appendix C

Raw comparison of lean and fat lines of sheep from Yan *et al.* (2012) study, with the presence and absence of fly-strike in the present study, and variations in the *FABP4* gene. The main difference between the fat and lean line of sheep in Yan et al. (2012) study is that the A variant was not found in the lean line of sheep.

	A	**B**	**C**	**D**
Fly-strike	25	62	53	13
No Fly-strike	43	68	33	6
Lean	0	3	89	8
Fat	51	31	4	13

Hardy Weinberg equation:

Allele frequencies of the *FABP4* gene for sheep with fly-strike. P=1. Chi-squared test = 0.012. The chi-squared test shows that the population of sheep with fly-strike is not Hardy Weinberg equilibrium.

Alleles	Observed	Expected	Difference
aa	0.022	0.027	0.005
ab	0.130	0.132	0.002
ac	0.098	0.113	0.015
ad	0.022	0.028	0.006
bb	0.185	0.164	0.021
bc	0.293	0.280	0.013
bd	0.065	0.070	0.005
cc	0.130	0.120	0.010
cd	0.054	0.060	0.006
dd	0	0.007	0.007

Allele frequencies of the *FABP4* gene for sheep without fly-strike. P=1, Chi-squared test = 0.054

Alleles	Observed	Expected	Difference
aa	0.043	0.082	0.034
ab	0.247	0.26	0.013
ac	0.161	0.126	0.035
ad	0.011	0.023	0.012
bb	0.301	0.205	0.096
bc	0.140	0.199	0.059

bd	0.043	0.036	0.007
cc	0.043	0.0.48	0.005
cd	0.011	0.018	0.007
dd	0	0.002	0.002